Molecular Strategies to Overcome Antibiotic Resistance

Luting Fang
Washington University in St. Louis

Follow this and additional works at: https://openscholarship.wustl.edu/art_sci_etds

Part of the Biochemistry Commons

Recommended Citation
https://openscholarship.wustl.edu/art_sci_etds/2364

This Dissertation is brought to you for free and open access by the Arts & Sciences at Washington University Open Scholarship. It has been accepted for inclusion in Arts & Sciences Electronic Theses and Dissertations by an authorized administrator of Washington University Open Scholarship. For more information, please contact digital@wumail.wustl.edu.
Molecular Strategies to Overcome Antibiotic Resistance
by
Luting Fang

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

January 2021
St. Louis, Missouri
# Table of Contents

List of Figures ........................................................................................................................................ v
List of Tables ........................................................................................................................................ xviii
Acknowledgments ................................................................................................................................. xxxi
Abstract of the Dissertation .................................................................................................................... xxxiv

Chapter 1. Introduction .............................................................................................................................. 1
  1.1 Preface .............................................................................................................................................. 2
  1.2 Bacteria ........................................................................................................................................... 2
  1.3 Antibiotics ....................................................................................................................................... 8
  1.4 Multidrug resistant bacteria ............................................................................................................. 11
  1.5 Application of adjuvants to overcome drug resistance .................................................................. 13
  1.6 Discovery of new antibiotic drug targets ....................................................................................... 14
  1.7 Anti-virulence strategies to mitigate drug resistance issue .............................................................. 16
  1.8 Figures and Tables ....................................................................................................................... 19
  1.9 References ..................................................................................................................................... 30

Chapter 2: Mechanistic Basis for ATP-Dependent Inhibition of Glutamine Synthetase by Tabtoxinine-β-Lactam .................................................................................................................. 35
  2.1 Preface .............................................................................................................................................. 36
  2.2 Abstract ......................................................................................................................................... 36
  2.3 Introduction ..................................................................................................................................... 36
  2.4 Results and Discussion .................................................................................................................. 41
  2.5 Outlook and Conclusions ............................................................................................................... 59
  2.6 Materials and Methods .................................................................................................................. 61
  2.7 Acknowledgements ....................................................................................................................... 75
  2.8 Figures and Tables ....................................................................................................................... 76
  2.9 References ..................................................................................................................................... 101

Chapter 3: Inhibition of Glutamine Synthetase GlnA1 Blocks Nitrogen Metabolism in Mycobacterium tuberculosis ................................................................................................................................. 111
  3.1 Preface .............................................................................................................................................. 112
  3.2 Abstract ......................................................................................................................................... 112
### Chapter 7: Fimsbactin and Acinetobactin Compete for the Periplasmic Siderophore Binding Protein BauB in Pathogenic *Acinetobacter baumannii* .............................................. 392

7.1 Preface .................................................................................................................. 393
7.2 Abstract ................................................................................................................ 393
7.3 Introduction ......................................................................................................... 394
7.4 Results and Discussion ....................................................................................... 398
7.5 Outlook and Conclusions ................................................................................. 412
7.6 Materials and Methods ..................................................................................... 414
7.7 Acknowledgements ............................................................................................ 419
7.8 Figures and Tables ............................................................................................. 420
7.9 References .......................................................................................................... 460

### Chapter 8: Conclusions and Perspectives ............................................................. 469

8.1 Preface .................................................................................................................. 470
8.2 Introduction ......................................................................................................... 470
8.3 Summary of Dissertation .................................................................................... 470
8.4 Investigation of folate pathway inhibitors ......................................................... 473
8.5 Exploration of other enzymes in Cl-tabtoxin BGC ............................................. 475
8.6 Fimsbactin A analogs ......................................................................................... 477
8.7 FimF investigation .............................................................................................. 479
8.8 Oxidized Pre-Acinetobactin as *A. baumannii* growth inhibitor ....................... 483
8.9 Conclusion .......................................................................................................... 483
8.10 Figures and Tables ............................................................................................ 485
8.11 References ........................................................................................................ 521
List of Figures

Chapter 1

Figure 1.1: Cell wall of (a) gram-positive, and (b) gram-negative bacteria.................................19

Figure 1.2: Structure of glycan strand (GlcNAc-MurNAc) in bacterial peptidoglycan (PG)...........20

Figure 1.3: Peptidyl strand of peptidoglycan in (a) Gram-positive and (b) Gram-negative bacteria. Red color shows the portion that is different between two structures.................................21

Figure 1.4: Peptide cross-bridge of (a) Gram-positive and (b) Gram-negative bacteria PG strands. ....22

Figure 1.5: Spore formation cycle in Streptomyces.6 .................................................................23

Figure 1.6: Structure of M. tuberculosis cell wall. PLG layer is shown in yellow color......................24

Figure 1.7: Timeline for discovery of new antibiotic classes in clinical use....................................24

Figure 1.8: Five classes of bacterial machinery comprise the targets of the major classes of antibiotics. (a) cell wall biosynthesis, (b) membrane function, (c) protein biosynthesis, (d) macromolecular synthesis (DNA and RNA), (e) folate biosynthetic pathway (interdicting the supply of deoxythymidylate for DNA synthesis).................................................................25

Figure 1.9: Antibiotic resistance mechanisms in bacteria. (a) exclusion by antibiotic efflux pumps, (b) modification of antibiotic targets, (c) inactivating modifications of the antibiotic.............26

Figure 1.10: Structure of (a) amoxicillin antibiotic, (b) clavulanic acid antibiotic adjuvant. The combination, marketed as Augmentin®, is used to overcome resistance by β-lactamases. ..........26

Figure 1.11: Mechanism of β-lactamase inactivating amoxicillin..................................................27

Figure 1.12: (a) Structure of β-lactam four membered ring, (b) structure of penicillin antibiotic, (c) structure of D-Ala-D-Ala (terminus of peptidoglycan, substrate of transpeptidase), (d) structure of tabtoxine-β-lactam (TβL)........................................................................................................27

Figure 1.13: (a) mechanism of transpeptidase catalyzing peptidoglycan crosslink. (R: peptidoglycan) (b) mechanism of penicillin inhibition of transpeptidase. (c) β-lactamase inactivation of penicillin. (d) tabtoxin stealth from β-lactamase. (Blue: positive charged pocked in β-lactamase active site, red: negatively charged portion in the molecules.) ........................................28

Figure 1.14: General schematic for siderophore-mediated iron transportation into bacteria with the help of Siderophores. The efflux pump and transporter are not intended to represent specific systems; rather, the intent is to highlight the need to excrete metal-free siderophores and import siderophore ferric iron complexes.................................................................29

Chapter 2
Figure 2. 1: Two classes of β-lactam antibiotics. Pencillin G is a transpeptidase (TPase) inhibitor, and tabtoxinine-β-lactam (TβL) is a glutamine synthetase (GS) inhibitor.................................81

Figure 2. 2: Reactions catalyzed by glutamine synthetase (GS): (A) canonical biosynthetic reaction and (B) phosphorylation of inhibitors generating tight-binding transition state analogues...........82

Figure 2. 3: Structures and abbreviations of compounds used in this work.........................................................83

Figure 2. 4: Representative 1H-NMR (300 MHz) of quantified tabtoxin (74 mM) after HILIC chromatography taken in D2O with an acetonitrile (10 mM) internal standard. For full purification and characterization of tabtoxin see our previous publication11.............................84

Figure 2. 5: SDS-PAGE analysis of purified N-His6-GS homologs used in this work. SDS-PAGE gels (Any kD, Bio-Rad) were loaded with protein ladder (Precision Plus Protein Dual Xtra Prestained Protein Standards, Bio-Rad) in lane 1 and Ni-NTA elutions for recombinant N-His6-GS from E. coli (54.1 kD), H. sapiens (44.2 kD), and S. aureus (53.0 kD) in lanes 2, 3, and 4, respectively. Gel was stained with Coomassie blue.................................85

Figure 2. 6: Michaelis-Menten plots for GS homologs......................................................................................86

Figure 2. 7: Inhibition kinetics for TβL, MetSox, and Glufos against recombinant GS from (A–C) E. coli, (D–F) S. aureus, and (G–I) H. sapiens show time and ATP dependence and fit the Kitz-Wilson model for mechanism-based enzyme inhibition. Error bars represent standard deviations for three independent trials.................................................................87

Figure 2. 8: Primary sequence alignments of E. coli, S. aureus, and H. sapiens GS performed using Clustal W.................................................................................................88

Figure 2. 9: TβL-Thr and AAG show L-Gln-dependent bacteriostatic activity towards E. coli ATCC 25922. Panels show growth curves of E. coli ATCC 25922 in chemically defined minimal medium supplemented with (A) 2 μM TβL-Thr, (B) 100 μM TβL-Thr, and (C) 2 μM AAG and variable amounts of L-Gln and L-Glu. Error bars represent standard deviations for three independent trials.................................................................................................89

Figure 2. 10: GS phosphorylates TβL, MetSox, and Glufos. Panels show extracted ion chromatograms for LC-MS analysis of denatured GS-inhibitor complexes, with (+) or without (-) ATP added, and after treatment with Fmoc chloride.................................................................90

Figure 2. 11: High-resolution MS and MS/MS of Fmoc-TβL and FmocTβL-Pi.................................93

Figure 2. 12: (a) 202-MHz 31P cross-polarization magic-angle spinning NMR spectrum of a mixture of glutamine synthetase, buffer, and ADP. The resulting phosphate peak is assigned a 0 ppm chemical shift. (b) 31P{15N} full-echo and (c) rotational-echo double-resonance (REDOR) difference for a mixture of glutamine synthetase, buffer, ATP, and 15N-labeled TβL after dipolar evolution for 32 rotor periods (4.48 ms). (d) 50.7 MHz 15N cross-polarization magic-angle spinning NMR spectrum of intact whole cells of Staphylococcus aureus whose cell walls labeled with D-[15N]alanine. The chemical shift reference is solid ammonium sulfate.
(To switch to a liquid ammonia nitrogen chemical shift scale, add 20 ppm.) (e) 50.3 MHz $^{15}$N cross-polarization magic-angle spinning NMR spectrum of the sample used for panel b.

Figure 2. 13: Model for glutamine synthetase complexes with the reaction tetrahedral intermediate Gln-P$_i$ (A and C) and T$\beta$L-P$_i$ (B and D). Panels A and B depict the glutamine synthetase active site formed between chain A and chain B with the top scoring docked poses of ADP, Gln-P$_i$, and T$\beta$L-P$_i$ shown as sticks with nitrogens in blue, oxygens in red, phosphorous in orange, and carbon in green (ADP) or teal (Gln-P$_i$ and T$\beta$L-P$_i$). The two active site Mn$^{2+}$ metals are shown as spheres colored sea green. The GS cartoon structure is shown in gray with selected amino acid site chains shown as navy blue line structures. The P–N through-space distances for Gln-P$_i$ (2.9 Å) and T$\beta$L-P$_i$ (4.2 Å) are highlighted as yellow dashed lines. Panels C and D show the two-dimensional interaction network between glutamine synthetase amino acid side chains and Gln-P$_i$ or T$\beta$L-P$_i$ for the top-scoring docked poses. The images in panels A and B were generated using PyMOL version1.7.

Figure 2. 14: Prep-HPLC chromatogram for purification of Fmoc-AAG. x-Axis represents retention time (min). y-Axis represents absorbance at 254 nm.

Figure 2. 15: LC-MS chromatograms of synthetic Fmoc-AAG. (A) Chromatogram of optical absorbance at 254 nm. (B) Total ion chromatogram (TIC). (C) Extracted ion chromatogram (EIC) for m/z = 546.2 corresponding to the [M+H]$^+$ ion of Fmoc-AAG.

Figure 2. 16: $^1$H-NMR (300 MHz) of AAG (piperidine salt) after Fmoc-deprotection in DMF. Spectrum was obtained in D$_2$O with an acetonitrile internal standard.

Figure 2. 17: LC-MS chromatograms of synthetic AAG. (A) Chromatogram of optical absorbance at 254 nm. (B) Total ion chromatogram (TIC). (C) Extracted ion chromatogram (EIC) for m/z = 324.1 corresponding to the [M+H]$^+$ ion of AAG.

Chapter 3

Figure 3. 1: Active site of GS. Yellow balls: metal ions, green balls: ammonium ion, red stick: glutamate, blue stick: ATP, pink stick: key side chains of GS residues that move to close the active site.

Figure 3. 2: Structures of GS inhibitors.

Figure 3. 3: Trojan Horse strategy for tabtoxin function as a prodrug.

Figure 3. 4: SDS-PAGE gel of purified N-His$_6$ EcGS (GlnE). (E. coli GlnE: 108kDa).

Figure 3. 5: SDS-PAGE analysis of purified N-His$_6$-MtBGs used in this work. SDS-PAGE gels (Any kD, Bio-Rad) were loaded with protein ladder (Precision Plus Protein Dual Xtra Prestained Protein Standards, Bio-Rad) in lane 1 and NiNTA elutions #1 from two batches of protein prep in lanes 7,6, elutions #2 and #3 in lanes 5,4 and 3,2 respectively. Gel was stained with Coomassie blue.
Figure 3. 6: Adenylylation level test of GS variants using total phosphate assay. ................................. 146

Figure 3. 7: Steady-state Michaelis-Menten kinetic plots for MtbGS performed in triplicate.................... 147

Figure 3. 8: Inhibition kinetics for TβL, MetSox, and Glufos against recombinant MtbGS show (a) ATP and (b) time dependence and (c) fit the Kitz-Wilson model for mechanism-based enzyme inhibition. Error bars represent standard deviations for three independent trials....................... 147

Figure 3. 9: (A) growth curve of M. tuberculosis with addition of prodrugs. (B) biofilm formation study of M. tuberculosis. (Data and figure provided by Stalling’s lab).............................. 148

Figure 3. 10: Tabtoxin (Tβl-Thr) isomerization to form Tβl-Thr ................................................................. 148

Figure 3. 11: Mice information and urine analysis from tabtoxin toxicity study. (Figure provided by Henderson’s lab). ........................................................................................................... 149

Chapter 4

Figure 4. 1: Important compound structures (blue is amino acid part for prodrugs). a) Cl-tabtoxin, b) tabtoxin, c) Cl-TβL, d) Tbl, e) Rhizoctin, f) Plumbemycin, g) Dehydroporphos, h) Clavulanic acid, i) SAM, j) Phosphinothricin-tripeptide, k) the only intermediate found in TβL biosynthetic pathway............................................................................................................. 214

Figure 4. 2: Trojan Horse strategy for tabtoxin interrupting the growth of the neighboring cells........ 214

Figure 4. 3: Biosynthetic building blocks of TβL. Black part is from aspartic acid, blue part is from the two carbon of pyruvate, red part is from the methyl group of L-methionine, and green part is unknown................................................................. 215

Figure 4. 4: Prep-HPLC trace for purification of Cl-tabtoxin (after ion-exchange column) from Streptomyces culture supernatant........................................................................................................ 216

Figure 4. 5: Cl-tabtoxin agar diffusion assay with the samples from peak #1 in the HPLC purification (see Figure 4.4) ....................................................................................................................... 217

Figure 4. 6: Analysis of recombinant N-His-tagged PepA (55kDa) by SDS-PAGE. (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder) ............. 218

Figure 4. 7: Spontaneous isomerization of TβL and tabtoxin (blue is amino acid part of the prodrug) .. 219

Figure 4. 8: In vitro dipeptide cleavage reactions with recombinant PepA. Conditions were screened to optimize pH and addition of MgCl₂ using L-Ala-L-Ala as a model substrate. a) PepA PH optimization (Starting material), b) PepA PH optimization (Product), c) PepA PH optimization, metal addition (Starting material), d) PepA PH optimization, metal addition (Product). y-axes represent extracted ion counts (EICs)................................................................. 220

Figure 4. 9: Further optimization of PepA dipeptide cleavage reaction. a) PepA finer PH scan (starting material), b) PepA finer PH scan (product). y-axes represent extracted ion counts (EICs). 221
Figure 4. 10: Substrate comparison for PepA cleavage of dipeptide prodrugs as measured by GS inhibition from released GS inhibitors. ................................................................. 222

Figure 4. 11: Substrate comparison for PepA cleavage of dipeptide prodrugs using LC-MS detection of Fmoc-tagged free GS inhibitor extracted ion counts. .................................................. 222

Figure 4. 12: Lysine Biosynthesis pathways in bacteria. .............................................................................. 223

Figure 4. 13: Functional pairing of TblF, TblE, and TblR enable prodrug formation and efflux .............. 224

Figure 4. 14: Minimum BGC for TβL biosynthesis in a. *Streptomyces* Cl-tabtoxin BGC found in this work b. Known *P. syringae* tabtoxin BGC. .............................................................................. 224

Figure 4. 15: Comparison of putative tabtoxin BGCs from top 14 strains that have high match with our strain via BLASTp and antiSMASH analyses. ................................................................. 226

Figure 4. 16: SDS-PAGE analysis of recombinant N-His<sub>6</sub>-tagged CabB (29kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Stained with Coomassie blue.) ..................................................................................... 227

Figure 4. 17: Ddh catalyzed reaction to produce THDPA, the substrate for CabB (stereochemistry not shown here). .................................................................................................................. 227

Figure 4. 18: SDS-PAGE analysis of recombinant N-His<sub>6</sub>-tagged Ddh (35kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ..................................................................................... 228

Figure 4. 19: Reaction progress curve for Ddh catalyzed conversion of meso-DAP to THDPA as monitored by formation of NADPH (340 nm). ................................................................................. 229

Figure 4. 20: LC-MS analysis of Ddh reaction confirms THDPA product formation. (top: extracted ion counts for product THDPA, bottom: extracted ion counts for starting material meso-DAP). y-axes represent extracted ion counts (EICs). ..................................................................................... 230

Figure 4. 21: Proposed CabB reactions (stereochemistry not shown here). .................................................. 231

Figure 4. 22: Ellman assay mechanism ........................................................................................................... 231

Figure 4. 23: Enzymatic activity of CabB towards succinylation of THDPA as judged by Ellman’s assay. ................................................................................................................................. 232

Figure 4. 24: Comparison of enzymatic activity of CabB towards succinylation or acetylation of THDPA as judged by Ellman’s assay. ......................................................................................... 233

Figure 4. 25: LC-MS analysis of CabB reactions with succinyl-CoA. Succinyl-THDPA is detected as product (m/z=290) (y-axes represent extracted ion counts (EICs)). a) Assay with CabB enzyme, succinyl-CoA, and THDPA substrate. b-d) Controls. e) Structure of product: succinyl-THDPA (stereochemistry not shown here). ..................................................................................... 233
Figure 4. 26: LC-MS analysis of CabB reactions with acetyl-CoA. Acetyl-THDPA is detected as product (m/z=232) (y-axes represent extracted ion counts (EICs)). a) Assay with CabB enzyme, acetyl-CoA, and THDPA substrate. b-d) Controls. e) Structure of product: acetyl-THDPA.

Figure 4. 27: Chemical tagging of CabB succinylated products using O-benzylhydroxylamine. Labeled succinyl-THDPA is detected as product (m/z=395) (y-axes represent extracted ion counts (EICs)). a) Assay with CabB enzyme, succinyl-CoA, and THDPA substrate. b-d) Controls. e) Structure of O-benzylhydroxylamine. f) Structure of oxime product (stereochemistry not shown here). Note: The two peaks corresponding to the oxime product reflect a diastereomeric mixture of cis and trans oximes.

Figure 4. 28: Chemical tagging of CabB acetylated products using O-benzylhydroxylamine. Labeled acetyl-THDPA is detected as product (m/z=337) (y-axes represent extracted ion counts (EICs)). a) Assay with CabB enzyme, acetyl-CoA, and THDPA substrate. b-d) Controls. e) Structure of oxime product (stereochemistry not shown here). Note: The two peaks corresponding to the oxime product reflect a diastereomeric mixture of cis and trans oximes.

Figure 4. 29: Predicted CblA methyltransferase structure generated using SWISS model (https://swissmodel.expasy.org/).

Figure 4. 30: SDS-PAGE analysis of recombinant N-His$_6$-tagged CblA (25.7kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel stained with Coomassie blue.)

Figure 4. 31: Proposed CblA-catalyzed methyltransferase reaction (stereochemistry not shown here).

Figure 4. 32: LC-MS analysis of CblA reactions exploring substrate preference for acetyl-THDPA versus succinyl-THDPA. CH$_3$-acetyl-THDPA (m/z=246) or CH$_3$-succinyl-THDPA (m/z=304) was detected as product (y-axes represent extracted ion counts (EICs)). a) and c) This is the complete assay where acetyl-CoA is added, b) and f) This is the complete assay where succinyl-CoA is added, c) and g) This is the control assay where neither succinyl-CoA nor acetyl-CoA is added. d) Structure of CH$_3$-acetyl-THDPA product. h) Structure of CH$_3$-succinyl-THDPA product (stereochemistry not shown here).

Figure 4. 33: LC-MS analysis of CblA control reactions. a)-d) Shows the amount of substrate as judged by extracted ion counts: acetyl-THDPA under different conditions; e)-h) Shows the amount of product as judged by extracted ion counts: CH$_3$-acetyl-THDPA under different conditions. a) and c) This is the complete assay with everything added; b) and f) This is the control assay with no CblA enzyme addition. c) and g) This is the control assay with no SAM added. d) and h) This is the control assay with no THDPA (substrate) added. y-axes represent extracted ion counts (EICs).

Figure 4. 34: Proposed CabD PLP-mediated aminotransferase reaction with methylated THDPA analogs as substrate (stereochemistry not shown here).
Figure 4. 35: SDS-PAGE analysis of recombinant *N*-His<sub>6</sub>-tagged CabD (42kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)

Figure 4. 36: LC-MS analysis of the CabD reaction using CH<sub>3</sub>-acetyl-THDPA as substrate. CH<sub>3</sub>-acetyl-DAP (*m/z*=469) was detected as product. a) Complete assay with addition of everything. b) Control reaction with no L-glutamate. c) Control with no THDPA. d) Control with no CabD enzyme. e) Control with no PLP. y-axes represent extracted ion counts (EICs).

Figure 4. 37: Possible CabD reaction products from different THDPA analog substrates (stereochemistry not shown here).

Figure 4. 38: LC-MS analysis of CabD reactions reveals substrate preference. a-d) Possible THDPA analogs as substrate. y-axes represent extracted ion counts (EICs). f) Summary of the extracted ion counts from a)-d).

Figure 4. 39: LC-MS analysis of CabD reactions using THDPA as substrate reveal *meso*-DAP contamination from DdH preparation. a) THDPA as substrate for CabD assay and b) no CabD enzyme control. y-axes represent extracted ion counts (EICs).

Figure 4. 40: Docking of Pi-Cl-Tβl in the active site of GS. Top molecule: ATP, purple spheres (metal ions), bottom molecule: Pi-Cl-Tβl.

Figure 4. 41: LC-MS analysis of CblF assay shows a preference for L-Ala-L-Ala production. L-Ala-L-Ala product was Fmoc-tagged using FmocCl to form Fmoc-Ala-Ala (*m/z*=383) to enable LC-MS detection. y-axes represent extracted ion counts (EICs).

Figure 4. 42: Representative NRPS assembly line. A: adenylation domain; C: condensation domain; T: thiolation domain (also known as peptidyl carrier domain, PCP); TE: thioesterase domain (release the product from the NRPS assembly line). E and MT (shown in red) are functional domain. (E: epimersation domain; MT: methyltransferase domain.)

Figure 4. 43: Mechanism for amino acid substrate chlorination via halogenase tailoring enzyme activity on the NRPS-tether substrate.

Figure 4. 44: Predicted three-dimensional structure of TAW3_06404, a putative halogenase in the C1-tabtoxin BGC. Model was generated using SWISS-MODEL (https://swissmodel.expasy.org/).

Figure 4. 45: SDS-PAGE analysis of recombinant *N*-His<sub>6</sub>-tagged Cl-Cupin (TAW3_06387) (18.8kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)

Figure 4. 46: SDS-PAGE analysis of recombinant *N*-His<sub>6</sub>-tagged TAW3_06400 (35.5kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained using Coomassie blue.)
Figure 4. 47: SDS-PAGE analysis of recombinant N-His<sub>6</sub>-tagged TAW3_06404 (34.9kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained using Coomassie blue.) ................................................................. 249

Figure 4. 48: SDS-PAGE analysis of recombinant N-His<sub>6</sub>-tagged TAW3_06406 (8.8kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ................................................................. 250

Figure 4. 49: SDS-PAGE analysis of recombinant N-His<sub>6</sub>-tagged TAW3_06407 (58.9kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ................................................................. 251

Figure 4. 50: Activation of NRPS T domain with CoASH and Sfp (phosphopantetheinyl transferase) enzyme to install the required phosphopantetheinyl prosthetic group. ..................................................... 251

Figure 4. 51: Possible NRPS substrate candidate structures explored in this work ........................................... 252

Figure 4. 52: PK/LDH/Myokinase coupled reaction to detect the AMP production by NRPS A-domain. OD<sub>350</sub> nm is used as reaction indicator. .............................................................................................................. 252

Figure 4. 53: Reaction progress curves for NRPS reactions measured using the assay shown in Figure 4.52. a) Substrate screen (black line is Formyl-L-lysine). b) A series of control reactions. 253

Figure 4. 54: SDS-PAGE analysis of recombinant MBP-TbIS. MBP-TbIS: 112kDa; MBP: 42kDa; TbIS: 70kDa. (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gels were stained with Coomassie blue.) ................................................................. 254

Figure 4. 55: Proposed TbIS reaction using TβL-COOH as substrate to form TβL product (stereochemistry is not shown here, ATP is a required substrate). ..................................................... 255

Figure 4. 56: Regioselective acetylation of diamines. a) ω-position selective acylation using lysine model molecule. B) ω-position selective acylation using TβL-COOH. (stereochemistry is not shown here) .............................................................................................................. 255

Figure 4. 57: 1H-NMR analysis of the products from nitrophenyl acetate react with lysine. a) lysine : nitrophenyl acetate ratio is 1:1. b) lysine : nitrophenyl acetate ratio is 1:2 ......................... 257

Figure 4. 58: LC-MS analysis of nitrophenylacetate-mediated acetylation of TβL-COOH to form acetyl-TβL-COOH. a) and b) Show the consumption of starting material TβL-COOH. c) and d) Show the formation of product acetyl- TβL-COOH. y-axes represent extracted ion counts (EICs). .............................................................................................................. 258

Figure 4. 59: Proposed TbIS-catalyzed reaction using acetyl-TβL-COOH as substrate to form acetyl-TβL (stereochemistry not shown here, ATP is required as a cosubstrate). ..................................................... 258
Figure 4.60: One of our current proposals for tabtoxin biosynthesis in *P. syringae*. The pathway is proposed to be analogous in Streptomyces up to TβL formation. (stereochemistry is not shown here) .................................................................................................................. 259

Figure 4.61: Predicted three-dimensional structure of CabD generated with SWISS-MODEL (https://swissmodel.expasy.org/). .................................................................................................................. 260

Figure 4.62: Predicted three-dimensional structure of CabB generated with SWISS-MODEL (https://swissmodel.expasy.org/). .................................................................................................................. 260

**Chapter 5**

Figure 5.1: Tetracycline development and parallel emergence of resistance mechanisms. ................. 298

Figure 5.2: Introduction to the tetracycline destructase family of FMO enzymes and structure of the first inhibitor, anhydrotetracycline (5). (A) Phylogenetic tree [aligned with Clustal Omega and viewed using iTOL software]. (B) X-ray crystal structure of chlortetracycline bound to Tet(X) (PDB ID 2y6r). (C) X-ray crystal structure of chlortetracycline bound to Tet(50) (PDB ID 5tui). .......................................................................................................................... 299

Figure 5.3: Michaelis−Menten kinetics of tetracycline destructase degradation of first generation tetracyclines. (A) Representative Michaelis−Menten plot of tetracycline destructase degradation of oxytetracycline. (B) Representative optical absorbance kinetic plots for the degradation of oxytetracycline by tetracycline destructase enzymes [as observed at 400 nm for Tet(50) and Tet(X)_3; 380 nm for Tet(X)]. (C) Apparent $K_m$, $k_{app}$, and catalytic efficiencies for the tetracycline destructase-mediated degradation of tetracycline, chlortetracycline, demeclocycline, and oxytetracycline. Error bars represent standard deviation for two independent trials. ........................................................................................................... 299

Figure 5.4: *In vitro* aTC inhibition of tetracycline destructase degradation of first-generation tetracycline antibiotics as observed via an optical absorbance kinetic assay. (A) aTc inhibition of Tet(50) degradation of tetracyclines. (B) aTc inhibition of Tet(X) degradation of tetracyclines. (C) aTc inhibition of Tet(X)_3 degradation of tetracyclines. (D) Apparent IC₅₀ for aTC inhibition (denoted for each substrate and enzyme). Error bars represent standard deviation for three independent trials. All data points possess error bars, though some are not visible at the plotted scale. .......................................................................................................................... 300

Figure 5.5: Lineweaver-Burk Plots for the *in vitro* inhibition of the tetracycline destructase-mediated degradation of tetracycline by anhydrotetracycline. .................................................................................................................. 301

Figure 5.6: *In vitro* inhibition of tetracycline destructase degradation of first-generation tetracycline antibiotics as observed via optical absorbance kinetic assay. Inhibitory activity of aTC library against (A) Tet(50) degradation of tetracycline, (B) Tet(X) degradation of tetracycline, (C) Tet(X)_3 degradation of tetracycline, (D) Tet(50) degradation of chlortetracycline, (E) Tet(X) degradation of chlortetracycline, and (F) Tet(X)_3 degradation.
of chlortetracycline. Error bars represent standard deviation for three independent trials. All data points possess error bars, though some are not visible at the plotted scale. 

Figure 5. 7: (A) Whole cell inhibition of *E. coli* expressing tetracycline destructase enzymes including calculated FICI and observed fold change enhancements. (B) Working model of the inhibition of tetracycline destructase enzymes by aTC-like small molecules (competitive inhibitor vs sacrificial substrate).

Figure 5. 8: Tet(X)-mediated degradation of aTC and aTC analogues. (A–F) Each panel depicts the degradation of the denoted aTC as observed via optical absorbance spectroscopy and plots of monitored extracted mass counts from LCMS. Each represents a reaction containing purified Tet(X) enzyme (or none, in the case of the control), aTc analogue, and an NADPH regenerating system [including MgCl₂]. The plots represent extracted ion counts normalized to an internal standard (Fmoc-alanine) and depicted as a percent of the total ion count [aTC+aTC−OH].

Figure 5. 9: Tet(X)-mediated degradation of oxytetracycline as a model for the degradation of aTC-like sacrificial substrates.

Figure 5. 10: aDem inhibition of tetracycline destructase-mediated degradation of first generation tetracyclines. (A) aDem inhibition of tetracycline destructase degradation of tetracycline. (B) aDem inhibition of tetracycline destructase degradation of demeclocycline. (C) Apparent IC₅₀ for the *in vitro* inhibition of tetracycline and demeclocycline degradation by tetracycline destructase enzymes with aTC and aDem. (D) Michaelis–Menten plot of dose-dependent aDem acceleration of Tet(50) consumption of NADPH, apparent *Kₘ*, and calculated catalytic efficiency. Error bars represent standard deviation for two to three independent trials. All data points possess error bars, though some are not visible at the plotted scale.

Figure 5. 11: *In vitro* Characterization of the reaction of aDem and Tet(50) in the presence of NADPH.

Figure 5. 12: (A) X-ray crystal structure of aTC bound to Tet(50) [PDB ID: 5TUF] and corresponding binding mode identifier; (B) time- and aDem-dependent degradation of NADPH by Tet(50) observed using a broad-scan optical absorbance kinetic assay; (C) hydrogen peroxide colorimetric detection experiments, from left to right: NADPH (no enzyme) control, no enzyme control (NADPH + aDem), Tet(50) + NADPH [no aDem] reaction mixture, and Tet(50) + NADPH + aDem reaction mixture.

Figure 5. 13: Broad-scan detection of aDem- and Tet-promoted consumption of NADPH by Tet(50). a, NADPH control: no enzyme, no substrate. b, aDem-NADPH, no enzyme control. c, Tet-NADPH, no enzyme control. d, Tet(50)-NADPH, no substrate control. e, Tet(50)-Tet-NADPH reaction. f, Tet(50)-aDem-NADPH reaction.

Figure 5. 14: Qualitative detection of aDem- and Tet-promoted hydrogen peroxide formation by Tet(50).
Figure 5. 15: (A) Michaelis–Menten plot and apparent kinetic parameters ($K_m$, $k_{app}$, and catalytic efficiencies) for the Tet(X)-mediated degradation of tigecycline. (B) Inhibitory activity of aTC and chlorinated aTC analogs against Tet(X) degradation of tigecycline. (C) Last generation tetracycline antibiotics tigecycline and eravacycline. Error bars represent standard deviation for three independent trials. All data points possess error bars, though some are not visible at the plotted scale. ................................................................. 311

Figure 5. 16: Toward extended library synthesis of aTC-like inhibitors of tetracycline destructase enzymes. (A) An adjuvant approach to combat enzymatic inactivation of tetracycline antibiotics and summary of preliminary structure–activity information. (B) Surface view of X-ray crystal structure of Tet(X) bound to tigecycline (PDB ID 4a6n) highlights importance of open active site to accommodate bulky D-ring substituents, suggesting further use of glycyclycline antibiotics may drive selective pressure of tetracycline destructase-involved resistance mechanisms. ................................................................. 311

Figure 5. 17: SDS-Page Gel Image – Purified Tetracycline Destructase Enzymes ...................................................... 312

Figure 5. 18: Applicable NMR Spectra of (4S,4aS,12aS)-7-chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydratetracene-2-carboxamide hydrochloride (aCTc, 6) ........................................................................................................ 315

Figure 5. 19: Applicable NMR Spectra of (4S,4aS,12aS)-7-chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydratetracene-2-carboxamide hydrochloride (aDem, 7) ........................................................................................................ 318

Figure 5. 20: Applicable NMR Spectra of (4S,4aS,12aS)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-7-iodo-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydratetracene-2-carboxamide hydrochloride (7-I-aTc, 8) ........................................................................................................ 321

Figure 5. 21: Applicable NMR Spectra of (4S,4aS,12aS)-9-bromo-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydratetracene-2-carboxamide hydrochloride (9-Br-aTc, 9) ........................................................................................................ 324

Figure 5. 22: LCMS traces for crude and preparative HPLC purified 7-I-aTc, 8 ...................................................... 326

Chapter 6

Figure 6. 1: Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal subunit to block accommodation of incoming tRNAs ........................................................................................................ 357

Figure 6. 2: An overview of tetracycline resistance mechanisms. a) Efflux b) ribosomal protection c) enzymatical inactivation ........................................................................................................ 358

Figure 6. 3: Structures of three generations of tetracyclines. Blue: first generation. Black: second generation. Red: third and fourth generation. ........................................................................................................ 359
Figure 6. 4: TDase-catalyzed inactivation tetracycline mechanism via C11as hydroxylation with the involvement of a flavin cofactor activated as the C4a-flavin peroxide. 360

Figure 6. 5: Chemical structure of flavin adenine dinucleotide (FAD). 360

Figure 6. 6: Reaction scheme for two-electron reduction of FAD. 361

Figure 6. 7: Proposed catalytic cycle for TDase-catalyzed oxidation of substrate (tetracycline) highlighting the FAD oxidation states. 361

Figure 6. 8: Optical absorbance assay monitoring for FAD oxidation state in TetX at 450 nm. a) normal assay with Ar pre-treatment. b) “no enzyme” control with Ar pre-treatment. c) “no NADPH” control with Ar pre-treatment. d) no Ar pre-treatment. 362

Figure 6. 9: Proposed TDase inactivation of aTcs via C11a hydroxylation. 362

Figure 6. 10: Addition of external oxygen gas enhances TDase-catalyzed oxidation rates. Chromatograms show LC-MS analysis of the aDem oxidized product from TetX with or without external addition of oxygen gas (2hr). a) normal assay, starting material. b) “no enzyme” control, starting material. c) normal assay, product. d) “no enzyme” control, product. e) summary of a)-d). 363

Figure 6. 11: Addition of external oxygen gas enhances TDase-catalyzed oxidation rates. Chromatograms in panels a-d) show LC-MS analysis of the IaTc oxidized product from TetX with or without external addition of oxygen gas (1.5 hr). Panel i) is a summary of the extracted ion counts for indicated conditions. 364

Figure 6. 12: Reaction progress curves for wild-type TetX and mutant TetX_T280A. The UV-Vis scan was taken with continuous monitoring at 380 nm absorbance to measure enzyme reaction speed. (31uM Tetracycline was as substrate for both enzymes.) 365

Figure 6. 13: Michaelis-Menten steady-state kinetic plots for TetX_T280A mutant. 366

Figure 6. 14: Michaelis-Menten steady-state kinetic plots for TetX_T280A mutant compared with TetX wild-type. 366

Figure 6. 15: IC_{50} curves for inhibition of TetX_T280A mutant by aTC using tetracycline (left) or tigecycline (right) as substrate. 367

Figure 6. 16: Comparison of aTC IC_{50} plots for TetX_T280A and wild-type TetX using tetracycline (left) or tigecycline (right) as substrate. 367

Figure 6. 17: Partial sequence alignment of TetX7 (top) and TetX (bottom). 368

Figure 6. 18: Michaelis-Menten plots for TetX7_V280A using variable tetracycline (left), tigecycline (middle), or NADPH (right, with tetracycline as substrate). 368

Figure 6. 19: Comparison of Michaelis-Menten plots for TetX7_V280A and TetX.
Figure 6. 20: IC\textsubscript{50} curves for aTC inhibition of TetX7\_V280A using tetracycline (left) or tigecycline (right) as substrate.................................................................369

Figure 6. 21: Comparison of aTC IC\textsubscript{50} curves for mutant TetX7\_V280A and wild-type TetX7 using tetracycline (left) or tigecycline (right) as substrate.................................................................369

Figure 6. 22: Structural alignment of TetX with Tet50. Green: Tet50 (PDB: 5TUF); Magenta: TetX(2XDO); Yellow: mutation site. Image created using PyMOL ‘align’ function. ........370

Figure 6. 23: Michaelis-Menten plots for Tet50\_R280A using variable tetracycline (left) and variable NADPH (right)..................................................................................371

Figure 6. 24: Comparison of Michaelis-Menten plots for Tet50\_R280A and wild-type Tet50 using variable tetracycline (left) and variable NADPH (right).................................371

Figure 6. 25: Structural aligned of wild-type TetX with mutant TetX\_T280A. a) Complete cartoon structures (green: TetX, pink: TetX\_T280A). b) Zoom in on active sites. Images were created using the PyMOL ‘align’ function. PDB Accession Codes: 3V3N for TetX\_T280A; 4A99 for wild-type TetX......................................................................................................372

Figure 6. 26: Phylogenetic tree figure showing sequence relationships of potentially new TDases compared to known TDases. (Credit: Dantas lab at Washington University School of Medicine).........................................................................................................................373

Figure 6. 27: Michaelis-Menten plots (Tet substrate) for the 4 enzymes picked from the tree in Figure 6.26 ..................................................................................................................374

Figure 6. 28: Michaelis-Menten plots for Tet\#4 using variable tetracycline (left), chlortetracycline (middle), and NADPH (right; with Tet as substrate). ........................................375

Figure 6. 29: Color changes observed in a 96-well plate with different combinations of tetracyclines and recombinant TDases enzymes. (Image provided by Alex Kong, summer intern in the Wencewicz lab.)...................................................................................................................375

Figure 6. 30: Chromogenic doxycycline derivatives as potential TDase color-changing diagnostic agents. a) compound #2, b) compound # 5, c) compound # 6.................................................................................376

Figure 6. 31: Michaelis-Menten plots for compounds 2, 5, and 6 (see Figure 6.30) as substrates for TetX (top row) and TetX7 (bottom row) using tetracycline as substrate..............................377

Figure 6. 32: Images of cuvettes containing chromogenic doxycycline analogs in the presence of Tdases. Left top corner number: compound. Right top corner: enzyme (X: TetX, 3: TetX7, -: no enz) ..............................................................................................................................................378

Figure 6. 33: Color change observed using tetracycline as substrate for TDases. Right corner: enzyme (X: TetX, 3: TetX7, -: no enz)..................................................................................378
Figure 6. 34: Optical absorbance spectra of compounds 2, 5, and 6 (Figure 6.30) before and after reaction with TDases. UV-Vis scans taken from 400-700 nm. Note, Tet3 is the same as TetX7. ....379

Figure 6. 35: IC$_{50}$ curve for aTC inhibition of Tet50_R280A using tetracycline as substrate. .....................380

Figure 6. 36: Comparison of aTC IC$_{50}$ curves for Tet50_R280A and Tet50 using tetracycline as substrate. ..........................................................380

Figure 6. 37: SDS-PAGE analysis of recombinant N-His$_6$-tagged Tet #1 (44kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ..........................................................381

Figure 6. 38: SDS-PAGE analysis of recombinant N-His$_6$-tagged Tet #4 (44kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ..........................................................382

Figure 6. 39: SDS-PAGE analysis of recombinant N-His$_6$-tagged Tet #5 (44kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ..........................................................383

Figure 6. 40: SDS-PAGE analysis of recombinant N-His$_6$-tagged Tet #13 (44kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ..........................................................384

Figure 6. 41: SDS-PAGE analysis of recombinant N-His$_6$-tagged TetX_T280A (44kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ..........................................................385

Figure 6. 42: SDS-PAGE analysis of recombinant N-His$_6$-tagged TetX7_V280A (45kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ..........................................................386

Figure 6. 43: SDS-PAGE analysis of recombinant N-His$_6$-tagged Tet50_R280A (46kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ..........................................................387

Chapter 7

Figure 7. 1: Siderophores from A. baumannii. (a) Structures of preacinetobactin (PreAcb), acinetobactin (Acb), fimsbactin (Fim), and baumannoferrin. PreAcb/Acb and Fim share a common motif derived from condensation of 2,3-DHB with L-Thr (PreAcb/Acb) or L-Ser (Fim). (b) DAD at 263 nm (black), EIC at m/z 347 (blue), and EIC at m/z 575 (red) chromatograms from LC-MS analysis of crude A. baumannii ATCC 17978 supernatant after acidification, treatment with XAD-7HP resin, and methanol elution. Baumannoferrin was not detected. .............423

Figure 7. 2: Predicted biosynthesis of acinetobactin and fimsbactin in A. baumannii ATCC 17978.78,79 Non-ribosomal peptide synthetase (NRPS) assembly lines for acinetobactin (top) and
Fimsbactin A (bottom) share a common precursor, 2,3-DHB, and a common phenolate oxazoline motif. Pre-acinetobactin (PreAcb) is the kinetic product released from the NRPS assembly line. PreAcb undergoes spontaneous isomerization to the thermodynamic product acinetobactin (Acb).

Figure 7.3: Structures and m/z values for [M+H]^+ molecular ions of fimsbactin A–F. Structural differences are highlighted in red.

Figure 7.4: Biosynthetic gene clusters for acinetobactin, fimsbactin, and baumannoferrin from A. baumannii ATCC 17978 along with annotated genes.

Figure 7.5: AntiSMASH analysis of putative fimsbactin A. baumannii producers identified from BLASTp analysis of A. baumannii genomes reveals conservation of acinetobactin and baumannoferrin biosynthetic gene clusters (BGCs). The acinetoferrin BGC is the reference in the antiSMASH database. Acinetoferrin and baumannoferrin BGCs share homology, so comparison for all strains was made to the acinetoferrin BGC.

Figure 7.6: Diode array optical absorbance detection (DAD) and extracted ion chromatograms (EICs) for fimsbactin A–F [M+H]^+ ions from LCMS analysis of A. baumannii ATCC 17978 culture supernatant extractions (Trial #1) using ESI ionization in positive ion mode. The x-axis represents retention time (min) for all chromatograms.

Figure 7.7: Diode array optical absorbance detection (DAD) and extracted ion chromatograms (EICs) for Acb [M+H]^+ ions from LCMS analysis of HPLC-purified Acb from A. baumannii ATCC 17978 using ESI ionization in positive ion mode. The x-axis represents retention time (min) for all chromatograms.

Figure 7.8: Diode array optical absorbance detection (DAD) and extracted ion chromatograms (EICs) for fimsbactin A–F [M+H]^+ ions from LCMS analysis of HPLC-purified fimsbactin A from A. baumannii ATCC 17978 (Trial #1) using ESI ionization in positive ion mode. The x-axis represents retention time (min) for all chromatograms.

Figure 7.9: Diode array optical absorbance detection (DAD) and extracted ion chromatograms (EICs) for fimsbactin A–F [M+H]^+ ions from LCMS analysis of HPLC-purified fimsbactin A from A. baumannii ATCC 17978 (Trial #2) using ESI ionization in positive ion mode. The x-axis represents retention time (min) for all chromatograms.

Figure 7.10: Diode array optical absorbance detection (DAD) and extracted ion chromatograms (EICs) for fimsbactin A–F [M+H]^+ ions from LCMS analysis of HPLC-purified fimsbactin A from A. baumannii ATCC 17978 (Trial #2) using ESI ionization in positive ion mode. The x-axis represents retention time (min) for all chromatograms.

Figure 7.11: High-resolution ESI MS (positive ion mode) of fimsbactin A purified by prep-HPLC from A. baumannii ATCC 17978 culture supernatant (Trial #1). Expected [M+H]^+ for C_{26}H_{31}N_{4}O_{11} 575.1984, found 575.2056.
Figure 7.12: $^1$H-NMR (600 MHz, DMSO-d6) spectrum of purified fimsbactin A purified by prep-HPLC from *A. baumannii* ATCC 17978. The x-axis is chemical shift given in parts per million (ppm). The y-axis is arbitrary peak intensity.

Figure 7.13: $^{13}$C-NMR (151 MHz, DMSO-d6) spectrum of purified fimsbactin A purified by prep-HPLC from *A. baumannii* ATCC 17978. The x-axis is chemical shift given in parts per million (ppm). The y-axis is arbitrary peak intensity.

Figure 7.14: Structures of Fim (*A. baumannii*), heterobactin (*Rhodococcus erythropolis*), and vibriobactin (*Vibrio cholerae*) siderophores. Metal chelating groups are shown in blue. The tetrahedral atom is highlighted by a salmon sphere.

Figure 7.15: Structures and microbial producers of amino acid-based siderophores fimsbactin A, heterobactin A, and JBIR-16, and spermidine-based siderophores vibriobactin, vulnibactin, fluvibactin, agroactin, parabactin, and protochelin. Iron chelating groups are shown in blue. The tetrahedral branch point for metal chelating ligands is highlighted by a yellow circle.

Figure 7.16: Titration of Fim with (A) Fe(acac)$_3$ and (B) FeCl$_3$ in methanol. Graphs depict fluorescence ($\lambda_{\text{excitation}} = 330$ nm; $\lambda_{\text{emission}} = 380$ nm) vs equivalents of ferric iron source showing a titration end point correlating with a 1:1 final stoichiometry for the [Fe(Fim)] ferric complex. Titration with FeCl$_3$ was performed as two independent trials. Error bars represent standard deviation from the mean.

Figure 7.17: Optical absorbance spectrum of the holo-[Fe(Fim)] complex at 100 μM in phosphate buffer (50 mM potassium phosphate pH 8.0, 150 mM NaCl, 1 mM DTT, 5% glycerol). The molar extinction coefficient ($\varepsilon$) of holo-[Fe(Fim)] was determined to be 4255 M$^{-1}$ cm$^{-1}$ at 445 nm. The inset shows the visible color of the [Fe(Fim)] solution at 100 μM.

Figure 7.18: Decay plots for EDTA competition assays with [Fe(Fim)]. Graphs depict absorbance at 500 nm (y-axis) vs time (x-axis) for (A) EDTA (1.2 equiv) plus [Fe(Fim)], (B) [Fe(Fim)], and (C) buffer only. Each experiment was performed in duplicate as independent trials. The apparent $K_F$ for [Fe(Fim)] was calculated using the final absorbance values after 150 minutes according to equations (1)–(11) shown on Equations 7.1. The final absorbance values at 500 nm were 0.3873 (trial #1 w/ EDTA), 0.3870 (trial #2 w/ EDTA), 0.4292 (trial #1 w/o EDTA), and 0.4357 (trial #2 w/o EDTA).

Figure 7.19: Titration of 100 μM [Fe(Acb)$_2$] with Fim reveals slow exchange of iron leading to complete formation of [Fe(Fim)]. Optical absorbance spectra were collected for each concentration after 20 min in phosphate buffer (50 mM potassium phosphate pH 8.0, 150 mM NaCl, 1 mM DTT, 5% glycerol). The final optical absorbance spectrum with 210 μM apo-Fim added was measured after 20 hours.

Figure 7.20: Influence of apo- and holo-siderophores on *A. baumannii* growth. Line graphs depict the growth of *A. baumannii* ATCC 17978 determined by measuring the optical density at 600 nm (OD$_{600}$) at 37 °C as a function of time in the presence of (a) Acb or [Fe(Acb)$_2$] and (b)
Fim or [Fe(Fim)]. Bar graphs depict the comparison of OD\textsubscript{600} values after 30 h in the presence of variable concentrations of (c) Acb or [Fe(Acb)\textsubscript{2}] and (d) Fim or [Fe(Fim)]. Error bars represent standard deviations from the mean for three independent trials, ****p < 0.0001.

Figure 7.21: Dose dependent growth promotion of A. baumannii ATCC 17978 by (A) apo-acinetobactin (Acb), (B) holo-acinetobactin (Acb\textsubscript{Fe}), (C) apo-fimsbactin (Fim), and (D) holo-fimsbactin (Fim\textsubscript{Fe}). Line graphs depict the growth of A. baumannii ATCC 17978 in M9 minimal medium supplemented with 175 μM 2,2′-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD\textsubscript{600}) as a function of time in the presence of variable siderophore concentrations. All experiments were performed in triplicate. Error bars are shown in Figure 7.22. Data from these plots were used to create the line and bar graphs shown in Figure 7.20 in the main text.

Figure 7.22: Dose dependent growth promotion of A. baumannii ATCC 17978 by (A) apo-acinetobactin (Acb), (B) holo-acinetobactin (Acb\textsubscript{Fe}), (C) apo-fimsbactin (Fim), and (D) holo-fimsbactin (Fim\textsubscript{Fe}). Line graphs depict the growth of A. baumannii ATCC 17978 in M9 minimal medium supplemented with 175 μM 2,2′-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD\textsubscript{600}) as a function of time in the presence of variable siderophore concentrations. Error bars represent standard deviations from the mean for three independent trials. Line graphs are shown without error bars for clarity in Figure 7.21. Data from these plots were used to create the line and bar graphs shown in Figure 7.20 in the main text.

Figure 7.23: Influence of apo- and holo-siderophore combinations on A. baumannii growth. Bar graphs depict the comparison of A. baumannii ATCC 17978 growth measured by optical density at 600 nm (OD\textsubscript{600}) values after 30 h at 37 °C in the presence of variable concentrations of (a) Fim and Acb, (b) Fim and [Fe(Acb)\textsubscript{2}], and (c) [Fe(Fim)] and Acb. Error bars represent standard deviations from the mean for two independent trials; ****p < 0.0001; ns = not significant.

Figure 7.24: Influence of apo- and holo-siderophore combinations on the growth of A. baumannii ATCC 17978. Line graphs depict the growth of A. baumannii ATCC 17978 in M9 minimal medium supplemented with 175 μM 2,2′-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD\textsubscript{600}) as a function of time in the presence of variable concentrations of siderophore mixtures. For all graphs, siderophore concentration gradients are provide on the x-axis and y-axis of the checkerboard. The black line graph represents bacterial growth without addition of siderophores. The red line graph represents bacterial growth in the presence of variable concentrations of (A) apo-Fim and apo-Acb, (B) holo-Fim\textsubscript{Fe} and apo-Acb, (C) apo-Fim and holo-Acb\textsubscript{Fe}, or (D) apo-Fim and holo-Fim\textsubscript{Fe}. Error bars represent standard deviations from the mean for two independent trials. Data from these plots were used to create bar graphs shown in Figure 7.23 in the main text.

Figure 7.25: Influence of apo-Fim holo-Fim\textsubscript{Fe} combinations on A. baumannii growth. Bar graphs depict the comparison of A. baumannii ATCC 17978 growth measured by optical density at 600 nm
(OD\textsubscript{600}) values after 30 hours at 37 °C in the presence of variable concentrations of (a) Fim and Acb, (b) Fim and Acb\textsubscript{2}Fe, and (c) FimFe and Acb. Error bars represent standard deviations from the mean for two independent trials. ns = not significant

Figure 7. 26: Siderophore-dependent fluorescence quenching of N-His\textsubscript{6}-BauB. Graphs depict intrinsic tryptophan fluorescence quenching (y-axis: λ\textsubscript{excitation} = 280 nm; λ\textsubscript{emission} = 320 nm) of 400 nM N-His\textsubscript{6}-BauB in the presence of variable siderophore concentrations (x-axis). Apparent K\textsubscript{d} values were calculated using a single-binding mode curve-fitting model in GraphPad Prism version 7.0b. Error bars represent standard deviations for two independent trials.

Figure 7. 27: [Fe(Acb)\textsubscript{2}] and [Fe(Fim)] compete for BauB binding. N-His\textsubscript{6}-BauB was immobilized on Ni-NTA resin and loaded with (a) [Fe(Acb)\textsubscript{2}] or (b) [Fe(Fim)], washed with phosphate buffer, and eluted with a competing holo-siderophore. Column elutions were analyzed by LC-MS for [Fe(Acb)\textsubscript{2}] (m/z = 347) and [Fe(Fim)] (m/z = 627) after each step. Extracted ion chromatograms (EIC) are shown for the initially bound holo-siderophore. EICs are representative for two independent trials.

Figure 7. 28: Structural comparison of [Fe(PreAcb)\textsubscript{2}]\textsuperscript{−}, [Fe(Acb)\textsubscript{2}]\textsuperscript{−}, and [Fe(Fim)]\textsuperscript{−} complexes. DFT calculated structures of the monoanionic (a) 1:1 [Fe(Fim)]\textsuperscript{−} and (b) 2:1 cis-[Fe(PreAcb)\textsubscript{2}]\textsuperscript{−} complexes (see Materials and Methods for DFT parameters). (c) Experimentally observed structure of the monoanionic cis-[Fe(Acb)\textsubscript{2}]\textsuperscript{−} complex bound to the siderophore-binding protein BauB (PDB 6MFL). (d) Overlay of all three structures highlighting similarity of geometry and placement of ligands (ox, oxazoline; cat, catecholate; hx, hydroxamate; im, imidazole) around the ferric iron center. (e) Surface view of the siderophore-binding pocket of BauB occupied by cis-[Fe(Acb)\textsubscript{2}]\textsuperscript{−} (PDB 6MFL).

Figure 7. 29: Schematic overview of the PreAcb/Acb and Fim iron acquisition pathways in A. baumannii. The Fim pathway has not been experimentally characterized and is hypothesized based on homology to related pathways in Gram-negative bacteria. Periplasmic BauB is highlighted to show interactions with both PreAcb/Acb and Fim connecting the two pathways through competition for the siderophore-binding protein. Antismash analysis of two deposited genomes of A. baumannii ATCC 19606 turned up acinetobactin and acinetoferrin/baumannoferrin as the only siderophore BGCs present in the genome. No fimsbactin BGCs were detected.

Figure 7. 30: Dose dependent influence of apo-Fim and holo-FimFe on A. baumannii ATCC 1960T growth. Line graphs depict the growth of wild-type A. baumannii ATCC 1960T in M9 minimal medium supplemented with 175 μM 2,2′-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD\textsubscript{600}) at 37 °C as a function of time in the presence of variable siderophore concentrations. Error bars represent standard deviations from the mean for three independent trials.

Figure 7. 31: Dose dependent growth promotion of A. baumannii ATCC 1960T strains by holo-FimFe. Line graphs depict the growth of wild-type (wt), s1-mutant (insertional mutant in basD, deficient in PreAcb/Acb biosynthesis), t6-mutant (insertional mutation in bauA, deficient in
PreAcb/Acb import to periplasm), and t7-mutant (insertional mutation in bauD, deficient in PreAcb/Acb import to cytoplasm) strains of A. baumannii ATCC 19606 in M9 minimal medium supplemented with 175 μM 2,2'-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD<sub>600</sub>) at 37 °C as a function of time in the presence of variable siderophore concentration. Error bars represent standard deviations from the mean for three independent trials.

Figure 7. 33: Nucleotide and amino acid sequence of N-His<sub>6</sub>-BauB in pET28bTEV (TEV-cleavable N-term hexahistidine-tag).<sup>91</sup> Start and stop codons are underlined. The TEV cleavage site is indicated by an arrow↓. The 969-bp bauB gene from Acinetobacter baumannii (Genbank Accession Number AAT52185) was used as the sequence template for subcloning.  

Chapter 8

Figure 8. 1: Reactions catalyzed by glutamine synthetase (GS) and dihydrofolate synthetase (DHFS).<sup>a</sup> a) Mechanism of GS catalyzed reaction. b) Mechanism of DHFS catalyzed reaction (stereochemistry is not shown here).  

Figure 8. 2: Structures of important compounds related to the development of new antifolates. c) also named as “proposed 3-HβL DHFS inhibitor”, d) also named as “3-(p-NH₂-phenyl)-3-HβL”.  

Figure 8. 3: A portion of the folate biosynthetic pathway in bacteria (only the steps we are interested in are shown in this figure).  

Figure 8. 4: a) Mechanism of ATP-dependent TβL inhibition of GS. b) Proposed mechanism of ATP-dependent inhibition of DHFS by our proposed 3-HβL inhibitor. (β-7,8-dihydropteroate also named as “proposed 3-HβL DHFS inhibitor”, “TbL” and “TbL-Pi” also named as “TβL” and “TβL-Pi”).  

Figure 8. 5: SDS-PAGE analysis of recombinant N-His<sub>6</sub>-tagged DHFS (45kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gels were stained with Coomassie blue.)  

Figure 8. 6: SDS-PAGE analysis of recombinant N-His<sub>6</sub>-tagged HPPK (18kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)  

Figure 8. 7: SDS-PAGE analysis of recombinant N-His<sub>6</sub>-tagged DHPS (31kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)  

Figure 8. 8: HPPK, DHPS and DHFS enzyme assay results. a) HPPK/DHPS coupled reaction to form dihydropteroate from natural substrate p-ABA and HMDP. b) HPPK/DHPS coupled reaction to form proposed DHFS inhibitor β-dihydropteroate from synthesized substrate β-p-ABA.
and natural substrate HMDP. c) The formation of dihydrofolate from DHFS catalysed reaction with glutamate and dihydropteroate as substrate. Credit: Brett Virgin-Downey...

Figure 8. 9: Proposed TβL biosynthetic pathway in P. syringae..........................................................................................................................495

Figure 8. 10: Comparison of Lysine and TβL biosynthetic pathways. (Red shows the difference between products and substrates from the enzymatic reaction, dashed line indicates the same steps between two pathways.)..........................................................................................................................496

Figure 8. 11: Natural Fimsbactin A and synthetic Fimsbactin Analogs. Common metal binding moieties are highlighted – catechol (blue) and hydroxymate (red). Each compound contains a similar tetrahedral center denoted with a * Credit: Dr. Tabbetha Bohac ........................................497

Figure 8. 12: Naturally produced siderophore Fimsbactin A and three synthetic analogs. Each analog contains a different sidechain substituent which are negatively (analog 1), neutral (analog 2) and positively (analog 3) charged, respectively, as neutral pH. Credit: Dr. Tabbetha Bohac ........................................498

Figure 8. 13: Influence of apo- and holo-fimsbactin analogs on A. baumannii growth. Bar graphs depict the growth of A. baumannii ATCC 17978 determined by measuring the optical density at 600 nm (OD_{600}) as a function of time in the presence of 15.625 μM concentration of either the apo- or holo- form of each Fimsbactin analog. Error bars represent standard deviations from the mean for three independent trials. ****p < 0.0001. Full growth curves at variable concentration seen in Figure 8.14. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019. ........................................................................................................................................498

Figure 8. 14: Influence of apo- and holo-fimsbactin analogs on A. baumannii growth. Line graphs depict the growth of A. baumannii ATCC 17978 determined by measuring the optical density at 600 nm (OD_{600}) as a function of time in the presence of variable concentration of either the apo- or holo- form of each Fimsbactin analog. Error bars represent standard deviations from the mean for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019. ........................................................................................................................................499

Figure 8. 15: Growth inhibition by each Fimsbactin analog-Ga complex and the growth recovery of variable Fimsbactin analog-Ga (0.975µM or 31.25µM) and 3.9µM FimA-Fe or Acb-Fe. Bar graphs depict the growth of A. baumannii ATCC 17978 determined by measuring the optical density at 600 nm (OD_{600}) as a function of time. Error bars represent standard deviations from the mean for three independent trials. ****p < 0.0001, ns: non-significant. Full growth curves at variable concentration seen in Figure 8.16, 8.17 and 8.18. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019. ........................................................................................................................................500

Figure 8. 16: a) Dose dependent growth inhibition by Fimsbactin analog 1-Ga complex. b) 3.9µM Fim-Fe and variable Fimsbactin analog 1-Ga complex. c) 3.9µM Acb-Fe and variable Fimsbactin analog 1-Ga complex. Line graphs depict the growth of A. baumannii ATCC 17978 determined by measuring the optical density at 600 nm (OD_{600}) as a function of time. Error
Figure 8. 17: a) Dose dependent growth inhibition by Fimsbactin analog 2-Ga complex. b) 3.9µM Fim-Fe and variable Fimsbactin analog 2-Ga complex. c) 3.9µM Acb-Fe and variable Fimsbactin analog 2-Ga complex. Line graphs depict the growth of *A. baumannii* ATCC 17978 determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time. Error bars represent standard deviations from the mean for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.

Figure 8. 18: a) Dose dependent growth inhibition by Fimsbactin analog 3-Ga complex. b) 3.9µM Fim-Fe and variable Fimsbactin analog 3-Ga complex. c) 3.9µM Acb-Fe and variable Fimsbactin analog 3-Ga complex. Line graphs depict the growth of *A. baumannii* ATCC 17978 determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time. Error bars represent standard deviations from the mean for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.

Figure 8. 19: Structures of siderophores from human pathogenic *A. baumannii*. a) Acinetobactin (Acb). b) Fimsbactin A (FimA). c) Fimsbactin F (FimF).

Figure 8. 20: Dose dependent growth promotion of *A. baumannii* ATCC 17978 by a) apo-Fimsbactin F and b) holo-Fimsbactin F. Line graphs depict the growth of *A. baumannii* ATCC 17978 in M9 minimal media supplemented with 175 µM 2,2'-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time in the presence of variable siderophore concentrations. All experiments were performed in triplicate. Data from these plots were used to create the bar graphs in c) influence of apo- and holo-siderophore on *A. baumannii* growth depicting the comparison of OD$_{600}$ values after 30 hours in the presence of variable concentrations of FimF and FimF_Fe. Error bars represent standard deviations from the mean for three independent trials. ****p<0.0001, ***p<0.001. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.

Figure 8. 21: Influence of apo- and holo-siderophore combinations on *A. baumannii* growth. Bar graphs depict the comparison of *A. baumannii* ATCC 17978 growth measured by optical density at 600 nm (OD$_{600}$) values after 30 hours in the presence of variable concentrations of a) FimA and FimF, b) FimAFe and FimF, and c) FimA and FimF_Fe. Error bars represent standard deviations from the mean for two independent trials. ****p<0.0001; **p<0.01; *p<0.1; ns = not significant. Figure 8.22 shows full growth curve data. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.

Figure 8. 22: Influence of apo- and holo-siderophore combinations on the growth of *A. baumannii* ATCC 17978. Line graphs depict the growth of *A. baumannii* ATCC 17978 in M9 minimal media supplemented with 175 µM 2,2'-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time in the presence of variable concentrations of siderophore mixtures. For all graphs, siderophore concentration gradients are provide on the x-axis and y-axis of the checkerboard. The black line graph represents bacterial growth without addition of siderophores. The red line graph represents bacterial growth in the...
presence of variable concentrations of (A) apo-FimA and apo-FimF, (B) holo-FimAFe and apo-FimF, or (C) apo-FimA and holo-FimF2Fe. Error bars represent standard deviations from the mean for two independent trials. Data from these plots were used to create bar graphs shown in Figure 8.21. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019. .......................................................... 509

Figure 8. 23: Influence of apo- and holo-siderophore combinations on A. baumannii growth. Bar graphs depict the comparison of A. baumannii ATCC 17978 growth measured by optical density at 600 nm (OD\textsubscript{600}) values after 30 hours in the presence of variable concentrations of a) Acb and FimF, b) AcbFe and FimF, and c) Acb and FimFFe. Error bars represent standard deviations from the mean for two independent trials. **p<0.01; *p<0.1; ns = not significant. Figure 8.24 shows full growth curve data. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabetha Bohac, 2019. ............................................................... 510

Figure 8. 24: Influence of apo- and holo-siderophore combinations on the growth of A. baumannii ATCC 17978. Line graphs depict the growth of A. baumannii ATCC 17978 in M9 minimal media supplemented with 175 µM 2,2'-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD\textsubscript{600}) as a function of time in the presence of variable concentrations of siderophore mixtures. For all graphs, siderophore concentration gradients are provide on the x-axis and y-axis of the checkerboard. The black line graph represents bacterial growth without addition of siderophores. The red line graph represents bacterial growth in the presence of variable concentrations of (A) apo-Acb and apo-FimF, (B) holo-AcbFe and apo-FimF, or (C) apo-Acb and holo-FimFFe. Error bars represent standard deviations from the mean for two independent trials. Data from these plots were used to create bar graphs shown in Figure 8.23. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabetha Bohac, 2019. ......................................................................................... 513

Figure 8. 25: Hypothesized potential relationship between FimA and FimF. We hypothesize Fimbsbactin can compete with some acinetobactin transport proteins, as we already demonstrated a competition for siderophore binding protein, BauB. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabetha Bohac, 2019. ......................................................................................... 514

Figure 8. 26: SDS-PAGE analysis of recombinant \(N\)-His\(_6\)-tagged FbsM (31kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ........................................................................... 515

Figure 8. 27: SDS-PAGE analysis of recombinant \(N\)-His\(_6\)-tagged BasA (33kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.) ........................................................................... 516

Figure 8. 28: BesA/FbsM test reaction using the module molecule nitrophenyl acetate. ......................... 517

Figure 8. 29: Chemical structures of pre-acinetobactin (PreAcb) and oxidized pre-acinetobactin (OxypreA). ........................................................................................................ 517

xxvi
Figure 8. 30: OxPreAcb₂Ga inhibits *A. baumannii* ATCC17978 growth. Growth curves of *A. baumannii* in M9 minimal medium supplemented with 125 μM 2,2'-dipyridyl (DIP) and gradient concentrations of OxPreAcb₂Ga. Error bars represent s.d. for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019. 518

Figure 8. 31: Growth studies of *A. baumannii* ATCC17978 in the presences of OxPreAcb-Ga (1-Ga) (7.8 μM) and Fims-Fe (31.25 μM, 0.975 μM); OxPreAcb-Ga (1-Ga) (7.8 μM) and (Acb)₂-Fe (31.25 μM, 0.975 μM); OxPreAcb-Ga (1-Ga) (7.8 μM) and OxPreAcb-Fe (31.25 μM, 0.975 μM); OxPreAcb (250 μM, 6.25 μM); OxPreAcb-Ga (250 μM, 7.8 μM). Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019...........................................518

Figure 8. 32: 7.8 μM OxPreAcb-Ga with variable Fims-Fe, Acb-Fe and OxPreAcb-Fe growth studies against *A. baumannii* ATCC17978. Growth in M9 minimal medium supplemented with DIP. Error bars represent s.d. for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019......................................................519

Figure 8. 33: OxPreAcb inhibits growth of *A. baumannii* by disruption of the siderophore pathway, as shown by the ability of OxPreAcb to bind periplasmic siderophore binding protein, BauB. Credit: Dr. Tabbetha Bohac ..................................................................................................................................................520
List of Tables

Chapter 2

Table 2.1: Strains and plasmids used in this work. ................................................................. 76
Table 2.2: Codon optimized glutamine synthetase genes used in this work. ........................... 78
Table 2.3: Apparent Michaelis-Menten parameters for GS from *E. coli*, *S. aureus*, and *H. sapiens* a. ...... 78
Table 2.4: Apparent inactivation parameters for TβL, MetSox, and Glufos against *E. coli* (EC), *S. aureus* (SA), and *H. sapiens* (H) GS a. ........................................................................................................ 78
Table 2.5: Primary protein sequence of GS homologs used in this work. ................................... 79
Table 2.6: Percent sequence identity of GS homologs. ............................................................... 80

Chapter 3

Table 3.1: GS isoforms sequence identity, generated from Cluster Omega. .............................. 141
Table 3.2: Nucleotide and protein sequence of recombinant N-His6-tagged *Mtbg*S use in this work. ... 141
Table 3.3: Results from percent adenylylation determination using total phosphate measurement. ...... 142
Table 3.4: Apparent *Km* values for *Mtbg*S from published studies (see main text for references). ...... 142
Table 3.5: Apparent Michaelis-Menten parameters for recombinant GS from *Mtbg*, *E. coli*, *S. aureus*, and *H. sapiens* a. ........................................................................................................ 143
Table 3.6: Apparent inactivation parameters for TβL, MetSox, and Glufos against *Mtbg*(TB) *E. coli* (EC), *S. aureus* (SA), and *H. sapiens* (H) GS a. ........................................................................................................ 143

Chapter 4

Table 4.1: Tabtoxin BGC from *P. syringae*. ............................................................................. 199
Table 4.2: Comparison of tabtoxin and Cl-tabtoxin BGCs in Pseudomonads and Streptomyces, respectively. a) Lysine biosynthesis pathway enzyme homologue in tabtoxin BGC. (Enzyme function is from Uniprot). b) Lysine biosynthesis pathway enzyme homologue in Cl-tabtoxin BGC. (Enzyme function is from Uniprot) ........................................................................................................ 201
Table 4.3: Comparison of *P. syringae* and Streptomyces BGCs................................................... 202
Table 4.4: Comparative genomic analysis of Cl-tabtoxin BGC with NCBI database. a) Top 14 strain hits for BLASTp analysis of *Streptomyces* 372A, NRRL 8045 and Cl-tabtoxin BGC antismash
result. (Black: contain this enzyme, white: don’t contain this enzyme). b) Name and accession number for these 14 strains

Table 4. 5: Flanking region of Cl-tabtoxin BGC. (red: Cl-tatoxin BGC, blue: interesting flanking region proteins.)

Table 4. 6: CblA DNA and protein sequences used in this study (gray is the difference between original and optimized CblA).

Table 4. 7: NRPS assay with PK/LDH/myokinase coupled reaction. a) Substrate candidates screened. b) Control reaction conditions (6400: TAW3_06400 protein; 6406: TAW3_06406 protein; 6407: TAW3_06407 protein).

Table 4. 8: Primers, nucleic acid sequences, and amino acid sequences of proteins used in this chapter.

Chapter 5

Table 5. 1: Relevant Sequences, Strains, Plasmids, and Primers for Tet(X)_3 [TE_7F_Contig_3].

Table 5. 2: Unabridged aTc analog inhibition of whole cell E. coli expressing tetracycline destructase enzymes. a, aTc analog inhibition of whole cell E. coli expressing Tet(50). b, aTc analog inhibition of whole cell E. coli expressing Tet(X). c, aTc analog inhibition of whole cell E. coli expressing Tet(X)_3.

Chapter 6

Table 6. 1: Tabulated Michaelis-Menten parameters for TetX_T280A and TetX.

Table 6. 2: Tabulated comparison of inhibition parameters for aTC against TetX_T280A and TetX.

Table 6. 3: Tabulated Michaelis-Menten parameters for TetX7_V280A and TetX7.

Table 6. 4: Tabulated comparison of cTC inhibition parameters for TetX7_V280A and TetX7.

Table 6. 5: Tabulated comparison of Michaelis-Menten parameters for Tet50_R280A and Tet50.

Table 6. 6: Tabulated comparison of aTC inhibition parameters for Tet50_R280A and Tet50.

Table 6. 7: Michaelis-Menten parameters (Tet substrate) for the 4 enzymes from the tree in Figure 6.26.

Table 6. 8: Michaelis-Menten parameters for Tet#4.

Table 6. 9: Michaelis-Menten parameters for compounds 2, 5, and 6 (see Figure 6.30) as substrates.

Chapter 7

Table 7. 1: NMR characterization data for purified fimsbactin A from this work compared with previously reported data from the original isolation and characterization of fimsbactin A.
Table 7. 2: Concentrations of test compounds used in combination for *A. baumannii* growth studies. For each combination of compounds 1 and 2, all possible concentrations were tested in duplicate as independent trials using a checkerboard arrangement in a 96-well plate. .......................... 422

**Chapter 8**

Table 8. 1: DNA and protein sequence of codon optimized genes and CabA and CabC proteins. ........... 486
Acknowledgments

My entire Ph.D. career and the successes I’ve had within it could not have been accomplished without my amazing advisor, Dr. Timothy Wencewicz. His creative ideas in science, his positive attitude towards difficult challenges, and his care for his students set a model that I look up to. I am lucky to have Tim as my advisor, and lucky to have all of my excellent colleagues in the Wencewicz lab. You all have been amazing throughout the years and made the toughest times bearable.

I thank my thesis committee Drs. John-Stephen Taylor, Meredith Jackrel, Gautam Dantas, and Jeffrey Henderson for providing useful scientific input to guide my research and dissertation. I thank my collaborators for the opportunities to work on a multitude of exciting projects. I thank the professors and the rest of the staff in the Chemistry Department for all their help.

I thank my parents and my whole family for supporting me to pursue my dream far away from them. It was extremely difficult to be away from my home and family for so long, but I’m thankful for the frequent Skype sessions to maintain my sanity. I thank my friends in St. Louis for making me feel cared and loved. Although COVID has set us apart in 2020, I’ll always remember the great meals and gatherings we shared.

I thank the supporting by the National Institute of Allergy and Infectious Diseases (NIAID-NIH R01-123394) for the tetracycline projects, the National Science Foundation through a CAREER Award (grant 1654611) for the siderophores projects, and the Children’s Discovery Institute at St. Louis Children’s Hospital (grant MI-PD-II-2018-748), the Research xxxi
Corporation for Science Advancement through a Cottrell Scholar Award (CS-24056), the Alfred P. Sloan Foundation through a Sloan Fellowship Award (FG201810935), and the Dreyfus Foundation through a Camille Dreyfus Teacher-Scholar Award (TC-19-079) for the β-lactam projects.

Luting Fang

*Washington University in St. Louis*

*January 2021*
Dedicated to my parents.
ABSTRACT OF THE DISSERTATION

Molecular Strategies to Overcome Antibiotic Resistance

by

Luting Fang

Doctor of Philosophy in Chemistry

Washington University in St. Louis, 2021

Professor John-Stephen Taylor, Chair

Antibiotics have greatly reduced the number of deaths caused by infectious diseases for the last 70 years, but as a result of overuse, antimicrobial resistance emerged. Resistance is the major reason why most traditional antibiotics currently have reduced clinical efficacy. However, there is still a drive to overcome this resistance in a variety of ways. There are two main avenues of research to overcome resistance; to discover and develop new drug candidates acting on new targets and pathways, and to determine the mechanisms of antibiotic resistance in order to combat it through multi-drug therapies, bringing back the effectiveness of antibiotics used in the past. My research uses both approaches to address the antibiotic resistance crisis with an emphasis on leveraging mechanistic enzymology to inspire the development of new antibacterial therapeutic strategies.
Chapter 1. Introduction
1.1 Preface

This chapter was written by Luting Fang (LF) and edited by Timothy Adam Wencwicz (TAW).

1.2 Bacteria

The year 2020 has been a challenging time for everyone in the world because of the COVID-19 pandemic. Millions of people have been infected by this viral disease, hundreds of thousands of people have died from it, and the number is still increasing dramatically. This worldwide pandemic brings people’s attention into public health, especially infectious diseases caused by viral and bacterial pathogens.

As one of the earliest life forms in the Earth, bacteria exist everywhere in this planet: air, water, soil, deep biosphere of the earth’s crust, acidic hot springs, and even radioactive waste. Living organisms cannot survive without the existence of bacteria since only certain bacteria contain the enzyme for the synthesis of vitamin B\textsubscript{12}, which is the DNA synthesis cofactor and involved in amino acid and fatty acid metabolism. In humans, bacteria can be found in the mouth, nose, on the skin, and especially in the gut. The number of bacteria in the human body (3.8x10\textsuperscript{23}) is larger than that of human cells (3x10\textsuperscript{23}). For millions of years, bacteria have affected humanity both in positive and negative ways. The human microbiota is crucial for our health, playing a part in digestion, protecting us from invader bacteria, as well as regulating moods. However, bacterial infection diseases such as tuberculosis and cholera haven been threatening people’s lives.

Gram-positive and Gram-negative bacteria
Bacteria can be classified as Gram-positive and Gram-negative bacteria based on the result of a Gram stain test, and depends on the structure of the cell envelope including the cell wall or peptidoglycan (PG). Gram-positive bacteria contain a thick PG layer in the cell envelope (Figure 1.1a), which retains the purple-colored crystal violet stain following the decolorization washing stage of the Gram staining protocol. On the other hand, Gram-negative bacteria show red or pink color due to a much thinner PG layer that is in the middle of inner membrane and outer membrane (Figure 1.1b), which is absent in Gram-positive bacteria. PG layer is unique to prokaryotes, and is the most important component for both classes of bacteria in the aspects of defining cell shape, protecting the cell under osmotic pressure, and keeping cell integrity during the growth and division phases of the cell cycle. As the name implies, PeptidoGlycan is comprised of Peptide and Glycan strands. The glycan strand is the repeat of two N-acetyl-glucosamine in the order of GlcNAc-MurNAc (Figure 1.2). The peptide strand connected to carbonyl in the MurNAc part of the glycan strand as a classic pentapeptide in the order of L-Ala₁-γ-D-Glu₂-X₃-D-Ala₄-D-Ala₅-COOH. The third residue differs between Gram-positive and Gram-negative bacteria. For Gram-positive bacteria, X₃ is lysine (Figure 1.3a) while for gram-negative bacteria, it is meso-diaminopimelyl (Figure 1.3b). This difference in X₃ makes the peptide cross bridge longer and more flexible for the Gram-positive PG layer (Figure 1.4). The amine group of the third residue in the pentapeptide can crosslink with the carboxyl group in the terminal residue or third residue (Gram-negative only) of another pentapeptide strand catalyzed by transpeptidase to form the macromolecular meshwork of PG layer. In Gram-negative bacteria, lipoproteins can attach to the PG layer peptide strand and reach into the outer membrane for specific functions as transporters, antigens, or adhesins, etc.

Streptomyces
An example of Gram-positive bacteria discussed in this dissertation is *Streptomyces*, which belongs to the *Actinobacteria* phylum according to scientific classification. *Streptomyces* is a big family of bacteria predominantly found in decaying vegetation and soil, and is famous for its “earthy” smell. A unique feature of *Streptomyces* as a soil bacterium is the ability to produce spores\(^6\) ([Figure 1.5](#)), which is a highly dormant form of life produced in response to extreme environments. The ability for *Streptomyces* to produce a wide variety of useful secondary metabolites, including antibiotics, also makes it unique and popular in drug discovery and industry applications. Over two-thirds of the natural antibiotics used in clinics including antibacterial, antifungal, antiviral, antihypertensive, immune suppressant, and antitumor compounds, are produced by *Streptomyces*\(^7,8\). Recently, several new secondary metabolites from *Streptomyces* have been discovered. For example, streptovaricin is effective against methicillin-resistant *Staphylococcus aureus* (MRSA), and metatricycloene contains a novel structure different from known drugs. The secondary metabolites found in *Streptomyces* are just the tip of the iceberg when looking at all natural products, both discovered and undiscovered. With more in-depth research on *Streptomyces* through genome mining, it was discovered that there are dozens of gene clusters encoding secondary metabolites in almost every *Streptomyces* species, but most of the compounds have not yet been unidentified\(^9\).

Rapid advancements in genomics (high-throughput genome sequencing), bioinformatics, and analytical tools (mass spectrometry and NMR) have made genome mining a popular approach for natural product drug discovery\(^10\). Genome sequencing and molecular biology techniques with *Streptomyces* can be challenging due to the high GC content of the genomic DNA. The first genome map of a *Streptomyces* strain was completed in 2002 using the Sanger sequencing approach\(^11\). The development of next and 3\(^{rd}\) generation sequencing technologies
expedited sequencing times to as short as 3 hours to finish the whole genome sequencing at a much lower price (~$2,000) compared to Sanger sequencing (> $35,000) and with impressive sequencing accuracy\textsuperscript{12}. Modern genome sequencing techniques provide the sequence of every single gene in the whole genome of a strain, which can be compared with all of the known proteins in the public databases. Since there are a large number of proteins that are involved in natural product biosynthesis deposited in the database, it is easy to identify homologous metabolic pathways within a given strain\textsuperscript{13}. Along with hardware and software improvements in genome sequencing technologies came the expansion and refinement of DNA sequence databases, computing algorithms, and web tools also accelerate the application of genome mining. The most popular web tool used to mine microbial genomes for natural products is “antibiotics and Secondary Metabolite Analysis SHell” (antiSMASH)\textsuperscript{14}, which was originally issued in 2011. This tool provides the predicted function of genes in the genome of a certain strain, and identifies gene clusters for natural products’ biosynthesis based on specific algorithms and sequence analysis compared with proteins deposited in the database\textsuperscript{15}.

People have discovered new drugs and novel natural product scaffolds using the genome mining approach. For example, Hornung has identified strains capable of producing halogenases, and discovered drugs with novel structures based on nuclear magnetic resonance spectroscopy (NMR) analysis\textsuperscript{16}. McAlpine used genome mining technology to discover new antibiotics against a variety of human pathogens\textsuperscript{17}. As one of the most suitable class for natural products’ discovery, \textit{Streptomyces} is popular in the genome mining approach\textsuperscript{18}. In the fourth chapter of this dissertation, the genome mining of a new strain of \textit{Streptomyces} is discussed, and it was discovered that the natural product from this strain has the potential to become an anti-tuberculosis drug.
Pseudomonas

An example of natural product discovery for Gram-negative bacteria is discussed in this dissertation for *Pseudomonas Syringae*; a plant pathogen that can be found in almost all plant species on land\(^\text{19}\). *P. Syringae* pathovars are unique for the ability to produce diverse phytotoxic compounds such as coronatine, syringomycin, and tabtoxin that cause chlorosis and plant death\(^\text{20}\). Coronatine is able to stimulate the open of the stomata as well as interpreting the salicylic acid-dependent defense in plant host cells, resulting in promoted bacterial entry into the host.\(^\text{21-23}\) Syringomycin is a lipopeptide that can influence the integrity of the cell membranes and lower cell surface tension.\(^\text{24, 25}\) Tabtoxin is an antimetabolite toxin that interrupts nitrogen metabolism. The ability to produce antimetabolites is unique for *P. Syringae* in the field of plant pathogens. Tabtoxin interrupts the nitrogen metabolism by inhibiting the glutamine synthetase enzyme that catalyzes the synthesis of glutamine in plant cells. *P. Syringae* pathovars also produce other antimetabolites such as mangotoxin and phaseolotoxin that cause arginine deficiency. The lack of nitrogen makes plants vulnerable to the infection with *P. Syringae*.\(^\text{26}\) Specifically, the decrease of amino acid level can influence plant protein synthesis and homeostasis to alter normal plant cell function, including defense against pathogens.\(^\text{27}\)

Genes encoding enzymes involved in the biosynthesis of antimetabolite toxins are usually integrated into specific sites in the genome of *P. Syringae* as coregulated operons also referred to as biosynthetic gene clusters (BGCs).\(^\text{28}\) Studies have shown that the evolution of these genes takes place through horizontal gene transfer and homologous recombination events.\(^\text{29}\) The BGC encoding tabtoxin production in *P. syringae* will be discussed in Chapter 4, along with the BGC encoding biosynthetic enzymes for a related but novel variant produced by *Streptomyces*.  

6
**Mycobacterium**

Unlike *Streptomyces* and *Pseudomonas* that have clear classifications, people debated about the classification of *Mycobacterium* as either Gram-positive or Gram-negative bacteria since it does not respond in standard ways to the traditional Gram staining protocol due to its unique cell envelope. Although *Mycobacterium* is technically classified as a Gram-positive in the genus of Actinobacteria, some people believe that it should belong to Gram-negative bacteria based on genome-based analysis and the presence of outer membrane in the cell wall. *Mycobacterium tuberculosis* is the causative pathogen of the tuberculosis (TB) disease, which has been threatening people’s life for 4,000 years, and still is one of the top causes of death in the world. One challenge for anti-TB drug development is that *M. tuberculosis* contains a waxy cell wall, which is impenetrable to antibiotics. Some successful anti-TB drugs target on cell wall biosynthesis such as isoniazid (INH) and ethionamide (ETH). The structure of *M. tuberculosis* cell wall (Figure 1.6) makes it distinguishable among the prokaryotes. Other than the thick PG layer that shares with all of the gram-positive bacteria, *M. tuberculosis* also contains extra components to make the cell wall strong and impenetrable, such as arabinogalactan, mycolic acids, and capsule-like material. Another unique component in *M. tuberculosis* cell wall is poly-L-glutamate/glutamine (PLG) (Figure 1.6, shown in yellow), which is only present in the pathogenic *Mycobacterium*. Studies showed that the PLG layer is crucial for cell wall strength against physical stress like sonication, as well as chemical stress such as lysozyme and SDS. It is also reported the presence of PLG is related to the extracellular glutamine synthetase. The biochemical property of glutamine synthetase and its influence on cell wall synthesis is further discussed in Chapter 3.
1.3 Antibiotics

The application of antibiotics can be dated back to more than 2000 years ago when ancient Egyptians applied moldy bread on infected wounds\textsuperscript{37}. However, the secret behind the ancient medicinal approach was not revealed until 1928. Alexander Fleming was the first one to notice and report the ability for molds (later identified as \textit{Penicillium rubens}) to produce something that can inhibit neighboring bacterial (in that case it was \textit{Staphylococcus aureus}) growth\textsuperscript{38}. This unexpected discovery opened up the natural antibiotic world, the first member of which was named penicillin by its discoverer Alexander Fleming. Since then, scientists from labs in universities as well as pharmaceutical companies have been working on the purification and optimizing yield of these seemingly magical compounds. The application of penicillin in the army saved a tremendous amount of lives during World War II and also marked the start of the “golden era” for antibiotics.

\textbf{Antibiotics development}

Since the success of penicillin, people have been focusing on the discovery of new antibiotics. 1940-1962 was the “golden era” of antibiotics (\textbf{Figure 1.7}). Most of the antibiotics we are using nowadays were discovered and approved during this period of time such as streptomycin, vancomycin, ampicillin, and tetracycline. The antibiotics were initially isolated from natural microorganisms. People collected soil samples from every corner of the earth in an attempt to discover new antibiotics. With the structural elucidation and structure-activity relation (SAR) development, analogues of natural antibiotics were synthesized in order to improve the efficiency, and those analogues are called semisynthetic antibiotics. People also discovered synthetic antibiotics from dyes such as the sulfonamides that can inhibit bacterial growth\textsuperscript{39}. 


Antibiotics changed the world by decreasing the death rate from infectious disease by 80% from 1900 to 1980\textsuperscript{40}, and lengthening human life expectancy by 23 years from 1910 to 2010\textsuperscript{41}.

The birth of any new antibiotic comes from the discovery and development of research laboratories. Once a candidate is selected from a large library, it is moved forward to pre-clinical studies to test toxicity and conduct \textit{in vitro} and \textit{in vivo} studies. Further safety and efficiency tests will be conducted on healthy people and patients in the traditional ‘phases’ of clinical research, which may take up to 6 years. Based on this information, the FDA will decide whether to approve the new drug or not.\textsuperscript{42} Generally, it can take up to one billion dollars and >10 years for a new antibiotic from birth to market.\textsuperscript{43}

The exhaustive discovery of natural products from any possible source on the earth and the emergence of resistance caused by excessive use of antibiotics limited the development of new antibiotics after 1962. Between streptogramins in 1962 and linezolid in 2000\textsuperscript{44}, there were no novel antibiotics approved by FDA in almost 40 years, which is called the “innovation gap” (\textbf{Figure 1.7}). Since the latest class of antibiotics, lipopeptide, discovered in 1987, there was no novel class of antibiotic discovered. The time between 1987 and now is so-called “discovery void”. Besides the resistance problem, innovation limitations, the development of vaccines and new therapies such as immunotherapy made antibiotic not as a hot topic as in the 1940s. However, people are still trying to discover new antibiotics through new methods such as genome mining. Government is also helping the antibiotic discovery by legislating for fast-tracking the approval process along with other incentives such as tax relief. With these efforts, there were 23 new approvals from 2000 to 2017. Despite the slow development, antibiotics are still the core and foundation of modern medicine.
Antibiotic mechanism of action

Most of the antibiotics we use today are broad-spectrum, effective against both Gram-positive and Gram-negative bacteria, without the need to identify the causative pathogens. The discovery of broad-spectrum antibiotics requires targets that are present in all kinds of pathogens. The most common targets used by clinical antibiotics are involved in cell wall biosynthesis, cell membrane integrity, protein biosynthesis, DNA/RNA metabolism, or folic acid biosynthesis (Figure 1.8).

β-lactam class antibiotics (discussed in Chapter 2), such as penicillin, and glycopeptide antibiotics, such as vancomycin, can interfere with the cross-linking step in cell wall biosynthesis, weaken the PG layer in the cell wall, and cause cells to undergo autolysis in a futile effort to repair damaged cell wall with depleted nutrient reserves. The integrity of the bacterial membrane is crucial to prevent small molecules or ions from leaking out of the cell, but lipopeptide antibiotics, such as daptomycin, can kill bacteria by destroying the integrity of the cell. The inhibition of protein biosynthesis can be caused by multiple steps. These steps include activation of amino acids, transportation of amino acid building blocks to the ribosome by tRNAs, condensation of peptide bonds, elongation of the polypeptide chain, and termination steps. Most antibiotics that inhibit protein biosynthesis target bacterial ribosomes; the cell’s protein factory. Examples of these antibiotics are tetracyclines (discussed in Chapter 5 and 6), macrolides, chloramphenicol, oxazolidinones, and aminoglycoside antibiotics (such as kanamycin). Without a sufficient pool of functional and properly folded proteins, bacteria will have trouble in growth, division, as well as survival and general cell maintenance. As with protein synthesis inhibitors, antibiotics that interrupt DNA replication or transcription into RNA can halt bacterial replication and even cause bacterial cell death. Polyketide antibiotics such as
rifamycin and synthetic antibiotic fluoroquinolones are those interfering with DNA/RNA metabolism. Folate biosynthesis is crucial in DNA biosynthesis and unique in bacteria, which can avoid the off-target effect on human. The synthetic sulfonamide antibiotics, inhibitors of dihydropteroate synthase, and trimethoprim, an inhibitor of dihydrofolate reductase, are two of the best known and clinically useful folate pathway inhibitors. A novel inhibitor of folate biosynthesis under development in our lab is discussed in Chapter 8.

1.4 Multidrug resistant bacteria

One reason for the end of antibiotics so called “golden era” is the emergence of drug resistance, which is caused at least in part by the excessive and improper use of antibiotics in hospitals and agricultural settings. The Centers for Disease Control and Prevention (CDC) reports that there are more than 2.8 million new cases of antibiotic resistance in the United States, and more than 35,000 deaths each year. It is estimated that by 2050, 10 million people will die from antibiotic resistance, even more than cancer. One thing that makes this situation worse is multidrug resistance. Multidrug resistance (MDR) bacteria are bacteria that are resistant to multiple antibiotics. This issue can be caused by either the bacterium utilizes a single mechanism for the resistance of multiple antibiotics such as efflux pumps, or the bacterium contains multiple resistance genes, each of which is responsible for resistance of one antibiotic. ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter) are the most common causes of nosocomial infections, and all of these species are MDRs. ESKAPE pathogens are notorious for infections in various human organs, frequent antibiotic resistance and lack of alternative antibiotics. The uncontrollable increase in MDR and the stagnant development of
antibiotics will soon bring our world back to pre-antibiotic era, when simple bacterial infections may lead to death. Therefore, there is a great need to study the mechanism of antibiotic resistance and find a solution to the MDR problem.

**Resistance mechanism**

The well-known biochemical mechanisms of antibiotic resistance include expression of efflux pumps, target protection and modification, and enzymatic antibiotic inactivation (Figure 1.9). An efflux pump is a membrane protein that can export antibiotics to the outside of the cell before reaching the target and maintain a low intracellular antibiotic concentration. Most efflux pumps are multidrug transporters and are one of the biggest causes of MDR problems. Target modification is usually caused by a genetic mutation on the bacterial chromosome in the gene encoding the antibiotic target protein. Since there is high specificity between antibiotics and protein targets, a subtle change of target will have a huge impact on antibiotic binding. In addition to target mutations, protection of target proteins through protein-protein interaction as and enzymatic modification of target proteins through phosphorylation, adenylylation, or other reversible group additions are possible. Some examples of these various target protection mechanisms include expression of penicillin binding protein (PBP) variants or point mutations in PBPs that have reduced affinity for beta-lactam antibiotics, alteration of cell wall precursors for vancomycin resistance, and ribosomal protector for tetracycline resistance. Enzymatic antibiotic inactivation is the most straightforward resistant mechanism by directly and irreversibly destroying the antibiotic drug scaffold. Antibiotic inactivation is usually carried out by enzymes that impart resistance to neighboring microbes in addition to the primary enzyme producer. β-lactamases are the most famous class of antibiotic-inactivating enzymes, which contain orthologs that can hydrolyze almost all known β-lactam antibiotics, such as penicillin. Aminoglycoside
modifying enzymes and chloramphenicol-acetyl-transferases are also members of this mechanism, along with relatively newly discovered tetracycline destructases, which will be discussed in Chapter 5 and 6.

1.5 Application of adjuvants to overcome drug resistance

Antibiotic adjuvants refer to molecules with low inherent antimicrobial activity, but are capable to increase the efficacy of traditional antibiotics by mitigating resistance mechanisms. The most successful clinical adjuvant antibiotic application is Augmentin®, which is the combination of amoxicillin and clavulanic acid. Amoxicillin (Figure 1.10 a) is a potent β-lactam class antibiotic that can inhibit cell wall biosynthesis by covalent inactivation of PBPs. However, the emergence of inducible β-lactamases causes widespread bacterial resistance against β-lactam antibiotics including amoxicillin. β-lactamases are able to hydrolyze the amoxicillin β-lactam ring to make this antibiotic inactive (Figure 1.11). Fortunately, as a β-lactamase inhibitor, clavulanic acid (Figure 1.10 b) can rescue amoxicillin from enzymatic inactivation through molecular self-sacrifice (β-lactamase reacts with clavulanic acid and enters an inhibited state unable to hydrolyze amoxicillin). In this case, clavulanic acid is used as adjuvant since it has low inherent antimicrobial activity by itself. The combined application of amoxicillin and clavulanic acid made Augmentin® the best-selling drug of 2001. With the success of Augmentin, people have put effort in antibiotic adjuvants development to overcome antibiotic resistance issue. Currently, there are three classes of antibiotic adjuvants including β-lactamase’s inhibitors, efflux pumps’ inhibitors, and outer membrane permeabilizers.

In this dissertation, we will discuss another example of an adjuvant, anhydrotetracycline, which is relatively new, and not yet used in clinic settings. As one of the most popular
antibiotics, tetracyclines are suffering from an increase in resistance occurrence. People have found all three mechanisms for this antibiotic resistance: efflux, target protection, and enzymatic inactivation. Amongst these mechanisms, enzymatic inactivation is the newest found, and is the most effective resistance mechanism. Tetracycline destructases are reported to be able to oxidize tetracycline class antibiotics to make them inactive\textsuperscript{52}. Our group discovered that anhydrotetracycline has the ability to inhibit tetracycline destructases and thus rescue tetracycline from enzymatic inactivation\textsuperscript{53}. Anhydrotetracycline is the biosynthetic precursor of tetracycline natural products, yet it lacks potent inherent antibacterial activity. Our group aims to synthesize improved analogues of anhydrotetracycline to optimize this adjuvant’s efficiency of saving tetracyclines from emerging tetracycline destructases\textsuperscript{54}.

1.6 Discovery of new antibiotic drug targets

The current pool of FDA-approved antibiotics acts on a small subset of essential and clinically viable cellular targets. To better manage resistance it is desirable to expand the pool of druggable antibiotic targets with clinical promise. One good example for this strategy is β-lactam antibiotics, which contain a unique β-lactam ring (Figure 1.12 a). Penicillin (Figure 1.12 b) is the superstar of this family of antibiotics. As mentioned earlier, the discovery and application of penicillin has saved a tremendous amount of lives. The target for the majority of β-lactam antibiotics is transpeptidase enzyme, which is involved in cell wall biosynthesis by crosslinking two peptide chains in the PG layer (Figure 1.13 a). Because of the structural similarity to the C-terminal moiety of PG, D-Ala-D-Ala (Figure 1.12 c), which is the natural substrate of transpeptidase, penicillin can be misrecognized and irreversible bind to transpeptidase on serine residue (Figure 1.13 b), resulting in the inhibition of cell wall biosynthesis.\textsuperscript{55} A positively
charged pocket in the active site of transpeptidase is crucial for locating the negatively-charged moiety of the substrate as well as penicillin, and thus position the β-lactam ring close to the serine residue in the active site. However, the resistance against penicillin as well as almost all other β-lactam family antibiotics have become a problem. β-lactamases are enzymes found in bacteria resistant to β-lactam antibiotics. Same as transpeptidase, β-lactamase also has positive charged pocket to locate the negatively charged moiety of β-lactam. Almost all of the β-lactams can be recognized and located to the active site of β-lactamases since the carboxylate group is required for the structural mimic to D-Ala-D-Ala. The water molecule in the active site can then protonate the nitrogen in the β-lactam ring to open the ring via hydrolysis (Figure 1.13 c).56

The discovery of tabtoxin (Figure 1.12 d) brings hope for the renaissance of the β-lactam ring as an antibiotic pharmacophore by acting on a unique drug target. Unlike the majority of β-lactams, tabtoxin does not inhibit transpeptidase domains of bacterial PBPs due in part to the lack of an ionizable anionic group attached to the β-lactam nitrogen. The absence of this negatively charged moiety also contributes the inability of β-lactamase to inactive tabtoxin (Figure 1.13 d). A molecular docking study showed that the serine residue, which is used to initially hydrolyze the β-lactam ring lacks the proper orientation for nucleophilic attack on the β-lactam carbonyl of tabtoxin. This study also shows tabtoxin can evade β-lactamase in vitro as well as in whole cell assays.57 Instead of targeting PBPs, tabtoxin inhibits the enzyme glutamine synthetase, which is a crucial enzyme in nitrogen metabolism for all bacteria. Based on the importance of glutamine synthetase in bacterial survival, the inhibitor of this enzyme has great potential to be developed into new antibiotics and expands the clinical utility of β-lactams. The study of tabtoxin inhibition of glutamine synthetase and potential as an antibiotic molecule is further discussed in Chapter 2.
1.7 Anti-virulence strategies to mitigate drug resistance issue

Antibiotics can be classified as bactericidal and bacteriostatic based on killing bacterial cells (bactericidal) or inhibiting bacterial proliferation and growth (bacteriostatic). Removal of antibiotic-sensitive bacteria from a given microenvironment, including an infection environment, can benefit the proliferation of resistant bacteria due to the availability of more space, more nutrients, and less growth pressure. Antibiotic resistance exists in nature, and without human interference can establish a natural balance, allowing for a stable environment for many types of bacteria. However, it is the wide application and large scale deployment of antibiotics that selectively amplifies the ratio of resistant to sensitive bacteria, and makes resistance such a big problem. Anti-virulence strategy, on the other hand, is a way to reduce the selection pressure by keeping bacterial growth pathway intact for sensitive bacteria while selectively blocking pathways in pathogenic bacteria that enable and drive host infection.

Anti-virulence agents

Anti-virulence agents are molecules that disarm virulence factors used by pathogenic bacteria to infect host tissues. Virulence factors are pathogen’s weapons to cause damage to the host and worsen infection. Virulence factors can be small molecules or proteins involved in pathogenesis such as invasion or adhesion, escape from the host immune system, and damage host tissues by toxic molecules. Without functional virulence factors, pathogenic bacteria can still survive and proliferate, but cannot cause damage or disease to the host. The application of anti-virulence to treat diseases can be dated back to 1893\textsuperscript{58}, but due to a limited understanding of virulence factors for specific pathogens, mechanism of pathogens causing disease, and the
discovery of highly efficient antibiotics, this strategy was not brought into public attention or considered as a clinically viable therapeutic strategy until 2007 when antibiotic resistance became a tricky issue.59

**Siderophores**

Siderophores, iron-chelating small molecules, are one example of a bacterial virulence factor.60 Since the first discovery of mycobactin, a growth promoter for Mycobacterium johnei, in the 1950s,61 there are more than 500 siderophores identified till 2010.62 Although diverse in structure, all siderophores are small molecules with negatively charged atoms presented in an orientation to favorable chelate ferric iron ions in multidentate fashion. Iron is an essential element for electron transport and DNA replication in the human body.63 However, as an innate immunity against bacterial infection, most of the iron ions in the human body are either sequestered intracellularly such as hemoglobin in erythrocytes, or bound within high-affinity proteins with, such as transferrin for the case of extracellular iron ions.64 In fact, there is a very low concentration equivalent to ~$10^{-24}$ M of free iron in the human body available for use in soluble form too low to sustain bacterial growth.65 Just like humans, bacteria also require iron to survive, maintaining an intracellular concentration of iron of as much as $10^{-5}$-$10^{-7}$M.65 With limited available free iron in host environment, bacteria produce siderophores to sequester iron from the host environment in a form suitable for selective uptake into bacterial cells to meet the nutritional requirements for survival within a host (Figure 1.14). Different bacteria can produce different siderophores to tailor the food supply and gain a competitive growth advantage. In Chapter 7, we are going to discuss the siderophores produced in A. baumannii, a pathogenic Gram-negative bacterium. By studying the pathways for iron transportation in A. baumannii, we can develop new strategies for antibiotic discovery that block virulence by inhibiting
siderophore-mediated iron uptake at the level of multiple transport proteins. Collectively, the research presented in this dissertation represents contemporary approaches to developing molecule-based solutions to the antibiotic resistance crisis.
1.8 Figures and Tables

a.

[Image of Gram-positive bacteria cell wall]

b.

[Image of Gram-negative bacteria cell wall]

**Figure 1.1**: Cell wall of (a) gram-positive, and (b) gram-negative bacteria.
Figure 1.2: Structure of glycan strand (GlcNAc-MurNAc) in bacterial peptidoglycan (PG).
Figure 1. 3: Peptidyl strand of peptidoglycan in (a) Gram-positive and (b) Gram-negative bacteria. Red color shows the portion that is different between two structures.
Figure 1.4: Peptide cross-bridge of (a) Gram-positive and (b) Gram-negative bacteria PG strands.
Figure 1. 5: Spore formation cycle in Streptomyces.⁶
Figure 1. 6: Structure of *M. tuberculosis* cell wall. PLG layer is shown in yellow color.

Figure 1. 7: Timeline for discovery of new antibiotic classes in clinical use.
Figure 1. 8: Five classes of bacterial machinery comprise the targets of the major classes of antibiotics. (a) cell wall biosynthesis, (b) membrane function, (c) protein biosynthesis, (d) macromolecular synthesis (DNA and RNA), (e) folate biosynthetic pathway (interdicting the supply of deoxythymidylate for DNA synthesis)
Figure 1.9: Antibiotic resistance mechanisms in bacteria. (a) exclusion by antibiotic efflux pumps, (b) modification of antibiotic targets, (c) inactivating modifications of the antibiotic.

Figure 1.10: Structure of (a) amoxicillin antibiotic, (b) clavulanic acid antibiotic adjuvant. The combination, marketed as Augmentin®, is used to overcome resistance by β-lactamases.
Figure 1.11: Mechanism of β-lactamase inactivating amoxicillin.

Figure 1.12: (a) Structure of β-lactam four membered ring, (b) structure of penicillin antibiotic, (c) structure of D-Ala-D-Ala (terminus of peptidoglycan, substrate of transpeptidase), (d) structure of tabtoxinine-β-lactam (TβL).
Figure 1.13: (a) mechanism of transpeptidase catalyzing peptidoglycan crosslink. (R: peptidoglycan) (b) mechanism of penicillin inhibition of transpeptidase. (c) β-lactamase inactivation of penicillin. (d) tabtoxin stealth from β-lactamase. (Blue: positive charged pocket in β-lactamase active site, red: negatively charged portion in the molecules.)
Figure 1. 14: General schematic for siderophore-mediated iron transportation into bacteria with the help of Siderophores. The efflux pump and transporter are not intended to represent specific systems; rather, the intent is to highlight the need to excrete metal-free siderophores and import siderophore ferric iron complexes.
1.9 References


asparagine synthetase in tomato leaves infected by the bacterial pathogen Pseudomonas syringae. *Plant Cell Physiol* 2004, 45 (6), 770-80.


36. Chandra, H.; Basir, S. F.; Gupta, M.; Banerjee, N., Glutamine synthetase encoded by glnA-1 is necessary for cell wall resistance and pathogenicity of Mycobacterium bovis. *Microbiology (Reading)* 2010, 156 (Pt 12), 3669-3677.


Chapter 2: Mechanistic Basis for ATP-Dependent Inhibition of Glutamine Synthetase by Tabtoxinine-β-Lactam
2.1 Preface

This chapter was adapted from an article of the same title (Patrick, G. J.; Fang, L.; Schaefer, J.; Singh, S.; Bowman, G. R.; Wencewicz, T. A. “Mechanistic basis for ATP-dependent inhibition of glutamine synthetase by tabtoxinine-β-lactam.” *Biochemistry* **2018**, *57*, 117-135) with permission from ACS Publications. Funding for this work was provided by Washington University in St. Louis.

2.2 Abstract

Tabtoxinine-β-lactam (TβL), also known as wildfire toxin, is a time- and ATP-dependent inhibitor of glutamine synthetase produced by plant pathogenic strains of *Pseudomonas syringae*. Here we demonstrate that recombinant glutamine synthetase from *Escherichia coli* phosphorylates the C3-hydroxyl group of the TβL 3-(S)-hydroxy-β-lactam (3-HβL) warhead. Phosphorylation of TβL generates a stable, noncovalent enzyme-ADP-inhibitor complex that resembles the glutamine synthetase tetrahedral transition state. The TβL β-lactam ring remains intact during enzyme inhibition, making TβL mechanistically distinct from traditional β-lactam antibiotics such as penicillin. Our findings could enable the design of new 3-HβL transition state inhibitors targeting enzymes in the ATP-dependent carboxylate-amine ligase superfamily with broad therapeutic potential in many disease areas.

2.3 Introduction

β-Lactams make up the most successful class of clinical antibiotics ever discovered.\(^1\) Canonical β-lactam antibiotics, such as penicillin G (*Figure 2.1*), are covalent inhibitors of
enzymes in the serine hydrolase superfamily, including bacterial transpeptidases (TPases) involved in cell wall assembly. The mechanistic basis for TPase inhibition is acylation of the active site, catalytic serine residue. Antibacterial β-lactams are structural mimics of the C-terminal d-Ala-d-Ala peptidyl stem of bacterial peptidoglycan. Formation of the TPase-β-lactam collision complex orients the active site serine residue for nucleophilic attack on the electrophilic β-lactam carbonyl, leading to formation of an unstable tetrahedral intermediate. Breakdown of the tetrahedral intermediate opens the β-lactam ring and generates a stable, covalent acyl enzyme adduct. Without functional TPases, bacteria are left unable to cross-link newly formed cell wall polysaccharides, resulting in the recruitment of lytic transglycosylases and initiation of a futile cycle ending in autolysis. To fight back, resistant bacteria express β-lactamases that exploit the hydrolytic liability of β-lactams. All major classes of β-lactam antibiotics, including penicillins, carbapenems, cephalosporins, and monobactams, share this common reactivity, driven by the inherent ring strain (~25 kcal/mol) of the β-lactam warhead, that is the source of both clinical success and failure.

Tabtoxinine-β-lactam (TβL) is a monocyclic 3-hydroxy-β-lactam (3-HβL) produced by plant pathogenic strains of Pseudomonas syringae and some soil Streptomyces (Figure 2.1). Unlike canonical β-lactam antibiotics, TβL does not inhibit bacterial TPases but instead is a potent, mechanism-based inhibitor of glutamine synthetase (GS). P. syringae pathovars, including tabaci, coronofaciens, phaseolicola, tomsato, and garcae, excrete the active phytotoxin TβL as a 1-Thr dipeptide prodrug known as tabtoxin (TβL-Thr). Unlike traditional β-lactams, both TβL and TβL-Thr are stable toward all major classes of β-lactamase enzymes. Bacterial resistance to TβL and TβL-Thr can be achieved through acquisition of the
The *ttr* gene, which encodes a cytoplasmic acetyltransferase that deactivates TβL through regioselective acetylation of the α-amino group using acetyl-CoA as the acyl donor.\textsuperscript{11,15} TβL and TβL-Thr undergo spontaneous rearrangement to the more thermodynamically stable δ-lactam isomers, tabtoxinine-δ-lactam (TδL) and tabtoxin-δ-lactam (TδL-Thr), respectively.\textsuperscript{11} The δ-lactam isomers and hydrolyzed β-amino acids are biologically inactive, indicating that the 3-HβL warhead of TβL plays a central role in GS inhibition.\textsuperscript{14} The mechanistic basis for GS inhibition by TβL is unknown. Given the clear differences in enzyme structure and function of bacterial TPases (serine hydrolase family\textsuperscript{16}) and GS (ATP-dependent amine-carboxylate ligase family\textsuperscript{17}), TβL represents a unique opportunity to understand how nature adapted the β-lactam ring to inhibit enzymes other than serine hydrolases.\textsuperscript{18}

Glutamine synthetase is critical for nitrogen metabolism in all forms of life.\textsuperscript{19} GS catalyzes the ATP-dependent conversion of L-Glu and NH\textsubscript{3} to L-Gln (Figure 2.2A). Bacterial GSs are functional as dodecamers formed by the face-to-face assembly of two hexameric rings.\textsuperscript{20} Bacterial GSs contain 12 active sites formed between each monomer with binding sites for two divalent cations, typically Mg\textsuperscript{2+} or Mn\textsuperscript{2+}.\textsuperscript{21} Active site residues are conserved among GS isoforms from different species, but the quaternary structure can vary. For example, human GSs are typically functional as decamers composed of two pentameric rings in a face-to-face arrangement.\textsuperscript{22} The binding of substrates, formation of intermediates, and release of products during the GS biosynthetic reaction are ordered.\textsuperscript{23,24} First, ATP binds in a site adjacent to the cation-binding sites. ATP binding increases the affinity for L-Glu binding, which takes place adjacent to the ATP site. L-Glu binding causes the active site to close via movement of a mobile loop with subsequent formation of the ammonium-binding site.\textsuperscript{25} Closing of the active site
prevents water entry and promotes the transfer of a phosphoryl from the \( \gamma \)-phosphate group of ATP to the \( \gamma \)-carboxylate of L-Glu, producing the reactive \( \gamma \)-glutamyl acyl phosphate intermediate (L-Glu-P\(_i\)) and ADP that stays bound through the remainder of the biosynthetic reaction. The Asp50 residue deprotonates the bound ammonium, and ammonia attacks the electrophilic \( \delta \)-carbonyl carbon of the \( \gamma \)-glutamyl acyl phosphate to form a GS-stabilized tetrahedral intermediate that resembles the late transition states for nucleophilic acyl substitution reactions.\(^{26}\) Breakdown of the tetrahedral intermediate releases P\(_i\) and generates L-Gln.\(^{27}\)

Inhibition of GS leads to accumulation of L-Glu and NH\(_3\) and blocks downstream production of amino acids, cofactors, nucleotides, and amino sugars.\(^{28,29}\) GS inhibitors have been explored as herbicides,\(^{30}\) antimicrobial agents,\(^{31}\) and treatments for neurological diseases.\(^{32}\) The two main types of GS inhibitors include molecules that bind competitively to the ATP- or L-Glu-binding site.\(^{31}\) Achieving selectivity for GS isoforms is challenging for both inhibitor types. ATP-competitive inhibitors incorporate hydrophobic heterocycles such as purines, aminomidazopyridines, and imidazoles. L-Glu-competitive inhibitors, including T\(\beta\)L, are polar \( \alpha \)-amino acids that structurally mimic L-Glu. Methionine sulfoximine (MetSox) and glufosinate (Glufos) are L-Glu-competitive inhibitors that have been mechanistically and structurally characterized as inhibitors of plant, animal, and bacterial GS. Glufos is sold commercially by BayerCropSciences as an herbicide that is paired with resistant transgenic crops under the trade name LibertyLink.\(^{30}\) GS inhibition by both MetSox and Glufos requires ATP and C2-(S) stereochemistry matching that of the L-Glu substrate.\(^{20,33,34}\) GS inhibition by MetSox and Glufos is time-dependent, irreversible, and noncovalent. MetSox is phosphorylated on the sulfoximine nitrogen to produce MetSox-P\(_i\) that stabilizes GS in a closed, inactive conformation with bound ADP.\(^{22,27,35}\) Similarly, Glufos is phosphorylated on the phosphinate oxygen to produce Glufos-P\(_i\)
that stabilizes GS in a closed, inactive conformation with bound ADP (Figure 2.2B).\textsuperscript{20} MetSox-P\textsubscript{i} and Glufos-P\textsubscript{i} are both tetrahedral transition state analogues.\textsuperscript{36} The phosphorylated inhibitors resemble the predicted structures of the late GS transition state leading to formation of the tetrahedral intermediate after nucleophilic attack of ammonia on the electrophilic acyl phosphate carbonyl and/or the early transition state leading to collapse of the tetrahedral intermediate with release of P\textsubscript{i}. The methyl groups of MetSox-P\textsubscript{i} and Glufos-P\textsubscript{i} occupy the GS ammonium-binding site, while the N-P\textsubscript{i}-sulfoxamine and O-P\textsubscript{i}-phosphinate groups interact with the active site metals and cationic amino acid side chains involved in stabilizing the GS tetrahedral intermediate.\textsuperscript{20,27} Although MetSox and Glufos are noncovalent GS inhibitors, the inhibition is essentially irreversible because MetSox-P\textsubscript{i} and Glufos-P\textsubscript{i} are tightly bound transition state mimics.\textsuperscript{37}

Because GS inhibition by T\textbeta{}L is time-dependent and requires ATP, we hypothesized that T\textbeta{}L is phosphorylated in the GS active site generating a stable, noncovalent GS-ADP-T\textbeta{}L-P\textsubscript{i} inhibitory complex (Figure 2.2B). To test our hypothesis, we directly detected T\textbeta{}L-P\textsubscript{i} released from the denatured GS-ADP-T\textbeta{}L-P\textsubscript{i} complex using high-resolution liquid chromatography and mass spectrometry and characterized the site of T\textbeta{}L phosphorylation within the intact GS-ADP-T\textbeta{}L-P\textsubscript{i} inhibition complex using \textsuperscript{31}P{\textsuperscript{15}N} full-echo and rotational-echo double-resonance (REDOR) solid state NMR with magic-angle spinning. We used computational modeling to show that T\textbeta{}L-P\textsubscript{i} with an intact \textbeta{}-lactam ring resembles the GS tetrahedral intermediate, suggesting that T\textbeta{}L-P\textsubscript{i} is a GS transition state inhibitor. We also quantitatively compared MetSox, Glufos, and T\textbeta{}L in kinetic assays for inhibition of recombinant GS from \textit{Homo sapiens}, \textit{Escherichia coli}, and \textit{Staphylococcus aureus} using the Kitz-Wilson model for mechanism-based inhibition. Our results show that T\textbeta{}L is mechanistically distinct from traditional \textbeta{}-lactam
antibiotics such as penicillin. Our findings enable the design of new 3-HβLs for inhibiting enzymes in the ATP-dependent carboxylate-amine ligase superfamily with broad therapeutic applications.

2.4 Results and Discussion

Purification of TβL-Thr and TδL

The dipeptide TβL-Thr and the corresponding δ-lactam isomer, TδL-Thr, can be isolated from culture supernatants of producing P. syringae strains grown in Woolley’s minimal medium (Figure 2.3). The corresponding GS inhibitor TβL and inactive δ-lactam isomer, TδL, can be obtained if small amounts of ZnCl₂ are added to the P. syringae fermentations. Presumably, the Zn²⁺ activates a periplasmic metallopeptidase that hydrolyzes the TβL-Thr dipeptide bond, resulting in the efflux of TβL to the extracellular space. The nonenzymatic isomerization of TβL-Thr and TβL to TδL-Thr and TδL, respectively, is difficult to suppress, and most attempts to isolate active phytotoxin result in a majority of the biologically inactive δ-lactam isomers. Limited access to pure, quantified amounts of TβL-Thr and TβL has restricted the measurement of quantitative GS inhibition parameters and biological activity. We solved this problem by rigorously purifying TβL-Thr from cultures of P. syringae pv. tabaci ATCC 11528 grown in Woolley’s medium. Glycerol freezer stocks of P. syringae ATCC 11528 stored at -80 °C stopped producing TβL-Thr at various times during our work. Loss of TβL-Thr production in P. syringae pathovars has been reported previously and is thought to be associated with spontaneous loss of genetic material from mobile genetic islands containing portions of the tabtoxin biosynthetic gene cluster. To restore TβL-Thr production, we used
a colony picking strategy and inoculated 5 mL cultures of Woolley’s medium. We monitored for TβL-Thr production using LC-MS and L-Gln-dependent antibacterial activity against *E. coli* ATCC 25922 in an agar diffusion assay on chemically defined minimal medium [7 g/L K$_2$HPO$_4$, 3 g/L KH$_2$PO$_4$, 0.47 g/L sodium citrate dehydrate, 0.1 g/L MgSO$_4$•7H$_2$O, 1 g/L (NH$_4$)$_2$SO$_4$, 4 g/L glucose, 20 mg/L thymine, 0.1 mg/L biotin, 2 mg/L thiamine, 2 mg/L nicotinic acid, 2 mg/L calcium pantothenate, 10 mg of MnSO$_4$•7H$_2$O and 30 g/L Bacto-agar (pH ~7.2)]. The most promising cultures were used to inoculate larger shake flask cultures (500 mL of Woolley’s medium per flask). TβL-Thr was purified from *P. syringae* supernatants by sequential cation exchange chromatography, preparative HILIC HPLC chromatography, and preparative RP-C18 HPLC. TβL-Thr stock solutions were quantified by $^1$H NMR and stored as the TFA salt at -80 °C to prevent isomerization to TδL-Thr (Figure 2.4).

Pure TβL was obtained by treating TβL-Thr with *P. syringae* ATCC 11528 cell lysate buffered to pH 6.5.$^{11}$ TβL was purified from the cell lysate by preparative HILIC HPLC with fraction collection guided by GS inhibitory activity. Final TβL concentrations were measured by titration with L-Thr and the ATP-dependent amino acid ligase TblF from the tabtoxin biosynthetic gene cluster. TblF activates the carboxyl group of TβL as an acyl phosphate and catalyzes amide bond formation with the α-amino group of L-Thr to give TβL-Thr with release of ADP and P$_i$. We coupled the TblF reaction to a double-enzyme reaction with pyruvate kinase (PK) and lactate dehydrogenase (LDH) to convert ADP and PEP to L-lactic acid with stoichiometric consumption of NADH.$^{45}$ The decrease in optical absorbance at 350 nm is proportional to the conversion of NADH to NAD$^+$, which allowed us to determine ADP
concentrations and by direct inference TβL concentrations. Quantified stock solutions of TβL were stored in pH 3.2 ammonium formate buffer at -80 °C to prevent isomerization to TδL.

**TβL is a Mechanism-Based Inhibitor of GS**

TβL inhibits GS from plants,\textsuperscript{10,40} animals,\textsuperscript{46} and bacteria.\textsuperscript{41} TβL inhibition of cytosolic and chloroplast forms of GS isolated from pea was shown to be time-dependent, ATP-dependent, irreversible, and competitive with respect to L-Glu.\textsuperscript{10,40} The use of semipurified TβL and unknown working TβL concentrations prevented the measurement of quantitative inhibition parameters. Another technical challenge in studying GS inhibitors is the specific activity of GS in vitro. GS activity in bacteria is tightly regulated through post-translational modification (tyrosine adenylylation), making the preparation of the homogeneous enzyme difficult.\textsuperscript{47,48} To overcome this challenge, we codon-optimized the *glnA* genes encoding GS from *E. coli*, *S. aureus*, and *H. sapiens* in a pET28b expression vector encoding an N-terminal hexahistidine tag with a thrombin cleavage site (Table 2.1 and Table 2.2). We recombinantly expressed the GS homologs in *E. coli* BL21 and purified the N-His\textsubscript{6}-tagged proteins by Ni-NTA affinity chromatography (Figure 2.5). The N-His\textsubscript{6}-tagged human GS was eluted from the Ni-NTA column, dialyzed, concentrated, flash frozen in liquid nitrogen, and stored at -80 °C. Human GS was used in all subsequent steps with an intact N-His\textsubscript{6} tag. The *E. coli* and *S. aureus* GSs are adenylated on a conserved tyrosine (residue 398 for *E. coli* GS and residue 375 for *S. aureus* GS) when expressed in *E. coli*.\textsuperscript{47} The adenylated forms of the *E. coli* and *S. aureus* GSs show low activity for catalyzing the biosynthetic reaction.\textsuperscript{49} While adhered to the Ni-NTA column, *E. coli* GS and *S. aureus* GS were treated with a buffered solution of phosphodiesterase from crude *C. adamanteus* snake venom to cleave adenylyl groups. This resulted in highly active unadenylated
preparations of N-His$_6$-tagged *E. coli* and *S. aureus* GS after elution from the Ni-NTA column. The N-His$_6$-tagged *E. coli* and *S. aureus* GS elutions were dialyzed, concentrated, flash-frozen in liquid nitrogen, and stored at -80 °C. The flash-frozen stocks of N-His$_6$-tagged GS from *E. coli*, *S. aureus*, and *H. sapiens* were used for all kinetic studies.

With highly pure, quantified solutions of TβL and active, homogenous preparations of GS enzymes in hand, we measured *in vitro* kinetic parameters for the GS inhibition reaction under steady state conditions. A coupled PK/LDH spectrophotometric assay for measuring ADP production was used to analyze GS inhibition kinetics. GS produces ADP and P$_i$ as byproducts, which enables real-time quantification of ADP with PK/LDH as a measure of GS activity for the biosynthetic reaction. We measured the Michaelis-Menten parameters (apparent $K_m$ and $k_{cat}$ for ATP and L-Glu) for the biosynthetic reaction (conversion of L-Glu, ATP, and NH$_3$ to L-Gln, ADP, and P$_i$) catalyzed by recombinant *E. coli*, *S. aureus*, and *H. sapiens* GS (Table 2.3 and Figure 2.6). All three enzymes gave a similar apparent $K_m$ for ATP (0.7 ± 0.1 mM for *E. coli* and *H. sapiens* GS and 1.8 ± 0.1 mM for *S. aureus* GS) under saturating L-Glu (50 mM) and NH$_3$ (10 mM). Apparent $K_m$ values for ATP reported in the literature vary from 0.1 to 0.7 mM for *E. coli* GS$^{24,26,33,51}$ and from 1.8 to 2.8 mM for *H. sapiens* GS,$^{52}$ both within our measured range. To the best of our knowledge, no kinetic parameters for *S. aureus* have been reported in the literature. GS from *E. coli* was faster (apparent $k_{cat}$ of 7300 ± 300 min$^{-1}$) and more efficient ($k_{cat}/K_m = 10400 ± 1500$) than both *H. sapiens* ($k_{cat} = 2600 ± 100$ min$^{-1}$; $k_{cat}/K_m = 3700 ± 500$ min$^{-1}$mM$^{-1}$) and *S. aureus* ($k_{cat} = 720 ± 20$ min$^{-1}$; $k_{cat}/K_m = 400 ± 30$ min$^{-1}$mM$^{-1}$) enzymes. The apparent $K_m$ values for L-Glu were measured in the presence of saturating ATP (10 mM) and NH$_3$ (10 mM). The final ATP concentration was always kept lower than that of MgCl$_2$ (25 mM) to avoid the inhibition by ATP that was previously reported for human,$^{52}$ rat liver,$^{53}$ and Chinese...
hamster liver GS.\textsuperscript{54} Literature values for the \textit{E. coli} GS apparent $K_m$ for L-Glu range from 0.8 to 6.6 mM.\textsuperscript{24,26,33,51} We measured an apparent $K_m$ value of \(7.2 \pm 0.8\) mM for the \textit{E. coli} enzyme. The apparent L-Glu $K_m$ values for the \textit{S. aureus} (52 $\pm$ 7 mM) and \textit{H. sapiens} (37 $\pm$ 5 mM) enzymes were significantly higher. We found two literature values for the human L-Glu ($K_m$ values of 3.0 $\pm$ 1.2 and 3.5 $\pm$ 0.7 mM) that were measured on the same batch of recombinant human enzyme expressed in \textit{E. coli} using a radiometric and colorimetric assay, respectively.\textsuperscript{52}

The relatively high apparent $K_m$ values for L-Glu are consistent with reported \textit{in vivo} concentrations of L-Glu on the order of 25–100 mM in certain tissues. Recently, the apparent $K_m$ for L-Glu was reported to be 26.3 $\pm$ 0.4 mM for GS isolated from \textit{Leishmania donovani}, a protozoan parasite that causes leishmaniasis in tropical regions.\textsuperscript{55} Under saturating ATP and NH$_3$ conditions, \textit{E. coli} GS ($k_{\text{cat}}/K_m = 1100 \pm 100$ min$^{-1}$mM$^{-1}$) was more efficient than \textit{H. sapiens} ($k_{\text{cat}}/K_m = 140 \pm 20$ min$^{-1}$mM$^{-1}$) and \textit{S. aureus} ($k_{\text{cat}}/K_m = 27 \pm 4$ min$^{-1}$mM$^{-1}$) variants.

Toxicity is a concern when GS inhibitors are being developed into commercial products such as herbicides or antibiotics.\textsuperscript{46} Toxicity arises because of the lack of selectivity of GS inhibitors for a specific GS isoform as well as broad off-target effects associated with the polypharmacology of ATP mimics and nonproteinogenic amino acids.\textsuperscript{56,57} We measured inhibition parameters for T$\beta$L, MetSox, and Glufos against recombinant N-His$_6$-tagged GS from \textit{E. coli}, \textit{S. aureus}, and \textit{H. sapiens} (Table 2.4 and Figure 2.7). The GS inhibition was time- and ATP-dependent for all combinations of inhibitors and enzymes. Longer preincubation times of the enzyme and inhibitor led a greater loss of GS activity consistent with a mechanism-based inhibition model.\textsuperscript{58} GS inhibition was irreversible for all three inhibitors. Treatment of \textit{E. coli} GS with T$\beta$L under biosynthetic reaction conditions provided a stable, soluble enzyme-inhibitor complex. GS activity could not be recovered even after dialysis of the GS-T$\beta$L inhibition
complex for several days at 4 °C and 37 °C. We used the Kitz-Wilson model for mechanism-based, irreversible enzyme inhibition to compare the potency of TβL, MetSox, and Glufos.\textsuperscript{58} Plots of $1/k_{\text{app}}$ (minutes) versus $1/[^{\text{inhibitor}}]$ (inverse micromolar) gave straight lines with $R^2$ values of $\geq 0.9$ for all enzyme and inhibitor combinations in support of a mechanism-based inhibition model (Figure 2.7C,F,I). The parameters $K_I$ (micromolar) and $k_{\text{inact}}$ (inverse minutes) were interpreted as a metric for the apparent binding affinity and rate of enzyme inactivation, respectively, and the $k_{\text{inact}}/K_I$ ratio (inverse minutes inverse micromolar) was used to compare inhibitor efficiency.

TβL, MetSox, and Glufos strongly inhibited \textit{E. coli}, \textit{S. aureus}, and \textit{H. sapiens} GS (Table 2.4 and Figure 2.7C,F,I). Inhibitor efficiency ($k_{\text{inact}}/K_I$) toward GS isoforms decreased in the following order: \textit{E. coli} $>$ \textit{S. aureus} $>$ \textit{H. sapiens}. The inhibitor binding ($K_I$) and rate of inactivation ($k_{\text{cat}}$) also followed the same general trend. Inhibitor potency did indicate a promising therapeutic window between \textit{E. coli} and \textit{H. sapiens} GS enzymes. TβL inhibited \textit{E. coli} GS with a $K_I$ of $1.7 \pm 0.4$ μM and a $k_{\text{inact}}$ of $0.3 \pm 0.1$ min\(^{-1}\), resulting in an inhibitor efficiency ($k_{\text{inact}}/K_I$) of $180 \pm 70$ min\(^{-1}\)μM\(^{-1}\). The inactivation parameters for TβL against human GS were as follows: $K_I = 130 \pm 40$ μM, $k_{\text{inact}} = 0.3 \pm 0.1$ min\(^{-1}\), and $k_{\text{inact}}/K_I = 2 \pm 1$ min\(^{-1}\)μM\(^{-1}\), corresponding to a 90-fold decrease in inhibitor efficiency. The inactivation parameters for TβL against \textit{S. aureus} GS ($K_I = 50 \pm 10$ μM, $k_{\text{inact}} = 0.4 \pm 0.1$ min\(^{-1}\), and $k_{\text{inact}}/K_I = 8 \pm 3$ min\(^{-1}\)μM\(^{-1}\)) were intermediary compared to those of \textit{E. coli} and \textit{H. sapiens} GS. The inactivation parameters for Glufos and MetSox were similar to those for TβL. Literature values of $K_I$ reported for Glufos and MetSox against unadenylated \textit{E. coli} GS are 1 μM and 2 μM, respectively.\textsuperscript{59,60} We determined the $K_I$ values against \textit{E. coli} GS to be $1.0 \pm 0.2$ μM and $3.9 \pm 1.1$ μM for Glufos and
MetSox, respectively. The closeness to literature $K_1$ values supports our use of the GS-PK-LDH coupled enzyme assay for measuring inhibition kinetics. Glufos was the most effective inhibitor against all three GS enzymes and gave a promising 120-fold difference in inhibitor efficiency for *E. coli* and *H. sapiens* GS. MetSox gave the largest therapeutic window in terms of inhibitor efficiency for *E. coli* and human GS (250-fold difference). Although $k_{\text{inact}}/K_1$ values for *E. coli*, *S. aureus*, and *H. sapiens* GS enzymes showed good separation, the $k_{\text{inact}}$ values are essentially equal, and low selectivity is predicted if therapeutic concentrations are high. *E. coli*, *S. aureus*, and *H. sapiens* GS enzymes show a low overall level of primary sequence homology but do share highly conserved active site residues ([Table 2.5, Table 2.6 and Figure 2.8]). Further computational, structural, and functional studies will be required to fully rationalize the observed binding affinities, inactivation rates, and overall inhibitor efficiencies for TβL, MetSox, and Glufos, representing three chemically distinct classes of l-Glu antimetabolites: 3-HβLs, sulfoximines, and phosphinates, respectively.

**GS is the Primary Cellular Target for TβL in E. coli**

Inhibition of central metabolic pathways with so-called antimetabolites is a classic method for perturbing cellular metabolism and is gaining traction in therapeutic and commercial applications. Antimetabolites such as TβL and other GS inhibitors dysregulate metabolic pathways through enzyme inactivation, causing changes in metabolic flux that activate and/or inhibit connected pathways both upstream and downstream of the target metabolite. MetSox is known to inhibit both glutamine synthetase and γ-glutamylcysteine synthetase. To support our *in vitro* GS inactivation measurements, we sought to validate that GS is the primary cellular target for TβL and Glufos in *E. coli*. To facilitate membrane transport, we used prodrug forms of
the GS inhibitors, $\text{TBL-THR}$ and synthetic Ala-Ala-Glufos (AAG) (**Figure 2.3**). We synthesized AAG via amide coupling of the Fmoc-L-Ala-L-Ala dipeptide with racemic Glufos followed by Fmoc deprotection with 20% piperidine in DMF (**Scheme 2.1**). Presumably, $\text{TBL-THR}$ and AAG are transported to the periplasm of *E. coli* cells via outer membrane di- and tripeptide permeases.$^{64-66}$ Peptidases in the periplasm and/or cytoplasm then cleave the dipeptide bonds, releasing the active GS inhibitors that reach cytoplasmic GS.$^{39,67}$ To control for metabolite concentrations, we used a chemically defined minimal medium $\{7 \text{ g/L K}_2\text{HPO}_4, 3 \text{ g/L KH}_2\text{PO}_4, 0.47 \text{ g/L sodium citrate dehydrate, 0.1 g/L MgSO}_4 \cdot 7\text{H}_2\text{O, 1 g/L (NH}_4\text{)}_2\text{SO}_4, 4 \text{ g/L glucose, 20 mg/L thymine, 0.1 mg/L biotin, 2 mg/L thiamine, 2 mg/L nicotinic acid, 2 mg/L calcium pantothenate, and 10 mg of MnSO}_4 \cdot 7\text{H}_2\text{O (pH ~7.2)}\}$ for growth studies with *E. coli* ATCC 25922. $\text{TBL-THR}$ and AAG both gave end point MIC$_{90}$ values of 0.5 $\mu$M in broth microdilution assays. Addition of L-Glu had no effect on the antibacterial activity of $\text{TBL-THR}$ and AAG. When the minimal medium was supplemented with 5 mM L-Glu, the MIC$_{90}$ values for $\text{TBL-THR}$ and AAG were 1 $\mu$M. Addition of 5 mM L-Gln to the minimal medium completely abolished the antibacterial activity of $\text{TBL-THR}$ and AAG (MIC$_{90}$ values of $>256$ $\mu$M). If cytoplasmic $\text{TBL}$ and Glufos have a polypharmacological antibacterial effect on *E. coli*, then L-Gln supplementation should not wholly restore bacterial growth. Treatment of *E. coli* with $\text{TBL-THR}$ and AAG results in glutamine auxotrophy consistent with GS being the primary cellular target for $\text{TBL}$ and Glufos in *E. coli*. The same Gln-auxotroph phenotype has been reported in *Mycobacterium tuberculosis* and *E. coli* treated with MetSox and bialaphos, the naturally occurring Glufos-Ala-Ala (GAA) tripeptide.$^{68,69}$ We confirmed this result in *E. coli* using a commercial source of bialaphos sodium salt. The order of Glufos tripeptide, synthetic AAG or natural GAA (**Figure 2.3**), does not
appear to influence cellular transport or peptidase cleavage to release the active GS inhibitor in *E. coli*.

We further probed the glutamine dependence of GS inhibitor antibiotics by measuring growth curves for *E. coli* ATCC 29522 in liquid minimal media in the presence of varying concentrations of L-Gln (Figure 2.9). With no L-Gln present, 2 μM TβL-Thr had a bacteriostatic effect on *E. coli* ATCC 29522 with full growth recovery after ~24 h (Figure 2.9A). *E. coli* growth was restored to near control levels when the medium was supplemented with 0.1 mM L-Gln. When L-Gln supplementation reached 2.5 mM, the growth curve matched the control with no TβL-Thr. When the concentration of TβL-Thr was increased to 100 μM, *E. coli* growth was suppressed for >50 h (Figure 2.9B). Addition of L-Gln antagonized the bacteriostatic activity, but to a lesser extent than the antagonism observed when using 2 μM TβL-Thr (Figure 2.9A). The growth of *E. coli* ATCC 29522 treated with 100 μM TβL-Thr improved with increasing L-Gln concentrations but never reached the full level of the control (Figure 2.9B). AAG had a similar bacteriostatic effect on the growth of *E. coli* ATCC 29522 with increased potency and decreased susceptibility to L-Gln antagonism (Figure 2.9C). The growth of *E. coli* was fully suppressed for >66 h when it was treated with 2 μM AAG. Bacterial growth was restored in the presence of 2 μM AAG with L-Gln supplementation, but higher concentrations of L-Gln were required compared to growth rescue in the presence of 2 μM TβL-Thr. The increased potency of AAG compared to that of TβL-Thr might be due to spontaneous isomerization of TβL-Thr to TδL-Thr in the growth medium. The isomerization half-life of TβL-Thr is 37 h at 23 °C in pH 7.2 phosphate buffer. We anticipate that the isomerization rate will increase at 37 °C in pH 7.2 phosphate-buffered minimal medium used for bacterial growth studies. *E. coli* growth started to
recovery in minimal medium after 26 h in the presence of 2 μM TβL-Thr. No growth recovery was observed after 48 h in the presence of 100 μM TβL-Thr. The concentration dependence and time to growth recovery are consistent with the isomerization of TβL-Thr to TδL-Thr in the growth medium. Because TβL and Glufos are both irreversible GS inhibitors, we do not believe that the growth recovery or L-Gln dependence is due to the inhibitor off rate. Intracellular GS turnover and GS overexpression might play a role in growth recovery. GS overexpression has been shown to be an effective mechanism for resistance to GS inhibitors in *Mycobacterium tuberculosis*. Some bacteria carry extra copies of *glnA* that encodes GS homologues with active site mutations that can reduce the inhibitory activity of MetSox and Glufos and potentially play a role in resistance to GS inhibitors. Spontaneous mutations in *glnA* coding sequences in *S. typhimurium, Bacillus subtilis, Exiguobacterium* sp., soybean, and rice have been reported to arise under GS inhibitor selection. The mutations were tracked to active site residues that reduce susceptibility to MetSox and/or Glufos inhibition *in vitro*. Careful consideration must be given to these potential resistance mechanisms along with endogenous L-Gln concentrations for applications of GS inhibitors as antibiotics and herbicides.

**GS Catalyzes Phosphorylation of TβL**

The mechanistic basis for GS inhibition by TβL is unknown (*Figure 2.2*). We have showed that TβL inhibition is irreversible, ATP-dependent, and time-dependent and fits the Kitz-Wilson kinetic model for mechanism-based enzyme inhibition, suggesting that TβL is phosphorylated in the GS active site similar to the related inhibitors MetSox and Glufos (*Figure 2.7*). We used LC-MS to detect the phosphorylated inhibitors TβL-P_i, MetSox-P_i, and Glufos-P_i released after denaturation of inhibition complexes with GS from *E. coli* (*Figure 2.10*).
Recombinant N-His$_6$ GS from *E. coli* was treated with TβL (Figure 2.10A), MetSox (Figure 2.10C), and Glufos (Figure 2.10D) in the presence and absence of ATP. The initial activity and inhibition of GS were confirmed by the coupled PK/LDH assay monitoring optical absorbance for consumption of NADH that was used for kinetic experiments. The GS-inhibitor complex was washed with buffer via centrifugal filtration to remove excess inhibitor prior to mild heat denaturation and protein precipitation with addition of KCl and MeOH. The clarified methanolic solution was treated with FmocCl to acylate the α-amino groups of the inhibitors to give Fmoc-TβL-P$_i$, Fmoc-MetSox-P$_i$, and Fmoc-Glufos-P$_i$. The Fmoc tagging step stabilized the phosphorylated inhibitors, increased the retention time during LC-MS analysis, and prevented the spontaneous isomerization of TβL or TβL-P$_i$ to TδL or TδL-P$_i$, respectively.$^{11}$ We also prepared a sample of isotopically labeled $^{15}$N-TβL by fermenting *P. syringae* ATCC 11528 in Woolley’s medium with K$^{15}$NO$_3$ as the sole nitrogen source. $^{15}$N-TβL-Thr and $^{15}$N-TβL were purified and quantified as described previously for unlabeled TβL-Thr and TβL. $^{15}$N-TβL was used as a positive control isotopomer to confirm the retention time and isotope distribution of TβL-P$_i$ (Figure 2.10B).

Treatment of the supernatant from the agglutinated TβL-treated GS solution with FmocCl followed by LC-MS analysis resulted in a clear peak in the extracted ion chromatogram (EIC) for Fmoc-TβL-P$_i$ ($m/z$ 491 for [M+H]$^+$) only when ATP was present during incubation of GS and TβL (Figure 2.10A). The molecular formula of Fmoc-TβL-P$_i$ was confirmed by high-resolution MS analysis in negative ion mode (HRMS$_1$ (ESI) calculated for C$_{22}$H$_{22}$N$_2$O$_9$P: 489.1068 [M-H]$^-$, found 489.1064) (Figure 2.11). As expected, MS/MS analysis of the Fmoc-TβL-P$_i$ parent ion (observed $m/z$ 489.1068 for [M-H]$^-$) showed loss of neutral Fmoc giving the
TβL-P$_i$ fragment ion (observed $m/z$ 267.0387 for [M-H]$^-$) and a neutral loss of TβL with formation of the inorganic phosphate (observed $m/z$ 96.9695 for [M-H]$^-$) and phosphite (observed $m/z$ 78.9589 for [M-H]$^-$) fragment ions.$^{77}$ The MS/MS fragmentation pattern Fmoc-TβL-P$_i$ is consistent with phosphorylation of the C3-OH group of the 3-HβL group but does not definitively establish the site of phosphorylation. The MS spectra implied that the β-lactam ring was intact, but it is not possible to distinguish between Fmoc-TβL-P$_i$ and the corresponding phosphorylated δ-lactam isomer without a standard sample. Fmoc tagging blocks isomerization of TβL to TδL and we suspect that Fmoc tagging also blocked the spontaneous isomerization of TβL-P$_i$ to the phosphorylated δ-lactam isomer. The same experiments conducted using $[^{15}N]$TβL produced the expected ion for Fmoc-[$^{15}$N]TβL-P$_i$ ($m/z$ 493 for [M+H]$^+$) with a retention time identical to that of Fmoc-TβL-P$_i$ (Figure 2.10B). We also confirmed that N-His$_6$-tagged GS from E. coli catalyzes the ATP-dependent conversion of MetSox to MetSox-P$_i$ (Figure 2.10C). LC-MS analysis of the denatured GS-MetSox-P$_i$ solution after treatment with FmocCl produced a strong peak for Fmoc-MetSox-P$_i$ in the EIC ($m/z$ 483 for [M+H]$^+$) only when ATP was included during enzyme incubation. Glufos-P$_i$ is an unstable phosphoanhydride that hydrolyzes rapidly upon denaturing the GS-Glufos-P$_i$ complex.$^{78}$ We observed a large enhancement in ion counts for Fmoc-Glufos ($m/z$ 483 for [M+H]$^+$) when ATP was present in the incubation of GS with Glufos (Figure 2.10D). Small amounts of Fmoc-Glufos were also present in the no ATP control reaction that might be a result of insufficient washing prior to denaturing, weak binding of Glufos to the GS active site, or co-purification of ATP in the GS active site resulting in a small amount of Glufos-P$_i$ production.
To gain insight into the mechanism of GS inhibition by TβL, we needed to establish the site of phosphorylation on TβL-P_i. We initially attempted to obtain an X-ray crystal structure of TβL-P_i bound to GS from *E. coli* without success. Although we could regularly obtain stable crystals of the GS-TβL-P_i inhibition complex, the quality of X-ray diffraction was not sufficient to determine the structure. During these crystallization attempts, we turned to solid state NMR as a proven method to provide structural information on enzyme-inhibitor complexes in heterogeneous mixtures. We hypothesized that rotational-echo double resonance (REDOR) could be used to restore dipolar couplings between 15N and 31P spin pairs of GS-bound TβL-P_i that are removed by magic-angle spinning. We scaled up production of GS from *E. coli* using the same recombinant expression system as described previously. During our scale-up, the phosphodiesterase from crude *C. adamanteus* (eastern diamondback rattlesnake) snake venom, used to cleave adenylation PTMs, was back-ordered from the supplier. Thus, we turned to a new source of phosphodiesterase from crude *C. atrox* (western diamondback rattlesnake) to cleave the adenyl groups. Treatment of N-His6-tagged GS bound to Ni-NTA resin with *C. atrox* phosphodiesterase cleaved the adenylyl groups as well as the N-His6-tag, resulting in elution of highly active GS. *C. atrox* venom is known to contain thrombin-like proteases capable of cleaving the N-His6 tag at the nearby thrombin cleavage site (Table 2.5). Untagged GS was further purified by size-exclusion chromatography (SEC) and catalytic activity towards the L-Glu to L-Gln biosynthetic reaction was confirmed using the PK/LDH coupled spectrophotometric assay. TβL inhibited the untagged, SEC-purified GS with time and ATP dependence with the same efficiency as the N-His6-GS. SDS-PAGE and matrix-assisted laser desorption ionization (MALDI) analysis confirmed the anticipated molecular weight of the GS monomer (51.9 kDa). Samples were prepared by treating *E. coli* GS (~63 mg) with excess
in the presence of ATP followed by extensive washing of the resulting GS-ADP-
complex with trehalose/HEPES buffer using centrifugal filtration. The sample was
cryoprotected using trehalose, PEG-8000, and dextran prior to controlled freezing at -10 °C
(ice/CaCl₂ bath) with gradual cooling to -78 °C (dry ice/acetone) and final freezing at liquid
nitrogen temperatures. Lyophilization for 72 h gave ~160 mg of a fluffy white powder that was
used for solid state NMR experiments. A control sample lacking [¹⁵N]TβL inhibitor and
replacing ATP with ADP was prepared in the same manner.

A control sample of GS, ADP, and buffer was used to establish a 0 ppm chemical shift
(δₚ) in the 202-MHz ³¹P cross-polarization magic-angle spinning NMR spectrum (Figure 2.12A). No signals associated with free ADP were observed. Next we tried a ³¹P{¹⁵N} REDOR experiment on the GS-ADP-[¹⁵N]TβL-P₁ complex. This experiment is always performed in two parts. The first is a so-called “full echo”, which is obtained with ³¹P π-pulses on the completion of each rotor period. This establishes a reference signal that takes account of homogeneous decay (T₂). Both free phosphate (P₁) and ADP peaks are observed, as well as a bound phosphate peak at −2 ppm (Figure 2.12B). The second part of the REDOR experiment inserts ¹⁵N π-pulses in the middle of each rotor period. These are so-called “dephasing” pulses. Their function is to defeat the spatial averaging of magic-angle spinning and allow ³¹P−¹⁵N dipolar coupling to appear as a diminution of the full echo resulting in S, a “dephased echo” (not shown). The REDOR difference is ΔS = S₀ − S (Figure 2.12C), which has a simple interpretation in terms of the heteronuclear dipolar coupling between ³¹P and ¹⁵N. This coupling yields rₚₙ, the internuclear separation. Because of the massive P₁ peak near 0 ppm in S₀, we chose to use the first spinning sideband of the bound phosphate peak to estimate S₀ and ΔS [Figure 2.12B,C (dotted line)].
Interference from the small (and broad) $P_i$ spinning sideband could be ignored. Integrals of both $\Delta S$ and $S_0$ first sidebands improved sensitivity and led to an $r_{PN}$ of $4.1 \pm 0.2$ Å.

Cross-polarization $^{15}$N NMR of a labeled bacterial cell wall sample established a chemical shift ($\delta_N$) reference spectrum for known amide (NH) and primary amine (NH$_2$) nitrogens (Figure 2.12D). The same experiment performed with the GS–ADP–[$^{15}$N]TβL–$P_i$ complex produced an amide nitrogen peak near 100 ppm and an isopropyl primary amine nitrogen peak near 50 ppm (Figure 2.12E), but no peaks associated with $sp^2$ nitrogens. Again spectral integration improved signal-to-noise ratios. The *E. coli* GS dodecamer has 12 functional active sites. On the basis of peak integrations, we estimate that approximately six of the GS active sites are occupied by [$^{15}$N]TβL–$P_i$. With only 50% active site occupancy, our samples of the GS–ADP–[$^{15}$N]TβL–$P_i$ complex might be heterogeneous, which might explain the difficulty in obtaining quality X-ray diffraction data for stable crystals. Previous studies showed that 11 of the 12 active sites of *E. coli* GS are occupied by Glufos–$P_i$ and MetSox–$P_i$ produced ~80% active site occupancy.$^{35,78}$ The substoichiometric inhibitor occupancy of GS active sites might be a result of communication between active sites, which is consistent with previous observations of progressively slower inhibitor binding and phosphorylation.$^{85}$

On the basis of structural models of GS bound to MetSox–$P_i$ and Glufos–$P_i$, there are three possible sites of phosphorylation on TβL: (1) β-lactam nitrogen, (2) β-lactam oxygen, and (3) C3-hydroxyl group. Amide O-phosphorylation is catalyzed by aminimidazole ribonucleotide synthetase in the purine biosynthetic pathway. Recently, a glutamine kinase that catalyzes N-phosphorylation of the L-Gln amide in a pathway for phosphoramide capsular polysaccharide biosynthesis in *Campylobacter jejuni*, a common foodborne pathogen, was discovered.$^{86}$ The $^{15}$N cross-polarization experiment rules out phosphorylation of the β-lactam
nitrogen and β-lactam oxygen, which would leave the $^{15}$N without a direct bond to a proton or with $\delta_N$ corresponding to full sp$^2$ hybridization. Furthermore, a $^{31}$P−$^{15}$N through-space distance of 4.1 Å rules out direct phosphorylation of nitrogen. The observed distance measurement and $^{15}$N cross-polarization experiments are consistent with phosphorylation of the TβL C3-hydroxyl group with an intact β-lactam ring. Phosphorylation at this site makes TβL-P$_i$ structurally related to MetSox-P$_i$ and Glufos-P$_i$ that mimic the tetrahedral GS transition state (Figure 2.2).

**TβL-P$_i$ is a GS Transition State Analogue**

Transition states for nucleophilic acyl substitution reactions resemble the tetrahedral intermediate.$^{36}$ MetSox-P$_i$, Glufos-P$_i$, and TβL-P$_i$ are structurally similar to the high-energy GS tetrahedral intermediate, Gln-P$_i$, and are predicted to bind with abnormally high affinity (MetSox-P$_i$ $K_i < 10^{-19}$ M against GS$^{37}$) and stabilize the normally short-lived GS transition state geometry. We built computational models for the GS tetrahedral intermediate, Gln-P$_i$ (Figure 2.13A), and TβL-P$_i$ (Figure 2.13B) with phosphorylation of the C3-hydroxyl group bound to dimetallic (Mn$^{2+}$) GS from *E. coli*. We used Avogadro to build structures of Gln-P$_i$ and TβL-P$_i$. We used a crystal structure of GS from *Salmonella typhimurium* (PDB entry 1FPY) as template to build a homology model of *E. coli* GS (98% identical sequence) using the Phyre2 software.$^{20,88}$ We used Autodock Vina to identify low-energy ligand-docked poses in the GS active site with bound ADP.$^{89}$ The ligand search space was validated using Glufos, which restored the original binding pose from the crystal structure (PDB entry 1FPY). Both Gln-P$_i$ (Fig. 13C) and TβL-P$_i$ (Fig. 13D) were found to bind as expected in the L-Glu binding site with the α-amino acid group interacting with Glu212A, Asn264A, Glu327A, and His269A.$^{27}$ Some key differences were observed upon comparison of interaction networks for the phosphorylated head.
groups of Gln-P_i and TβL-P_i. The phosphate of Gln-P_i interacts with Mn^{2+}, His210A, and Tyr179A. The Gln-P_i oxyanion and nitrogen cation are predicted to interact with Tyr179A. The stereochemistry and conformation of TβL-P_i resemble those of Gln-P_i with the phosphate groups occupying similar chemical space. The β-lactam oxygen and nitrogen aligned closely with the oxyanion and nitrogen cation, respectively, of Gln-P_i, suggesting that the 3-HβL warhead mimics the structure, polarity, and chirality of the GS transition state. TβL-P_i showed more extensive interactions with amino acid side chains including residues from chain B. The phosphate of TβL-P_i interacted with Mn^{2+}, His210A, and Tyr179A similar to that of Gln-P_i. The β-lactam oxygen was stabilized by hydrogen bonding to Arg359A, and the β-lactam NH group donated a hydrogen bond to Asp50B. Interaction of TβL-P_i with Asp50B is an interesting observation for several reasons. Asp50B is highly conserved on the central, mobile flap of GS chain B that is involved in active site closure upon L-Glu binding. Asp50B also forms part of the ammonium-binding site and is thought to deprotonate NH_4^+ prior to nucleophilic attack on the δ-carbonyl carbon of the γ-glutamyl acyl phosphate leading to Gln-P_i formation. Asp50B is not a strong enough base to deprotonate the β-lactam NH group (pKa ~25), so a stable H-bond might result holding GS in a closed conformation resembling the transition state. The strained β-lactam imparts amine-like character on the β-lactam nitrogen (pK_a of protonated twisted amides of ~4.5^{90-92}), which might also play a role in the stabilization of the Asp50 interaction and overall stereo-electronic resemblance of the GS transition state. The stable H-bond between Asp50B and the β-lactam NH might be a distinguishing feature for TβL compared to MetSox and Glufos, which both place a less polar CH_3 group in this chemical space. Efforts to substitute the Glufos CH_3 group with more polar groups such as amines result in slower binding and decreased
inactivation rates. The tetrahedral γ-phosphate structure of Glufos increases the rate of phosphoryl transfer due to similarity in structure to the GS transition state and tetrahedral intermediate but slows the rate of initial GS binding due to differences in structure from the trigonal planar substrate L-Glu γ-carboxylate. Detailed kinetic and structural studies of TβL, similar to those reported for Glufos and MetSox, are needed to fully appreciate the contribution of GS binding and rate of phosphoryl transfer to the observed $K_i$ and $k_{\text{inact}}$.

The (S)-C3-hydroxyl group of TβL is positioned in the GS active site to attack the ATP γ-phosphate group to achieve phosphoryl transfer. The C3-hydroxyl is predicted to be protonated at physiological pH, but α-orientation to the β-lactam carbonyl might decrease the pKa by at least an order of magnitude, via hydrogen bonding and inductive effects, making it a better mimic of the L-Glu carboxylate and increasing the nucleophilicity. Thus, decreasing the pKa of the C3-hydroxyl group might increase the rate of GS binding and phosphoryl transfer. Interestingly, a TβL analogue that is chlorinated on the γ-carbon (α to the C3-OH) was isolated from a strain of Streptomyces species 372A. The purpose of chlorination at this point is unknown: enhanced binding through favorable polarity effects in the GS active site or increased rate of phosphoryl transfer to the C3-hydroxyl group. Chlorination likely decreases the pKa of the C3-hydroxyl proton through inductive effects, making deprotonation more thermodynamically favorable and kinetically faster. Reactivation of GS inhibited by MetSox-Pi was shown to have a strong pH dependence, implying that the pKa values of active site amino acid residues and the inhibitor are important for the formation and stability of the inhibition complex. Furthermore, a γ-hydroxylated variant of Glufos was reported to increase the rate of GS activation by 50%, which might be the result of faster binding and/or phosphoryl transfer. Inhibitor potency toward GS isoforms seem to correlate with $K_m$ values for L-Glu (Table 2.3 and
Table 2.4). A higher apparent $K_m$ value for L-Glu correlated with an increase in $K_i$ for TβL, MetSox, and Glufos, which is consistent with a competitive inhibition model. Both $K_i$ and $k_{inact}$ are important and physiologically relevant metrics for mechanism-based inhibitors. Lessons learned from tetrahedral transition state analogues, including phosphinates (ATP-dependent ligase inhibitors) and tertiary alcohols (protease inhibitors), might prove to be useful for the synthetic optimization of 3-HβLs as inhibitors of ATP-dependent ligases.

2.5 Outlook and Conclusions

We have established the mechanistic basis for the ATP-dependent inhibition of GS by TβL using enzyme kinetics, mass spectrometry, solid state NMR, and computational modeling. TβL is competitive with L-Glu, and GS catalyzes phosphoryl transfer from ATP to the C3-hydroxyl group of the β-lactam warhead. Phosphorylated TβL-Pi resembles the GS tetrahedral transition state and forms a stable inhibition complex with bound ADP that is noncovalent and essentially irreversible. TβL is mechanistically distinct from traditional β-lactam antibiotics, such as penicillin, that covalently inhibit transpeptidases in the serine hydrolase superfamily by a strain-driven, β-lactam ring-opening acylation mechanism. The TβL β-lactam ring remains intact during GS inhibition and serves as a tetrahedral template that matches the conformation, polarity, and chirality of the GS transition state. The 3-HβL warhead of TβL might be broadly applicable as a tetrahedral scaffold for designing transition state analogues of enzymes that stabilize tetrahedral intermediates, including proteases and enzymes in the ATP-dependent carboxylate-amine ligase superfamily. The phosphinate warhead of the related GS inhibitor Glufos has been repurposed many times for such targeted applications. Targeting the active site of ATP-dependent ligases might be advantageous over the commonly exploited ATP site, which can
generate off-target toxicity. Dipeptide prodrugs of TβL show broad-spectrum bacteriostatic antibacterial activity. GS is the primary cellular target for TβL in *E. coli*, and treatment induces L-Gln auxotrophy. TβL and the related inhibitors MetSox and Glufos show moderate selectivity for inhibiting bacterial GS over human GS. Toxicity associated with inhibition of human GS, competition with endogenous L-Gln, cell permeability, and emerging resistance mechanisms are challenges for developing GS inhibitors as antimicrobial agents. Applications of GS inhibitors for treating infectious diseases might be limited to scenarios in which endogenous L-Gln levels are low, such as L-Gln depletion associated with sepsis, because L-Gln strongly antagonizes antimicrobial activity. MetSox was shown to be effective and synergistic with isoniazid in a guinea pig lung infection model of *M. tuberculosis*, and long exposure to small doses of MetSox was well tolerated. Prospects for synergistic antibiotics combinations with GS inhibitors are attractive because GS plays a central role in nitrogen metabolism feeding important downstream pathways targeted by established clinical antibiotics. Glutamine synthetase activity has been associated with bacterial virulence and is required for some multidrug resistant bacterial phenotypes. Improved structural analogues of TβL, MetSox, and Glufos structures and new prodrug formulations might enhance the therapeutic window and cellular uptake of GS inhibitors. Methods reported here will be useful for establishing SAR for GS inhibition *in vitro* and in whole cell antibacterial assays. Target binding (*K*_\text{t}) and the rate of enzyme inactivation (*k*_\text{inact}) are important metrics for mechanism-based inhibitors that expand dimensions for structural optimization. TβL, MetSox, and Glufos represent three chemically distinct classes (3-HβL, sulfoximine, and phosphinate, respectively) of mechanism-based GS inhibitors with the potential to offer differential selectivity toward GS isoforms in applications as herbicides, pesticides, antimicrobials, and therapeutics for treating human diseases.
2.6 Materials and Methods

Strains, Materials, and Instrumentation

*E. coli* ATCC 29522 and *P. syringae* pv. *tabaci* ATCC 11528 were purchased from ATCC ([Table 2.1](#)). *E. coli* BL21-Gold(DE3) was purchased from Agilent. *E. coli* TOP10 cells were purchased from Invitrogen. Both *E. coli* BL21-Gold(DE3) and *E. coli* TOP10 cells were made electrocompetent by standard procedures. Electroporation was accomplished using a MicroPulser electroporator and 0.2 cm gap sterile electroporation cuvettes from Bio-Rad. All bacteria were stored as frozen glycerol stocks at -80 °C. *GlnA* coding sequences from *E. coli*, *S. aureus*, and *H. sapiens* were purchased codon-optimized for expression in *E. coli* BL21 from GenScript in a pET28a vector with a thrombin cleavable *N*-His₆ tag ([Table 2.2](#) and [Table 2.5](#)). DNA purification was performed with kits purchased from Qiagen. All plasmid sequences were confirmed by sequencing performed by Genewiz. Nickel-nitriloacetic acid (Ni-NTA) agarose was purchased from Invitrogen. Any kD sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels were purchased from Bio-Rad. Proteins were dialyzed using 10K molecular weight cutoff (MWCO) SnakeSkin dialysis tubing purchased from Thermo Fisher Scientific. Centrifugal filters (30K MWCO) used for protein concentration were purchased from Millipore. Crude phosphodiesterase from *Crotalus adamanteus* and *Crotalus atrox* was purchased from Sigma-Aldrich as a lyophilized powder. A mixture of pyruvate kinase (PK) and lactate dehydrogenase (LDH) enzymes from rabbit muscle was purchased from Sigma-Aldrich as a buffered glycerol solution (900–1400 units/mL LDH and 600–1000 units/mL PK). All aqueous solutions were prepared with water purified using a Milli-Q system. All buffer and salt solutions were sterilized by sterile filtration through a 0.2 μm filter. Luria broth and Difco.
nutrient broth were sterilized in an autoclave. Woolley’s medium was filter sterilized. All pH measurements were recorded using an Orion Star A111 pH meter and a PerpHecT ROSS micro combination pH electrode from Thermo Fisher Scientific. All buffers, salts, media, solvents, and chemical reagents were purchased from Sigma Aldrich unless otherwise stated. D$_2$O and K$^{15}$NO$_3$ were purchased from Cambridge Isotope Laboratories. Racemic Glufos was purchased from Oakwood Chemical. Fmoc-protected L-Ala-L-Ala dipeptide was purchased from ChemImpex. Glufos-L-Ala-L-Ala tripeptide (GAA), also known as bialaphos, was purchased as a 1 mg/mL aqueous solution from PhytoTechnology Laboratories. L-Ala-L-Ala-Glufos tripeptide (AAG) was synthesized (Scheme 2.1 and Figure 2.14–17).

$^1$H-NMR spectra were obtained using a Varian Unity Plus 300 MHz NMR spectrometer. LC-MS samples were prepared in 0.45 µ PTFE mini-UniPrep vials from Agilent. LC-MS was performed using an Agilent 6130 quadrupole with a model G1313 autosampler, a model G1315 diode array detector, and 1200 series solvent module. LC-MS separations were performed using a 5 µ Gemini C18 column (50 mm x 2 mm) from Phenomenex fit with a guard column. LC-MS mobile phases were 0.1% formic acid in (A) water and (B) acetonitrile. Samples were loaded in 10% B holding for 2 min, and a linear gradient was then formed to 100% B over 15 min followed by ramping to 10% B over 3 min at a flow rate of 0.5 mL/min. LC-MS data were processed using G2710 ChemStation software. Preparative high-performance liquid chromatography (HPLC) was performed using a Beckman Coulter SYSTEM GOLD 127P solvent module and a model 168 diode array detector using a Luna 10 µ C18(2) 100 Å column (250 mm x 21.2 mm) from Phenomenex fit with a guard column (15 mm x 21.2 mm). Mobile phases for RP-C18 prep-HPLC were 0.1% TFA in (A) water and (B) acetonitrile. Samples were loaded and eluted in 100% A (held for 15 min) at a flow rate of 10 mL/min. The column was
washed with 50% B for 10 min and re-equilibrated with 100% A prior to the next run. For preparative HILIC, the column was a Luna 5 μ HILIC 200 Å column fit with a guard column (15 mm x 21.2 mm). HILIC HPLC mobile phases were 5 mM ammonium formate (pH 3.2) (pH adjusted with aqueous HCl) in (A) a 90:10 acetonitrile/water mixture and (B) a 50:50 acetonitrile/water mixture. Samples dissolved in a 1:1 EtOH/water mixture were loaded in 20% B holding for 10 min, and a linear gradient was formed to 60% B over 20 min followed by a re-equilibration to 20% B at a flow rate of 12 mL/min. HPLC data were processed using 32 Karat software, version 7.0. Protein purification was performed on an AKTA Explorer 100 FPLC with ultraviolet–visible (UV–vis) detection using a Sephadex 75 26/60 HiLoad prep grade gel filtration column purchased from GE Healthcare. DNA and protein concentrations were determined using a NanoDrop 2000 UV–vis spectrophotometer from Thermo Fisher Scientific. Protein extinction coefficients were determined using the ExPasy ProtParam tool. UV–vis spectrophotometry was performed in 1 cm quartz cuvettes on an Agilent Cary 50 fit with an autosampler and water Peltier thermostat system. Bacterial growth studies were performed using polystyrene 96-well plates with polystyrene lids with OD₆₀₀ measurements that were taken using a SpectraMax Plus 384 plate reader from Molecular Devices. High-resolution LC-MS/MS spectra were collected using a Q-Exactive instrument (Thermo-Fisher Scientific) equipped with a custom-built Eksigent microLC instrument at the Donald Danforth Plant Science Center (St. Louis, MO). The solvents were 0.1% formic acid in (A) water and (B) acetonitrile. The column was a 0.5 mm x 150 mm Supelco C8 column. A flow rate of 15 μL/min was held constant while a solvent gradient (2% B held for 3 min, then ramped to 100% B over 11 min, then held at 100% B for 4 min, then ramped to 2% B over 1 min, and re-equilibrated at 2% B for 6 min) was formed. The mass spectrometer was operated in polarity switching mode and scanned from m/z
200 to 500 at a resolution setting of 70000 (at m/z 200) for MS₁ and a resolution of 17500 for MS₂.

**Expression and Purification of GS**

Constructs of codon-optimized *GlnA* in a pET28a vector encoding *N*-His₆-tagged GS from *E. coli*, *S. aureus*, and *H. sapiens* were transformed into electrocompetent *E. coli* BL21-Gold(DE3) via electroporation ([Tables 2.2 and Table 2.5](#)). Overnight cultures of *E. coli* BL21 grown at 37 °C harboring the appropriate plasmid were grown in LB broth containing 50 μg/mL kanamycin, and 200 μL was used to inoculate 1L batches of sterile Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 5 g/L glycerol, 17 mM KH₂PO₄, and 72 mM K₂HPO₄) containing 50 μg/mL kanamycin. The cultures were grown at 37 °C while being agitated to an OD₆₀₀ of ~0.9 and then cooled to 16 °C prior to induction with 1 mL of 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM final concentration). Cultures were then grown for ~18 h at 16 °C while being agitated. Cells were harvested by centrifugation (5000 rpm for 20 min at 4 °C). Cells and protein solutions were kept at ~4 °C for all remaining steps. Cell pellets were suspended in 40 mL of lysis buffer (50 mM K₂HPO₄, 500 mM NaCl, 5 mM β-mercaptoethanol, 20 mM imidazole, and 10% glycerol, adjusted to pH 8.0) and flash-frozen in liquid nitrogen. After thawing, cells were mechanically lysed using an Avestin EmulsiFlex-C5 high-pressure homogenizer. The cell lysate was clarified via ultracentrifugation (45000 rpm for 35 min) and incubated for 30 min with Ni-NTA resin preconditioned with lysis buffer. The Ni-NTA resin was washed twice with 40 mL aliquots of lysis buffer. For human enzyme, the resin was eluted in five separate washes with 10 mL of elution buffer (50 mM K₂HPO₄, 500 mM NaCl, 5 mM β-mercaptoethanol, 300 mM imidazole, and 10% glycerol, adjusted to pH 8.0). Fractions
containing pure N-His$_6$-GS as judged by SDS-PAGE analysis (Figure 2.5) were combined, dialyzed into 100 mM HEPES (pH 7.4), concentrated via centrifugal filtration, flash-frozen in liquid nitrogen, and stored at -80 °C to give stocks ready for in vitro biochemical assays. For E. coli and S. aureus enzymes, the resin was washed several times with snake venom buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, and 10 mM MgCl$_2$, adjusted to pH 8.0) and then treated with 20 mL of snake venom buffer containing 1 mg/mL crude phosphodiesterase from C. adamanteus while being gently rocked at 20 °C for 12 h. The resin was washed thoroughly with lysis buffer and then eluted in five separate washes with 10 mL of elution buffer. Fractions containing pure N-His$_6$-GS as judged by SDS-PAGE analysis (Figure 2.5) were combined, dialyzed into 100 mM HEPES (pH 7.4), concentrated via centrifugal filtration, flash-frozen in liquid nitrogen, and stored at -80 °C. Typical protein preparations yielded 3–5 mL of E. coli, S. aureus, and human N-His$_6$-GS at concentrations of ~45–150 μM (~6–30 mg/L of induced E. coli BL21 culture).

Purification of TβL and TβL-Thr

Caution: TβL, MetSox, Glufos, and formulations thereof are potentially toxic if ingested. Personal protective equipment and caution should be used when handling solutions containing these compounds. P. syringae pv. tabaci ATCC 11528 is a known plant pathogen and requires a U.S. Department of Agriculture permit for laboratory use. Live cultures should be sterilized using a calibrated autoclave prior to disposal.

A colony selection protocol was followed prior to starting TβL-Thr production cultures. A glycerol stock of P. syringae pv. tabaci ATCC 11528 was used to inoculate a 5 mL culture of Difco Nutrient Broth grown overnight at 26 °C. The overnight culture was diluted
1000–100000-fold onto Difco nutrient agar plates that were grown overnight at 26 °C. Single colonies (~20) were selected and grown in fresh Difco Nutrient Broth overnight at 26 °C. A portion of each culture was used to prepare a fresh glycerol stock stored at -80 °C and to inoculate 5 mL cultures in Woolley’s medium (10 g/L sucrose, 5 g/L KNO₃, 0.8 g/L K₂HPO₄, 0.8 g/L NaH₂PO₄·H₂O, 0.2 g/L MgSO₄·7H₂O, 0.1 g/L CaCl₂·2H₂O, 20 mg/L FeSO₄·7H₂O) that were grown at 26 °C for 96 h. Each 5 mL culture was analyzed for OD₆₀₀, pH, activity of the filter-sterilized supernatant against E. coli in an agar diffusion antibacterial susceptibility assay, the presence of TβL-Thr in the supernatant detected by LC-MS, and DNA precipitation upon 1:1 dilution in EtOH (more DNA precipitation correlated with more TβL-Thr production). The glycerol stock of the most promising P. syringae culture was used to inoculate a fresh overnight culture in Nutrient Broth at 26 °C. The Nutrient Broth culture was used as inoculum (200 μL) for 0.5 L cultures in Woolley’s medium in 2.8 L baffled flasks grown for 96 h while being shaken (225 rpm) at 26 °C. Cells were pelleted by centrifugation for 20 min at 5000 rpm and 4 °C. The supernatant was diluted 1:1 with EtOH to precipitate DNA, and 1 M HCl was added to allow the pH to reach ~4 to slow TβL-Thr isomerization. The remainder of the TβL-Thr purification was performed as described in our previous publication [ion exchange chromatography using Dowex 50WX8-200 cation exchange, then preparative HPLC using a HILIC column (typically elutes at retention time of 14–17 min), and finally preparative HPLC using a RP-C18 column (typically elutes at retention time of 7–9 min)].¹¹ Stock solutions of TβL-Thr were quantified by ¹H-NMR using an acetonitrile internal standard and stored frozen at -80 °C as lyophilized solids or as an aqueous solution at pH <4 (Figure 2.4). [¹⁵N]TβL-Thr was prepared exactly as described for TβL-Thr using Woolley’s medium prepared with K¹⁵NO₃. Purification of [¹⁵N]TβL-Thr was
performed only through preparative HPLC using a HILIC column (typically elutes at retention time of 12–14 min) prior to conversion to $[^{15}\text{N}]\text{T}\beta\text{L}$. Both $[^{15}\text{N}]\text{T}\beta\text{L}$-Thr and $\text{T}\beta\text{L}$-Thr were converted to $[^{15}\text{N}]\text{T}\beta\text{L}$ and $\text{T}\beta\text{L}$, respectively, by treatment with lysate from $P. \text{syringae pv. tabaci}$ ATCC 11528 grown in Woolley’s medium supplemented with 10 $\mu$M ZnCl$_2$, as described in our previous publication.$^{11}$ $[^{15}\text{N}]\text{T}\beta\text{L}$ and $\text{T}\beta\text{L}$ were purified by preparative HPLC using a HILIC column, and stock solutions were quantified by titration with L-Thr and the amino acid ligase TblF from the TβL-Thr biosynthetic gene cluster, as described in our previous publication.$^{11}$ Standardized solutions of $[^{15}\text{N}]\text{T}\beta\text{L}$ and $\text{T}\beta\text{L}$ were stored at -80 °C at pH <4.

GS Michaelis–Menten Kinetics

All experiments were performed at 37 °C in quartz cuvettes under steady state conditions with continuous monitoring at 350 nm in an Agilent Carey 50 UV–vis spectrophotometer. Glufos was racemic, and reported concentrations reflect only the L-enantiomer.$^{34}$ For all experiments, the total reaction volume was 500 $\mu$L and contained 100 mM HEPES (pH 7.4), 100 mM KCl, 25 mM MgCl$_2$, 10 mM NH$_4$Cl, 0.5 mM PEP, 0.2 mM NADH, 0.2 unit of PK, 0.3 unit of LDH, and 10 nM GS. For determination of the apparent $K_m$ for ATP, reaction mixtures contained 50 mM L-Glu and variable concentrations of ATP ranging from 0.5 to 5.0 mM. For determination of the apparent $K_m$ of L-Glu, reaction mixtures contained 10 mM ATP and variable concentrations of L-Glu ranging from 1 to 55 mM. GS was added last to initiate the reaction. Reaction velocities ($k_{obs}$ in absorbance per minute) were determined by calculating the slope of the linear region of the 350 nm absorbance versus time plot with background correction for control reactions lacking GS.$^{107}$ Kinetic constants were determined
from \( k_{\text{obs}} \) versus substrate concentration data using a nonlinear, least-squares fitting method with GraphPad Prism, version 7.0a fit to the Michaelis–Menten equation (eq 1)

\[
k_{\text{obs}} = \frac{k_{\text{cat}}[S]}{K_m + [S]}
\]

(1)

where \( k_{\text{cat}} \) is the maximal velocity, \([S]\) is the substrate concentration, and \( K_m \) is the substrate concentration corresponding to \( k_{\text{obs}} = 1/2k_{\text{cat}} \). All reactions were performed in triplicate as independent trials (Figure 2.6).

**GS Kitz–Wilson Kinetics**

All experiments were performed at 37 °C in quartz cuvettes under steady state conditions with continuous monitoring at 350 nm in an Agilent Carey 50 UV–vis spectrophotometer. MetSox was purchased from Sigma-Aldrich as the L-enantiomer. TβL isolated from *P. syringae* cultures was the L-enantiomer. The total reaction volume for experiments with MetSox and Glufos was 500 μL. To conserve sample, the total reaction volume for experiments with TβL was 400 μL. Each reaction mixture contained 100 mM HEPES (pH 7.4), 100 mM KCl, 25 mM MgCl₂, 10 mM NH₄Cl, 0.5 mM PEP, 0.2 mM NADH, 0.2 unit of PK, 0.3 unit of LDH, 50 mM L-Glu, and enough GS to give a starting rate close to -0.05 absorbance unit/min at 350 nm, yielding a straight line for ~10 min without consuming all NADH (GS stocks are prepared in advance from frozen concentrated stocks the day of inhibition assay). Control reactions with either no L-Glu or no inhibitor were used to correct for nonspecific ATPase activity and NADH degradation. An appropriate preincubation time of the enzyme and inhibitor was established by investigating the time dependence of GS inhibition by TβL, MetSox, and racemic Glufos. The percent GS activity relative to a no inhibitor control was
measured for inhibitor concentrations ranging from 1 to 100 μM using preincubation times of 0–60 min. No ATP controls were included to demonstrate the requirement of ATP for GS inhibition. Kinetic inhibition constants were determined from \( k_{\text{app}} \) versus inhibitor concentration data using a linear fitting method with GraphPad Prism, version 7.0a, fit to the Kitz–Wilson equation for irreversible enzyme inhibition (eq 2)\(^{58}\)

\[
\frac{1}{k_{\text{app}}} = \frac{1}{k_{\text{inact}}} + \frac{K_I}{k_{\text{inact}}} \frac{1}{[I]} \tag{2}
\]

where \( k_{\text{inact}} \) is the rate constant for conversion of the reversible enzyme-inhibitor complex to the irreversible complex, \([I]\) is the inhibitor concentration, and \(K_I\) is the dissociation constant for the initial reversible enzyme-inhibitor complex. The parameter \( k_{\text{app}} \) is defined by equation 3

\[
k_{\text{app}} = \frac{t}{\ln \left( \frac{v_I}{v_0} \right)} \tag{3}
\]

where \(t\) is the preincubation time, \(v_I\) is the rate with inhibitor, and \(v_0\) is the rate without inhibitor. Preincubation times for Kitz–Wilson kinetic studies were chosen to give linear plots of \(1/k_{\text{app}}\) versus \(1/[I]\) with inhibitor concentrations above and below the \(K_I\) value. Inhibitor concentrations were varied between 0.33 and 100 μM, and preincubation times were typically between 2 and 10 min. All reactions were performed in triplicate as independent trials.

**Bacterial Growth Assays**

A filter-sterilized, chemically defined minimal media [7 g/L \(K_2HPO_4\), 3 g/L \(KH_2PO_4\), 0.47 g/L sodium citrate dehydrate, 0.1 g/L \(MgSO_4\cdot7H_2O\), 1 g/L \((NH_4)_2SO_4\), 4 g/L glucose, 20 mg/L thymine, 0.1 mg/L biotin, 2 mg/L thiamine, 2 mg/L nicotinic acid, 2 mg/L calcium]
pantothenate, and 10 mg of MnSO₄•7H₂O (pH ~7.2) was used for all growth assays with *E. coli* ATCC 29522. Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method following guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI). Each well of a 96-well plate was filled with 50 μL of sterile minimal medium; 50 μL of TβL-Thr or AAG solution in minimal medium was added to the first well of the 96-well plate and diluted 2-fold down each row, and 50 μL of the *E. coli* ATCC 25922 inoculum (5 x 10⁵ cfu/mL in minimal medium) was added to each well giving a final volume of 100 μL/well. The final concentration gradient of TβL-Thr and AAG was from 128 to 0.0625 μM. The same procedure was used for studies with L-Glu or L-Gln supplementation with the appropriate amount of amino acid added to the minimal medium. Controls with no antibiotic or kanamycin (MIC = 8 μM) were included on every plate. Plates were covered with lids and incubated at 37 °C for 18–24 h until the MIC could be judged as the lowest concentration of antibiotic required to inhibit visible bacterial growth relative to the no antibiotic control. All MICs were measured in triplicate from independent rows on the same 96-well plate.

The same chemically defined minimal medium was used to measure growth curves of *E. coli* ATCC 29522 in the presence of 5 mM L-Glu or variable concentrations of L-Gln (0.1–5 mM). TβL-Thr was evaluated at final concentrations of 2 μM and 100 μM. AAG was evaluated at a final concentration of 2 μM. The final working volume in each well of the 96-well plate was 100 μL. Covered plates were incubated at 37 °C for ≤ 70 h with OD₆₀₀ measurements taken every 2 h with plate agitation prior to each measurement. All OD₆₀₀ measurements were taken in triplicate from independent wells on the same 96-well plate.

*Detection of TβL-P₅ by LC-MS*
GS-inhibitor complexes were formed by incubating purified N-His$_6$-GS from *E. coli* (84 μM) with 100 μM inhibitor (TβL, [¹⁵N]TβL, MetSox, and racemic Glufos), 100 mM HEPES (pH 7.4), 10 mM ATP, 100 mM KCl, and 25 mM MgCl$_2$ at a final volume of 500 μL for 1 h at 37 °C. Full inhibition of GS was confirmed using the PK/LDH coupled spectrophotometric kinetic assay. Control experiments without ATP in the reaction mixture were also performed for each inhibitor. The solution was then applied to a 30K MWCO spin column and concentrated to a final volume 200 μL. The solution was then diluted with 300 μL of water and again concentrated to 200 μL. This washing was repeated 10 times to remove all unbound inhibitor and co-substrates. A 200 μL solution of the GS-inhibitor complex was transferred to a microcentrifuge tube and treated with 300 μL of MeOH and 20 mM KCl (final concentration) to induce protein precipitation. The mixture was incubated at 60 °C for 1 h, and the solution became cloudy. Solid particulates were removed via centrifugation, and the clear, colorless supernatant was transferred to a fresh 30K MWCO spin column. After the sample had been spun for 30 min at 5K rpm, the flow through (~500 μL) was collected and treated with 50 μL pH 8 sodium borate buffer, 105 μL acetonitrile, and 20 μL of 20 mM FmocCl. After 30 min at room temperature, the sample was analyzed by low-resolution LC-MS in positive ion mode to detect the presence of the phosphorylated and Fmoc-tagged inhibitors (Fmoc-TβL-Pi, Fmoc-[¹⁵N]TβL-Pi, Fmoc-MetSox-Pi, and Fmoc-Glufos-Pi). During our analysis, we found that Fmoc-Glufos-Pi was unstable and observed only peaks corresponding to the m/z value for Fmoc-Glufos as the corresponding [M+H]$^+$ ion. For low-resolution LC-MS experiments, we observed the following retention times and m/z values corresponding to [M+H]$^+$ ions: Fmoc-TβL-Pi [retention time of 12.5 min; MS (ESI) calculated for C$_{22}$H$_{24}$N$_2$O$_9$P 491.1 [M+H]$^+$, found 491.1], Fmoc-[¹⁵N]TβL-Pi.
[retention time of 12.7 min; MS (ESI) calculated for C_{22}H_{24}^{15}N_{2}O_{9}P 493.1 [M+H]^+, found 493.1], Fmoc-MetSox-P; [retention time of 9.7 min; MS (ESI) calculated for C_{20}H_{24}N_{2}O_{8}PS 483.1 [M+H]^+, found 483.1], and Fmoc-Glufos [retention time of 9.3 min; MS (ESI) calculated for C_{20}H_{23}NO_{6}P 404.1 [M+H]^+, found 404.1]. The Fmoc-TβL-P sample was further analyzed by high-resolution LC-MS/MS in positive and negative ion modes. In negative ion mode, we observed the following m/z values corresponding to [M-H]^- ions for Fmoc-TβL-P: HRMS1 (ESI) calculated for C_{22}H_{22}N_{2}O_{9}P 489.1068 [M-H]^-, found 489.1064; HRMS2 (ESI) calculated for loss of Fmoc C_{7}H_{12}N_{2}O_{7}P 267.0387 [M-H]^-, found 267.0381. In positive ion mode, we observed m/z values corresponding to [M+H]^+ ions for Fmoc-TβL: HRMS1 (ESI) calculated for C_{22}H_{24}N_{2}O_{9}P 491.1214 [M+H]^+, found 491.1230; HRMS2 (ESI) calculated for loss of Fmoc C_{7}H_{13}N_{2}O 189.0870 [M+H]^+, found 189.0877 (Figure 2.11).

Sample Preparation for Solid-State NMR

GS from *E. coli* was overexpressed in *E. coli* BL21 as described previously. During large-scale production of GS, the crude phosphodiesterase from *C. adamanteus* (eastern diamondback rattlesnake) was back-ordered from the supplier so crude phosphodiesterase from *C. atrox* (western diamondback rattlesnake) was substituted at this step. Treatment of N-His\textsubscript{6}-GS with *C. atrox* phosphodiesterase cleaved the adenyl groups along with the N-His\textsubscript{6} tag at the thrombin cleavage site. GS was dialyzed into SEC buffer [50 mM potassium phosphate, 150 mM NaCl, 1 mM DTT, and 5% glycerol (pH 8)] and purified on an AKTA Explorer 100 FPLC instrument with UV–vis detection using a Sephadex 75 26/60 HiLoad prep grade gel filtration column eluting with SEC buffer at a flow rate of 2 mL/min. Fractions containing pure GS as judged by SDS-PAGE analysis and activity in the biosynthetic assay were combined, dialyzed
into 100 mM HEPES buffer at pH 7.4, and concentrated via centrifugal filtration to ~6–8 mg/mL. A 10 mL aliquot of 62.8 mg GS in 100 mM HEPES buffer (pH 7.4) was treated with excess [\[^{15}\text{N}\]T\(\beta\)L in the presence of 10 mM ATP, 100 mM KCl, 25 mM MgCl\(_2\), and 10 mM NH\(_4\)Cl at 37 °C for 2 h (final volume of 20 mL). Full GS inhibition was confirmed using the coupled PK/LDH assay under biosynthetic reaction conditions. The solution was transferred to a 30K MWCO spin filter and diluted with trehalose buffer [5 mM HEPES and 15 mM trehalose (pH 7.4)] prior to centrifugation at 5K rpm and 4 °C. To fully desalt the sample, the dilution and centrifugation process was repeated 10 times until the sample was ultimately concentrated to ~9 mL in trehalose buffer. The sample was treated with PEG 8000 (28.1 mg), 100 kDa dextran (6 mg), and 500 kDa dextran (6 mg) prior to controlled freezing. The sample was frozen by being chilled in a -10 °C ice/CaCl\(_2\) bath, freezing in a -70 °C dry ice/acetone bath, and further freezing in a liquid nitrogen bath.\(^{41}\) The frozen sample was lyophilized for 72 h to provide a fluffy white powder that was used in solidstate NMR experiments. The final sample components were the GS-ADP-[\[^{15}\text{N}\]T\(\beta\)L-P\(_\text{i}\) inhibition complex (62.8 mg), trehalose (46.2 mg), PEG 8000 (28.1 mg), 100 kDa dextran (6 mg), 500 kDa dextran (6 mg), and HEPES (10.3 mg). A control sample was prepared under identical conditions with omission of [\[^{15}\text{N}\]T\(\beta\)L and replacement of ATP with ADP. The control sample contained the GS-ADP complex (71.5 mg), trehalose (46.2 mg), PEG 8000 (28.1 mg), 100 kDa dextran (6 mg), 500 kDa dextran (6 mg), and HEPES (10.3 mg).

**Solid-State NMR Parameters**

Experiments were performed at 12 Tesla with a six-frequency transmission line probe having a 12 mm long, 6 mm inner diameter analytical coil, and a Chemagnetics/Varian ceramic spinning module.\(^{79}\) Samples were spun using a thin wall Chemagnetics/Varian (Fort Collins,
CO, and Palo Alto, CA) 5 mm outer diameter zirconia rotor at 7143 Hz, with the speed under active control and maintained within ±2 Hz. A Tecmag (Houston, TX) Libra pulse programmer controlled the spectrometer. Two-kilowatt American Microwave Technology (AMT) power amplifiers were used to produce radiofrequency pulses for $^{31}$P (202.3 MHz) and $^{15}$N (50.7 MHz). The $^1$H (500 MHz) radio-frequency pulses were generated by a 2 kW Creative Electronics tube amplifier driven by a 50 W AMT amplifier. All final-stage amplifiers were under active control. The $\pi$-pulse lengths were 6 $\mu$s for $^{31}$P and $^1$H, and 9 $\mu$s for $^{15}$N. Proton-phosphorous (or nitrogen) matched cross-polarization transfers were made in 2 ms at 56 kHz. Proton dipolar decoupling was 100 kHz during data acquisition. The S and $S_0$ alternate-scan strategy compensated for short- and long-term drifts in REDOR experiments. Standard XY-8 phase cycling was used for all refocusing observe-channel $\pi$ pulses (inserted at the end of each rotor period during dipolar evolution) and dephasing $\pi$ pulses (inserted in the middle of each rotor period) to compensate for finite pulse imperfections. Typically, CPMAS spectra from 100 mg samples were the result of the accumulation of 20000-40000 scans at room temperature. The $P\{N\}$experiment involved the accumulation of 300000 scans (two weeks) for each spectrum.

**Computational Modeling of the GS-ADP-TβL-P$_i$ Complex**

Avogadro was used to construct the phosphorylated GS tetrahedral intermediate Gln-P$_i$ and TβL-P$_i$.$^{87}$ The GS active site was defined using the crystal structure of GS from *Salmonella typhimurium* [Protein Data Bank (PDB) entry 1FPY].$^{20}$ On the Basis of sequence alignments from Phyre2,$^{88}$ this structure was 98% identical to that of *E. coli* GS and is a suitable model for studying receptor-ligand interactions. Autodock Vina$^{89}$ was used for docking each ligand to the GS active site with ADP and both Mn$^{2+}$ ions included in the active site. The ligand
search space was defined using a box with dimensions 22 Å x 22 Å x 22Å centered on the Mn$^{2+}$ ion farthest from ADP. To confirm the usability of this search space, the Glufos ligand from the crystal structure (PDB entry 1FPY) was docked giving the original binding pose. Docking results were visualized using The PyMOL Molecular Graphics System version 1.7 from Schrödinger, LLC.

### 2.7 Acknowledgements

We thank A. D’Avignon (formerly at Department of Chemistry, Washington University in St. Louis; currently Sanford Burnham Medical Research Institute, Orlando, FL), J. Kao (Department of Chemistry, Washington University in St. Louis), and B. Marsden (Department of Chemistry, Washington University in St. Louis) for assistance in the acquisition of solution NMR spectra. We thank Dr. Brad Evans at the Proteomics & Mass Spectrometry Facility at the Donald Danforth Plant Science Center (St. Louis, MO) for assistance with the acquisition of the QTRAP LC-MS/MS spectra (supported by the National Science Foundation under Grant DBI-0521250). We thank Margaret Reck (Department of Chemistry, Washington University in St. Louis) for assistance purifying tabtoxin from *P. syringae* cultures. We thank Dr. Joe Jez and Cynthia Holland (Department of Biology, Washington University in St. Louis) for diligent efforts crystallizing GS and analyzing diffraction patterns at the Advanced Photon Source at Argonne National Laboratory.
### 2.8 Figures and Tables

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Inducible Gene/Marker</th>
<th>Origin/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas syringae</em> pv. <em>tabaci</em> ATCC 11528</td>
<td>None</td>
<td>Tabtoxin producer</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 29522</td>
<td>None</td>
<td>Antibiotic Susceptibility Testing</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>None</td>
<td>Cloning strain</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>None</td>
<td>Protein expression strain</td>
<td>Agilent</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>pET28a</td>
<td><em>GlnA</em> from <em>E. coli</em></td>
<td>This Work</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>pET28a</td>
<td><em>GlnA</em> from <em>S. aureus</em></td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>pET28a</td>
<td><em>GlnA</em> from <em>H. sapiens</em></td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>pET28a</td>
<td><em>GlnA</em> from <em>E. coli</em></td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>pET28a</td>
<td><em>GlnA</em> from <em>S. aureus</em></td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>pET28a</td>
<td><em>GlnA</em> from <em>H. sapiens</em></td>
<td>This work</td>
</tr>
</tbody>
</table>

*Table 2.1*: Strains and plasmids used in this work.
Gene

Source

Codon Optimized Nucleotide Sequence

GlnA

E. coli

ATGAGCGCGGAGCACGTGCTGACCATGCTGAACGAGCACGAAGTGAAGTTCGTTGACCTGCGTTTTAC
CGATACCAAGGGTAAAGAGCAGCACGTGACCATCCCGGCGCACCAAGTTAACGCGGAATTCTTTGAGG
AAGGTAAAATGTTTGACGGCAGCAGCATCGGTGGCTGGAAAGGCATTAACGAAAGCGACATGGTGCT
GATGCCGGATGCGAGCACCGCGGTTATCGACCCGTTCTTTGCGGATAGCACCCTGATCATTCGTTGCGA
TATTCTGGAGCCGGGTACCCTGCAGGGTTATGACCGTGATCCGCGTAGCATCGCGAAACGTGCGGAAG
ACTATCTGCGTAGCACCGGTATTGCGGATACCGTGCTGTTTGGTCCGGAGCCGGAATTCTTTCTGTTCG
ACGATATCCGTTTTGGTAGCAGCATTAGCGGCAGCCACGTTGCGATCGACGATATTGAGGGTGCGTGG
AACAGCAGCACCCAATACGAAGGTGGCAACAAGGGTCACCGTCCGGCGGTGAAAGGTGGCTATTTTCC
GGTGCCGCCGGTTGACAGCGCGCAGGATATCCGTAGCGAGATGTGCCTGGTTATGGAACAAATGGGTC
TGGTGGTTGAAGCGCACCACCATGAAGTTGCGACCGCGGGTCAGAACGAGGTTGCGACCCGTTTCAAC
ACCATGACCAAGAAAGCGGACGAAATCCAAATTTACAAGTATGTGGTTCACAACGTTGCGCACCGTTT
CGGCAAGACCGCGACCTTTATGCCGAAACCGATGTTCGGCGACAACGGTAGCGGCATGCACTGCCACA
TGAGCCTGAGCAAGAACGGTGTGAACCTGTTTGCGGGTGATAAATACGCGGGCCTGAGCGAGCAGGC
GCTGTACTATATCGGTGGCGTTATTAAGCACGCGAAAGCGATCAACGCGCTGGCGAACCCGACCACCA
ACAGCTACAAGCGTCTGGTGCCGGGTTATGAGGCGCCGGTTATGCTGGCGTATAGCGCGCGTAACCGT
AGCGCGAGCATCCGTATTCCGGTGGTTAGCAGCCCGAAAGCGCGTCGTATTGAAGTTCGTTTTCCGGAT
CCGGCGGCGAACCCGTATCTGTGCTTTGCGGCGCTGCTGATGGCGGGTCTGGATGGCATCAAGAACAA
AATTCACCCGGGCGAGGCGATGGACAAGAACCTGTATGATCTGCCGCCGGAGGAAGCGAAAGAAATT
CCGCAAGTGGCGGGCAGCCTGGAGGAAGCGCTGAACGAGCTGGACCTGGATCGTGAATTTCTGAAAG
CGGGTGGCGTTTTCACCGACGAAGCGATCGATGCGTACATTGCGCTGCGTCGTGAGGAAGACGATCGT
GTGCGTATGACCCCGCACCCGGTTGAGTTCGAACTGTACTATAGCGTTTAA

GlnA

S. aureus

ATGCCGAAACGTACCTTCACCAAGGAAGACATTCGTAAATTTGCGGAGGAAGAGAACGTGCGTTACCT
GCGTCTGCAGTTCACCGATATCCTGGGTACCATTAAGAACGTGGAAGTTCCGGTTAGCCAACTGGAAA
AAGTTCTGGACAACGAGATGATGTTCGATGGTAGCAGCATTGAGGGCTTTGTGCGTATCGAAGAGAGC
GACATGTACCTGCACCCGGACCTGGATACCTGGGTGATTTTTCCGTGGACCGCGGGTCAGGGCAAGGT
TGCGCGTCTGATCTGCGACGTGTATAAAACCGATGGTACCCCGTTTGAGGGCGATCCGCGTGCGAACC
TGAAACGTGTTCTGAAGGAAATGGAGGACCTGGGTTTCACCGATTTTAACCTGGGCCCGGAACCGGAG
TTCTTTCTGTTCAAACTGGACGAAAAGGGCGAGCCGACCCTGGAACTGAACGACGATGGTGGCTACTT
TGACCTGGCGCCGACCGATCTGGGTGAAAACTGCCGTCGTGATATTGTTCTGGAACTGGAGGACATGG
GTTTTGATATCGAAGCGAGCCACCATGAGGTTGCGCCGGGTCAGCACGAAATCGACTTCAAATATGCG
GATGCGGTGACCGCGTGCGACAACATTCAAACCTTTAAACTGGTGGTTAAGACCATCGCGCGTAAGCA
CAACCTGCACGCGACCTTCATGCCGAAACCGCTGTTTGGTGTTAACGGTAGCGGCATGCACTTCAACGT
GAGCCTGTTTAAGGGTAAAGAGAACGCGTTCTTTGATCCGAACACCGAAATGGGCCTGACCGAGACCG
CGTACCAATTCACCGCGGGTGTTCTGAAGAACGCGCGTGGCTTTACCGCGGTTTGCAACCCGCTGGTG
AACAGCTATAAACGTCTGGTGCCGGGTTACGAAGCGCCGTGCTATATTGCGTGGAGCGGCAAGAACCG
TAGCCCGCTGATCCGTGTTCCGAGCAGCCGTGGCCTGAGCACCCGTATTGAAGTTCGTAGCGTGGATCC
GGCGGCGAACCCGTACATGGCGCTGGCGGCGATTCTGGAAGCGGGTCTGGATGGCATCAAGAACAAA
CTGAAGGTTCCGGAGCCGGTGAACCAGAACATTTACGAAATGAACCGTGAAGAGCGTGAGGCGGTTG
GTATCCAAGACCTGCCGAGCACCCTGTATACCGCGCTGAAGGCGATGCGTGAAAACGAGGTGATCAAG
AAAGCGCTGGGCAACCACATCTATAACCAGTTCATTAACAGCAAAAGCATCGAATGGGACTACTATCG
TACCCAAGTGAGCGAATGGGAGCGTGATCAGTACATGAAGCAATATTAA

GlnA

H. sapiens

ATGACCACCAGCGCGAGCAGCCACCTGAACAAGGGCATCAAACAGGTGTACATGAGCCTGCCGCAGG
GTGAAAAGGTTCAAGCGATGTATATCTGGATTGACGGTACCGGCGAGGGTCTGCGTTGCAAGACCCGT
ACCCTGGATAGCGAACCGAAATGCGTGGAGGAACTGCCGGAGTGGAACTTCGACGGCAGCAGCACCC
TGCAAAGCGAAGGTAGCAACAGCGATATGTACCTGGTTCCGGCGGCGATGTTCCGTGACCCGTTTCGT
AAGGATCCGAACAAACTGGTGCTGTGCGAAGTTTTCAAGTATAACCGTCGTCCGGCGGAGACCAACCT
GCGTCACACCTGCAAACGTATCATGGACATGGTTAGCAACCAGCACCCGTGGTTTGGCATGGAGCAAG
AATACACCCTGATGGGCACCGATGGTCACCCGTTCGGTTGGCCGAGCAACGGTTTTCCGGGTCCGCAG
GGTCCGTACTATTGCGGTGTGGGTGCGGACCGTGCGTATGGCCGTGATATCGTTGAAGCGCACTACCG
TGCGTGCCTGTATGCGGGCGTGAAAATTGCGGGTACCAACGCGGAAGTGATGCCGGCGCAGTGGGAAT
TCCAAATCGGTCCGTGCGAGGGCATTAGCATGGGTGACCACCTGTGGGTTGCGCGTTTTATTCTGCACC
GTGTGTGCGAGGACTTCGGCGTTATCGCGACCTTTGATCCGAAGCCGATTCCGGGTAACTGGAACGGC
GCGGGTTGCCACACCAACTTCAGCACCAAGGCGATGCGTGAGGAAAACGGTCTGAAATACATCGAGG
AAGCGATTGAAAAGCTGAGCAAACGTCACCAATACCACATCCGTGCGTATGACCCGAAAGGTGGCCTG
GATAACGCGCGTCGTCTGACCGGCTTCCACGAGACCAGCAACATTAACGACTTTAGCGCGGGTGTGGC
GAACCGTAGCGCGAGCATCCGTATTCCGCGTACCGTTGGCCAGGAGAAGAAAGGTTACTTCGAAGACC

77


Table 2.2: Codon optimized glutamine synthetase genes used in this work.

<table>
<thead>
<tr>
<th>GS</th>
<th>ATP</th>
<th>L-Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$k_{cat}$ (min$^{-1}$)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.7 ± 0.1</td>
<td>7300 ± 300</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>1.8 ± 0.1</td>
<td>720 ± 20</td>
</tr>
<tr>
<td><em>H. sapiens</em></td>
<td>0.7 ± 0.1</td>
<td>2600 ± 100</td>
</tr>
</tbody>
</table>

*a All standard deviations were determined for three independent trials.

Table 2.3: Apparent Michaelis-Menten parameters for GS from *E. coli*, *S. aureus*, and *H. sapiens*.

<table>
<thead>
<tr>
<th>GS</th>
<th>TBL</th>
<th>MetSox</th>
<th>Glufos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$ (μM)</td>
<td>$k_{inact}$ (min$^{-1}$)</td>
<td>$k_{inact}/K_i$ (x10$^3$ min$^{-1}$μM$^{-1}$)</td>
</tr>
<tr>
<td>EC</td>
<td>1.7 ± 0.4*</td>
<td>0.3 ± 0.1</td>
<td>180 ± 70</td>
</tr>
<tr>
<td>SA</td>
<td>50 ± 10</td>
<td>0.4 ± 0.1</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>H</td>
<td>130 ± 40</td>
<td>0.3 ± 0.1</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

*a All standard deviations were determined for three independent trials.

Table 2.4: Apparent inactivation parameters for TBL, MetSox, and Glufos against *E. coli* (EC), *S. aureus* (SA), and *H. sapiens* (H) GS.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Primary Protein Sequences of N-His₆-GS Homologs*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlnA</td>
<td>E. coli</td>
<td>MGSSHHHHHHSSGLVPRGSHGlnA * E. coli MGSSHHHHHHSSGLVPRGSH MSAEHVTMLNEHVKEFVGLRFDKGEQHTIAPHQVNAEEFEGKMF DGSIGWGKINESMDMLPASTAVIDPFAADSTLJIRCDILEPGLQYDRPDRAKRAEDYLRSTGIAD TVLGFPEPEFFFLDDIRFGSSISGSHVAIDIEGAWNDSSTQYEKGNGKGRPAVKGGYFPVPPVDSAQDIRSEM CLVMEQGMLVVEAHHHEVATAQGNVEATRFTNMTKKADEIQYKYYVHNAHRFGKTATMPKPMFGD NGSQMHCHMSLSKGNVLFLAGDKYAGLSEQALYYIGGVKHKHAINALANPTTNSYKRLVPGYEAPVML AYSARNSASIRIPVSSPPKARRIEVRFPFDPAANPYLCFAALLMGALDGKNIHPGEAMDKNLYDLPEEA KEIPVVAGSLEELNEDLQDEFLKAGGVTDEAYAIALRREEDRVRMTPHPVEFELYYSV</td>
</tr>
<tr>
<td>GlnA</td>
<td>S. aureus</td>
<td>MGSSHHHHHHSSGLVPRGSHGlnA * S. aureus MGSSHHHHHHSSGLVPRGSH MPKRTFTKEDIRKFAEENVRYLQLQFTDILGTKNVEVPVSQLEKVLDNE MMFDGSSEIEEFVRIEDSMYLDHDLWTWIFPWTAQQGKVARLICDVKYKTDGTFEPPARLALKRVEK EDLGFDTFLGPEPEFLLKDEKPGTLELNDDGYYFDLAPTDLQNCRRDLVELEDGFDEISHEVA PGQHEIDFKYADAVTACDNITFCLIKVTKIARKHNLHATFMPKPLFGVNGSGMHFNVSLFKGKNAFFDPN TEMGLETAYQFTAGVNLKARGTFAVCNPLVSYKLRVPGYAEPCAYLASHGKNRPLRIVPSSRGLSTRIEV RSVDAANPYMALAAILEAGLDGKIKNLKVPEPVQNIYEMRREEAEVQIQDLPLSTLYTALKAMRENEV KKLQGNNHYQFINSKSEWIDYRTYRSEWSERDQYMKY</td>
</tr>
<tr>
<td>GlnA</td>
<td>H. sapiens</td>
<td>MGSSHHHHHHSSGLVPRGSHGlnA * H. sapiens MGSSHHHHHHSSGLVPRGSH MTTSASSHLKIGKQVYSMLPQGEKVQAMYWDDGETGELRCKTRTLDE PKCVEELPEWNPDSQSTLSQEGSNDSMYLPAAMFRDPFRKDPKLNVLCEVFKNRRPAAETNLHTCKRIM DMYVSNHPWFGQMEQYYTLMGTDGHPGWSPNGQPGPQYPYYCGVAGDRAYRGIDVEAHYACLAYAG VKIAITAIENMPAQWQFQGPGCEGSMQDHLWVARFILHWCDEFGV1ATDFKIPGWSWAGCETCHNST KAMREEENGLYIEEAIKLISKRHOHYRAYSHPGGLDNARLLTGGHETNINDFASGVAIRNSASIRIPRTVG QEKKGYFEDRRSPASCDDPSVTEALIRCTCLLLNETDEPFPQYKN</td>
</tr>
</tbody>
</table>

*Hexahistidine motif with thrombin cleavage site highlighted in yellow.

Table 2.5: Primary protein sequence of GS homologs used in this work.
Percent sequence identities were determined using Clustal W.

Table 2.6: Percent sequence identity of GS homologs.
Figure 2. 1: Two classes of β-lactam antibiotics. Pencillin G is a transpeptidase (TPase) inhibitor, and tabtoxinine-β-lactam (TβL) is a glutamine synthetase (GS) inhibitor.
Figure 2. Reactions catalyzed by glutamine synthetase (GS): (A) canonical biosynthetic reaction and (B) phosphorylation of inhibitors generating tight-binding transition state analogues.
Figure 2.3: Structures and abbreviations of compounds used in this work.
Figure 2.4: Representative $^1$H-NMR (300 MHz) of quantified tabtoxin (74 mM) after HILIC chromatography taken in D$_2$O with an acetonitrile (10 mM) internal standard. For full purification and characterization of tabtoxin see our previous publication$^{11}$. 
Figure 2.5: SDS-PAGE analysis of purified N-His$_6$-GS homologs used in this work. SDS-PAGE gels (Any kD, Bio-Rad) were loaded with protein ladder (Precision Plus Protein Dual Xtra Prestained Protein Standards, Bio-Rad) in lane 1 and Ni-NTA elutions for recombinant N-His$_6$-GS from *E. coli* (54.1 kD), *H. sapiens* (44.2 kD), and *S. aureus* (53.0 kD) in lanes 2,3, and 4, respectively. Gel was stained with Coomassie blue.
Figure 2.6: Michaelis-Menten plots for GS homologs.
Figure 2.7: Inhibition kinetics for TβL, MetSox, and Glufos against recombinant GS from (A–C) E. coli, (D–F) S. aureus, and (G–I) H. sapiens show time and ATP dependence and fit the Kitz-Wilson model for mechanism-based enzyme inhibition. Error bars represent standard deviations for three independent trials.
Figure 2.8: Primary sequence alignments of *E. coli*, *S. aureus*, and *H. sapiens* GS performed using Clustal W.
Figure 2.9: TβL-Thr and AAG show L-Gln-dependent bacteriostatic activity towards *E. coli* ATCC 25922. Panels show growth curves of *E. coli* ATCC 25922 in chemically defined minimal medium supplemented with (A) 2 μM TβL-Thr, (B) 100 μM TβL-Thr, and (C) 2 μM AAG and variable amounts of L-Gln and L-Glu. Error bars represent standard deviations for three independent trials.
Figure 2. 10: GS phosphorylates TβL, MetSox, and Glufos. Panels show extracted ion chromatograms for LC-MS analysis of denatured GS-inhibitor complexes, with (+) or without (-) ATP added, and after treatment with Fmoc chloride.
Fmoc-TiL [M+H]^+
Chemical Formula: C_{22}H_{32}N_8O_9^+
Exact Mass: 411.1551
Figure 2. 11: High-resolution MS and MS/MS of Fmoc-\(\beta\)L and Fmoc\(\beta\)L-Pi.
Figure 2.12: (a) 202-MHz $^{31}\text{P}$ cross-polarization magic-angle spinning NMR spectrum of a mixture of glutamine synthetase, buffer, and ADP. The resulting phosphate peak is assigned a 0 ppm chemical shift. (b) $^{31}\text{P}^{[15}\text{N}]$ full-echo and (c) rotational-echo double-resonance (REDOR) difference for a mixture of glutamine synthetase, buffer, ATP, and $^{15}\text{N}$-labeled TβL after dipolar evolution for 32 rotor periods (4.48 ms). (d) 50.7 MHz $^{15}\text{N}$ cross-polarization magic-angle spinning NMR spectrum of intact whole cells of *Staphylococcus aureus* whose cell walls labeled with D-$^{[15}\text{N}]$alanine. The chemical shift reference is solid ammonium sulfate. (To switch to a liquid ammonia nitrogen chemical shift scale, add 20 ppm.) (e) 50.3 MHz $^{15}\text{N}$ cross-polarization magic-angle spinning NMR spectrum of the sample used for panel b.
Figure 2.13: Model for glutamine synthetase complexes with the reaction tetrahedral intermediate Gln-P_i (A and C) and TβL-P_i (B and D). Panels A and B depict the glutamine synthetase active site formed between chain A and chain B with the top scoring docked poses of ADP, Gln-P_i, and TβL-P_i, shown as sticks with nitrogens in blue, oxygens in red, phosphorous in orange, and carbon in green (ADP) or teal (Gln-P_i and TβL-P_i). The two active site Mn^{2+} metals are shown as spheres colored sea green. The GS cartoon structure is shown in gray with selected amino acid site chains shown as navy blue line structures. The P–N through-space distances for Gln-P_i (2.9 Å) and TβL-P_i (4.2 Å) are highlighted as yellow dashed lines. Panels C and D show the two-dimensional interaction network between glutamine synthetase amino acid side chains and Gln-P_i or TβL-P_i for the top-scoring docked poses. The images in panels A and B were generated using PyMOL version 1.7.
Figure 2. 14: Prep-HPLC chromatogram for purification of Fmoc-AAG. x-Axis represents retention time (min). y-Axis represents absorbance at 254 nm.
Figure 2.15: LC-MS chromatograms of synthetic Fmoc-AAG. (A) Chromatogram of optical absorbance at 254 nm. (B) Total ion chromatogram (TIC). (C) Extracted ion chromatogram (EIC) for m/z = 546.2 corresponding to the [M+H]$^+$ ion of Fmoc-AAG.
Figure 2. 16: $^1$H-NMR (300 MHz) of AAG (piperidine salt) after Fmoc-deprotection in DMF. Spectrum was obtained in $D_2O$ with an acetonitrile internal standard.
Figure 2.17: LC-MS chromatograms of synthetic AAG. (A) Chromatogram of optical absorbance at 254 nm. (B) Total ion chromatogram (TIC). (C) Extracted ion chromatogram (EIC) for m/z = 324.1 corresponding to the [M+H]^+ ion of AAG.
AAG was synthesized in two steps from Fmoc-L-Ala-L-Ala and (±) Glufos. Fmoc-L-Ala-L-Ala (177 mg, 0.46 mmol) and iPr$_2$EtN (0.45 mL) were dissolved in CH$_3$CN (20 mL) and H$_2$O (5 mL). The clear, yellow solution was stirred at rt for 20 min before addition of (±) Glufos (30 mg, 0.15 mmol). After 27 h, the reaction mixture was concentrated under reduced pressure. The crude material was dissolved in 40 mL of 50:50 CH$_3$CN:H$_2$O and purified by RP-C18 prep-HPLC using a linear gradient of 5% (B) to 95% (B) over 20 min. Fmoc-AAG eluted at ~17 min and the identity was confirmed by LC-MS (retention time = 9.7 min, MS (ESI) calculated for C$_{26}$H$_{33}$N$_3$O$_8$P: 546.2 [M+H]$^+$, found 546.2). Fractions containing pure Fmoc-AAG were combined and concentrated under reduced pressure before treatment with 20 mL of 20% piperidine in DMF (formation of white vapor was observed). After 3 h at rt, the DMF/piperidine were removed via rotary evaporation to give the AAG piperidine salt (14.3 mg, 0.37 mmol) in 29% overall yield. AAG purity was analyzed by LC-MS (retention time = 0.7 min, MS (ESI) calculated for C$_{11}$H$_{23}$N$_3$O$_6$P: 324.1 [M+H]$^+$, found 324.1) and $^1$H-NMR (P-Me, 1.10 ppm, d, $J = 13.4$ Hz$^{109,110}$).

![Synthesis of L-Ala-L-Ala-Glufos (AAG).](image)

**Scheme 2.1** Synthesis of L-Ala-L-Ala-Glufos (AAG).
2.9 References


Chapter 3: Inhibition of Glutamine Synthetase GlnA1 Blocks Nitrogen Metabolism in *Mycobacterium tuberculosis*
3.1 Preface

This chapter was written by Luting Fang (LF) and edited by Timothy Adam Wencewicz (TAW). Funding for this research was provided by the Children’s Discovery Institute at St. Louis Children’s Hospital through Interdisciplinary Research Initiative grant MI-PD-II-2018-748. All BSL3 experiments using Mycobacterium tuberculosis were performed in the laboratory of Professor Christina Stallings at Washington University School of Medicine. All mouse experiments and GCMS metabolomics experiments were performed in the laboratory of Professor Jeffrey Henderson at Washington University School of Medicine.

3.2 Abstract

*Mycobacterium tuberculosis* (*Mtb*), the causative agent of Tuberculosis, remains to be one of the world’s leading causes of death. The emergence of multi-drug resistant (MDR) and extremely-drug resistant (XD) *Mtb* has stressed the urgent need for new second-line anti-*Mtb* agents that compensate for resistance to front-line agents (isoniazid, rifampin, ethambutol, and pyrazinamide). Glutamine synthetase (GS) is a promising anti-*Mtb* target that is essential for nitrogen metabolism in the infected phagosome. *Mtb* expresses four GS homologs encoded by the *glnA1-A4* genes. Only *glnA1* is required for survival in the host and the GlnA1 GS homolog is excreted into the phagosomal matrix. Inhibition of phagosomal GlnA1 (pGlnA1) by methionine sulfoximine (MetSox) was shown to be synergistic with isoniazid in clearing *Mtb* in a guinea pig infection model. Here, we explored the anti-*Mtb* activity of di- and tri-peptide pro-drug formulations of three GS inhibitors, MetSox, glufosinte (Glufos), and tabtoxinine-β-lactam (TβL), that gain access to cytoplasmic GlnA1 (cGlnA1) via peptide permeases followed by
peptidase cleavage in the cytoplasm. MetSox, Glufos, and TβL feature three distinct ‘warheads’ (sulfoximine, phosphinate, 3-hydroxy-β-lactam, respectively) appended to an L-Glu backbone. We utilized the Kitz-Wilson model for time-dependent mechanism-based inhibition to determine inhibition parameters for the three GS inhibitors to reveal a general trend of inhibition efficiency of phosphinate (Glufos) > 3-h sulfoximine (MetSox). We discovered that pro-drug formulations of the GS inhibitors potently inhibit Mtb replication in Gln-deficient media while free GS inhibitors have no growth inhibitory effects to validate cGlnA1 as a promising target in Mtb.

3.3 Introduction

Tuberculosis (TB) is one of the top 10 causes of death in the world and is the leading cause from a single infectious agent. According to the latest data from World Health Organization (WHO), there were 1.5 million deaths from TB in 2018. WHO reports that about a quarter of the world’s population carries the latent form of TB. The causative pathogen of TB is Mycobacterium tuberculosis (Mtb), a bacterium that resides and multiplies within a membrane-bound phagosome in human mononuclear phagocytes, most typically in lung macrophages. Treatment for TB typically takes 6 months through a combination of four first-line anti-Mtb drugs including isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA). Unfortunately, as a result of inappropriate use of anti-TB medicines, multidrug-resistant TB (MDR-TB) and extremely drug-resistant TB (XDR-TB) has emerged and is considered to be a serious threat to the clinical viability of front line therapeutics. Bedaquiline and pretomanid were recently FDA approved in 2012 and 2019, respectively, as novel treatments for MDR- and XDR-TB infections. More novel second-line anti-Mtb agents effective against resistant strains will be needed to keep up with the global spread of MDR- and XDR-Mtb.
One factor that increases the difficulty for developing new anti-*Mtb* drugs is that *Mtb*’s unique waxy cell wall can prevent antibiotic penetration required to reach the drug target. However, *Mtb* secretes a variety of to the extracellular space or intraphagosomes in human mononuclear phagocytes which are the bacterium’s primary host cells. Glutamine synthetase (GS) [L-glutamate: ammonia ligase (ADP-forming) EC 6.3.1.2] is one of the most abundant secreted proteins that is released extracellularly since its typical location is inside of the bacterial cytoplasm. Notably, only pathogenic mycobacteria, for example, *Mtb* and *Mycobacterium bovis*, release GS extracellularly in large amounts. However, nonpathogenic mycobacteria, such as *Mycobacterium smegmatis* and *Mycobacterium phlei*, as well as nonmycobacterial microorganisms like *Legionella pneumophila* and *Escherichia coli*, do not release GS extracellularly. It was reported that in pathogenic mycobacteria, there is a correlation between the release of GS and the presence of a poly-L-glutamate/glutamine (PLG) component in the cell walls (Figure 1.6) where nonpathogenic mycobacteria lack this PLG component. This correlation suggests that GS’s extracellular presence not only is a vital factor for PLG synthesis and incorporation into *Mtb* cell walls, but also is crucial to virulence. Study showed a lack of PLG component and decrease in cell wall strength caused by limiting the expression of GS in *M. bovis*. Besides virulence, PLG formation has been associated with biofilm formation, which could decrease antibiotic potency and lead to persistent infection. Given the fact that *Mtb* in human phagosome has limited access to both intracellular and extracellular sources of L-glutamine caused by impermeability, extracellular GS plays a crucial role in supplying substrate glutamine required for biosynthesis of the PLG cell wall component. Hence, inhibition of extracellular GS represents an attractive therapeutic strategy for treating *Mtb* infections where the GS inhibitors do not need to permeate the waxy *Mtb* cell envelope.
In addition to supplying glutamine for extracellular PLG biosynthesis and incorporation into the *Mtb* cell wall, extracellular *Mtb*GS also influences ammonia levels (and pH) in phagosomes of infected host cells\(^\text{15}\), allowing the pathogen to prevent phagosome-lysosome fusion\(^\text{16}\) and phagosome acidification\(^\text{8, 17}\). Although the extracellular GS aids in the survival of *Mtb* inside of the phagosome, it also makes the bacteria susceptible to antibiotics since drugs targeting at GS do not need to get into the cell by penetrating the formidable cell wall of *Mtb*. Besides that, low similarity between *Mtb* and human GS also contributes to the focus of GS being a promising target for new anti-tuberculosis drug design (only about 24% amino acid sequence identity (Table 3.1)). Inhibitors against GS already show good inhibition on *Mtb* growth both *in vitro* (human macrophage) and *in vivo* (guinea pigs) based on earlier studies\(^\text{10, 14, 18}\).

GS is a critical enzyme in bacterial nitrogen metabolism due to its role in catalyzing the condensation of ammonia and glutamate into glutamine (Reaction 3.1), which functions as the nitrogen donor for many crucial molecules such as pyrimidines, purines, amino acids, carbamoyl phosphate and glucosamine-6-phosphate\(^\text{19}\). In *Mtb*, other than glutamate synthetase assimilating ammonia under nitrogen-limiting growth conditions, GS is the only known enzyme to facilitate the biosynthesis of glutamine\(^\text{20}\). For now, four types of GSs expressed by different organisms were discovered\(^\text{21}\), and *Mtb* can only express GS type I (GSI) enzyme, which is a dodecamer (twelve identical 53-kDa subunits) composed of two hexameric rings in a face-to-face arrangement. Unlike bacteria, mammalian such as human express Type II GS that typically functions as a decamer formed of two pentameric rings stacked on top of each other\(^\text{22}\). Although the quaternary structures are different, active sites are conserved in these two species of GSs, which are formed between each of the two adjacent subunits\(^\text{23, 24}\). The active site of GS is shaped
as a “bifunnel” with the narrow ends connected in the middle, where two to three metal ions (Mg$^{2+}$ or Mn$^{2+}$) are located$^{25}$. The ATP comes into the active site from the top funnel, followed by the entrance of a glutamate molecule and an ammonium ion from the bottom funnel. The active site is then closed by side chain movement and shielded from water to enable Reaction 3.1 happen but not unwanted hydrolysis (Figure 3.1). At last, glutamine is released from the active site by side chain movement and a new cycle will start.

As noted earlier, the function of GS in bacteria is to fix and assimilate ammonia to glutamine in order to supply a nitrogen source for metabolism. Unlike bacteria, humans are able to obtain glutamine from their diet, so GS in humans has different roles, which vary depending on its localization. For example, GS in the brain regulates concentration of toxic ammonia and supplies neurons with glutamine, which was converted from neurotoxic glutamate.$^{26, 27}$ On the other hand, GS in the liver is responsible for detoxifying the ammonia that escapes from the urea cycle. Although the purpose of GS is different, the enzyme catalytic mechanism is the same across different forms of GS,$^{28, 29}$ so ideal inhibitors of the $Mtb$GS must not have substantial effects on the human GS$^{18}$.

As for now, the most popular GS inhibitors are glutamate analogues, with L-methionine-S-sulfoximine (MetSox or MSO) and phosphinothricin (Glufos) (Figure 3.2 a,b) being the most famous representatives.$^{30}$ MetSox was first discovered in nitrogen chloride treated zein$^{31}$, and Glufos is a natural product of $Streptomyces viridochromogenes$$^{32}$. They are phosphorylated within the GS active site and function as the natural transition state (Glu-Pi) analogues$^{33, 34}$. As a result of the inability for ammonia to replace phosphate in these analogues, the enzyme cannot open up the active site to start a new cycle and consequently comes to a dead end. Although high doses of MSO can cause convulsions as well as death in some kinds of experimental animals$^{35-39}$,
administering low concentrations of MSO can mitigate the epileptic seizures.\textsuperscript{40} MSO shows high selectivity for the \textit{Mtb}GS, which is about 100-fold more active than the human GS\textsuperscript{10, 18}. Studies \textit{in vitro} and \textit{in vivo} have detailed the efficacy of MSO and its ability to inhibit growth of \textit{Mtb} by selectively blocking the formation of the PLG component in \textit{Mtb} cell walls\textsuperscript{10, 18, 41}. Compounds that are able to interact with the ATP binding pocket such as aminomethylenebisphosphonic acid derivatives have also been developed as inhibitors (\textbf{Figure 3.2 d})\textsuperscript{42, 43}. More recently, it was discovered that aromatic derivatives of imidazole functionality can be served as inhibitors of bacterial GS by competing with ATP (\textbf{Figure 3.2 e})\textsuperscript{44, 45}.

Here, we will describe our investigations of a novel inhibitor of \textit{Mtb}GS: Tabtoxinine-\beta-lactam (Tβl) (\textbf{Figure 3.2 e}), which is another glutamate antimetabolite like Glufos and MetSox with a structurally unique beta-lactam ‘warhead’. Tβl is a monocyclic 3-hydroxy-\beta-lactam (3-HβL) naturally produced by plant pathogenic strains that are pathovars of tobacco, such as \textit{coronofaciens}, \textit{garcae}, and \textit{P. syringae pv tabaci}\textsuperscript{46}, as well as some soil \textit{Streptomyces}\textsuperscript{47, 48}. These strains produce and secrete a dipeptide prodrug called tabtoxin, commonly known as wildfire toxin\textsuperscript{49}, which is an inactive precursor of the free Tβl metabolite\textsuperscript{47, 50}. The structure of tabtoxin consists of a nonclassical amino acid Tβl along with a traditional amino acid, typically L-Thr, but also L-Ser as a minor variant. Tabtoxin can release free Tβl by peptidase action either when being secreted through the producer strain’s periplasm or during the uptake process by plant cells (\textbf{Figure 3.3}).\textsuperscript{51-53} Tβl is different from traditional \beta-lactam antibiotics in that it does not inhibit bacterial transpeptidases, which is an attractive trait that results in its evasiveness from \beta-lactamases. Instead, Tβl is a potent and mechanism-based inhibitor against GS\textsuperscript{54, 55} by being misrecognized as glutamate and causes the time- and ATP-dependent irreversible inactivation.\textsuperscript{54-56}

In Chapter 2, we showed that Tβl inhibits GS by the same mechanism as MetSox and Glufos
by undergoing phosphoryl group transfer from ATP on the 3-hydroxyl group of the 3-HβL ‘warhead’ in the GS enzyme active site to form a noncovalent but stable phosphorylated transition state analogue.\(^5\)

The \textit{in vitro} data presented in Chapter 2 proves that Tβl efficiently inhibits E.coli GS with a weaker inhibitory effect on human GS. We hypothesized that Tβl could be a potent inhibitor of \textit{Mtb}GS and developed into an anti-TB drug. In this Chapter, we investigate Tβl’s inhibitor potency against recombinant \textit{Mtb}GS \textit{in vitro} as well as the ability of the prodrug form, tabtoxin, to inhibit whole cell \textit{Mtb} growth in liquid culture and biofilms. We also performed \textit{in vivo} toxicity tests using a mouse model with both high and low dose of prodrug tabtoxin revealing no induction of neurological problems. Given the good selectivity and low toxicity for \textit{Mtb}GS compared to human GS, we conclude that Tβl is promising \textit{Mtb}GS inhibitor and prodrug tabtoxin is a promising anti-TB agent selectively targeting GS to disrupt global nitrogen metabolism in pathogenic \textit{Mtb}.

\section*{3.4 Results and Discussion}

\textit{Heterologous Expression of Recombinant MtbGS (GlnA1)}

As mentioned earlier, \textit{Mtb} only expresses one type of GS, which is GS type I (GSI), and there are four \textit{glnA} genes (\textit{glnA1}, \textit{glnA2}, \textit{glnA3}, and \textit{glnA4}) in \textit{Mtb} encoding the GSI orthologs (GlnA1, GlnA2, GlnA3, and GlnA4). GlnA1 accounts for the majority of GS activity and is the only one essential GS ortholog required for \textit{Mtb} growth in hosts.\(^8,58,59\). At least four mechanisms are involved in GlnA1 regulation, one of which is adenylylation/deadenylylation on a conserved Tyr residue. Specifically, the site for adenylylation in \textit{Mtb}GS is Tyr406 side chain in each subunit of the enzyme,\(^60-64\), which was proven by the loss of adenylylation in Y406F mutant.\(^65\).
Instead of GSI, humans express GS type II enzyme, which is not regulated by the adenylylation mechanism because GSII lacks an adenylylation loop\textsuperscript{28}, making adenylylated bacterial GS an interesting topic. The common belief is that the activity of GS is inversely related to the adenylylation degree\textsuperscript{66} depending on the availability of nitrogen and carbon\textsuperscript{67}. Adenylylated GS tends to predominate under carbon-limited and nitrogen-rich conditions, while the deadenylylated GS predominates in nitrogen limited conditions\textsuperscript{67}. The number of adenylylated residues may vary between 0 to 12 since each subunit of the dodecamer has a chance for adenylylation. Adenylyltransferase (ATase), encoded by the gene \textit{glnE}, is one of the proteins used in regulation of GS by adenylylation. Disruption of \textit{glnE} in \textit{Mtb} results in a lethal phenotype\textsuperscript{68, 69}. \textit{E. coli} GlnE was shown to catalyze the adenylylation, group transfer of adenosine monophosphate (AMP) from ATP, of the Tyr406 side chain on each \textit{Mtb}GS subunit of the dodecamer, which aligns with the homologous residue Tyr397 in \textit{E. coli} GS. Global adenylylation of \textit{Mtb}GS resulted in a strong decrease in the catalytic activity of the enzyme for the biosynthetic reaction\textsuperscript{70-72}. Although the net regulatory effect of transcriptional control and post-translational modification is similar between \textit{Mtb} and \textit{E. coli} GS, \textit{E. Coli} GlnE is not particularly efficient in the adenylylation of recombinant \textit{Mtb}GS expressed in an \textit{E. coli} heterologous host. Treatment of recombinant \textit{Mtb}GS with \textit{E. coli} GlnE in the presence of excess ATP resulted in adenylylation of ~25% of \textit{Mtb}GS subunits where analogous conditions yielded 100% adenylylation of \textit{E. coli} GS\textsuperscript{65}.

We obtained fully deadenylylated \textit{E. coli} GS (DA-\textit{Ec}GS) by expressing the \textit{E. coli} GS from \textit{E. coli} BL21-Gold (DE3), followed by purification and hydrolysis of adenylylated subunits with snake venom phosphodiesterase as described in Chapter 2\textsuperscript{57}. \textit{E. coli} GlnE, which was used to obtain fully adenylylated \textit{E.coli} GS (A-\textit{Ec}GS), was expressed from \textit{E.coli} BL21-Gold (DE3)
with an N-hexahistidine tag and purified by Ni-NTA (Figure 3.4). The incubation of DA-EcGS and GlnE gave apparent quantitative conversation to A-EcGS, which showed extremely low activity for the GS biosynthetic reaction. *Mtb*GS (Table 3.2) was expressed from pCRT7/CT vector with a N-terminal hexahistidine tag from *E. coli* BL21-Gold (DE3) and purified by Ni-NTA affinity chromatography (Figure 3.5) yielding 31 mg of pure protein per liter of bacterial culture. The estimated size of purified protein is about 54kDa according to SDS-PAGE analysis, which is in good agreement with the theoretical size for each subunit of the *Mtb*GS dodecamer. Unlike *E. coli* GS, no further adenylylation/deadenylylation modification was attempted on the recombinant N-His$_6$-tagged *Mtb*GS used in all studies for this Chapter.

The adenylylation levels of purified enzymes were determined by measuring total phosphate content after GS hydrolysis. Theoretically, if the enzyme is fully adenylylated such as A-EcGS, there should be 12 μM of phosphate from 1 μM of enzyme since each subunit of the dodecamer has one adenylylation site, with one phosphate group generated from each adenyl moiety. The result showed 15.72 μM phosphate per μM of A-EcGS enzyme, or 1.31 μM phosphate per μM of active site, which means 131% adenylylation. HEPES was used as negative control and showed -4.4% adenylylation. In the case of *Mtb*GS, we observed -4.2% adenylylation, which is essentially the same as the HEPES control (Figure 3.6 and Table 3.3). Thus, we concluded that the recombinant *Mtb*GS used in this study was fully deadenylylated. This low level of adenylylation for *Mtb*GS recombinantly expressed in *E.coli* is consistent with the study that showed a lack of ability for *E. coli* GlnE to adenylylate *Mtb*GS. Fully adenylylated *E.coli* GS was expressed from an engineered *E.coli* strain, but only 25-35% of adenylylation was observed when *Mtb*GS was expressed in the same strain. Lack of *Mtb*GS adenylylation implied that the enzyme should have fully activity towards catalyzing the biosynthetic GS reaction.
Steady State Kinetics of \(Mtb\)GS (\(\text{GlnA1}\))

\(\text{GS}\) is best known for catalyzing conversion of L-glutamate to L-glutamine in the biosynthetic \textbf{Reaction 3.1}. The mechanism of this biosynthetic reaction is described as a two-step model based on early studies (\textbf{Reaction 3.2}).\textsuperscript{73-76} Once ATP binds to the active site, one of the two metal cations (\(\text{Mg}^{2+}\) or \(\text{Mn}^{2+}\)) in the active site is used to coordinate ATP’s \(\gamma\)-phosphate oxygens to assist in phosphoryl transfer to glutamate’s \(\gamma\)-carboxylate group to form \(\gamma\)-glutamyl phosphate, which is a high-energy activated acyl phosphate. In the second step, ammonia attacks the carbonyl of the acyl phosphate intermediate to form a tetrahedral intermediate that resembles the late transition state for nucleophilic acyl substitution. Breakdown of the tetrahedral intermediate releases inorganic phosphate and proves the carboxamide group of L-glutamine. Movement of the amino acid residue side chains in the GS active site at the interface of GS monomers plays an important role in facilitating this two-step reaction mechanism. For example, the initial binding of ATP causes the side chain Asp-50’ movement, and thus increases the affinity for glutamate binding as well as aids in formation of the ammonium binding site\textsuperscript{76-78}.

Early kinetic characterization studies on \(Mtb\)GS from different sources were carried out with different analytical methods (\textbf{Table 3.4}). Horwitz’s group purified the protein directly from the supernatants of \(Mtb\) cultures. The activity of the enzyme was defined based on the rate of \(\text{P}_i\) formation. Their study showed an apparent \(K_m\) of \(Mtb\)GS for \(\text{L-glutamate}\) of \(2.7\pm0.2\ \text{mM}\) at the optimized pH of 7.5 with \(\text{Mg}^{2+}\).\textsuperscript{8} Bhatnagar and coworkers were able to obtain recombinant \(Mtb\)GS expressed and purified from an \textit{E.coli} heterologous host. Bhatnagar’s group inserted an \textit{Mtb glnA1} gene into a pQE30 vector and expressed the protein as a \(N\)-His\textsubscript{6} tagged construct in \textit{E.coli} M15. The hexahistidine tag was not removed prior to their in vitro evaluations of enzyme kinetics. Enzyme activity was measured by a colorimetric method showing the formation of
inorganic phosphate.\textsuperscript{79} Their result showed a \( K_m \) for \textit{L-glutamate of 2.56 mM and ATP of 2.43 mM}.\textsuperscript{80} Similarly, Sriram’s group expressed \textit{glnA1} in a pEQ2 vector in \textit{E.coli} BL21 (DE3) with a \( N\text{-His}_6 \) tag. They also measured apparent enzyme activity based on inorganic phosphate formation, which corresponded to an apparent \( K_m \) for \textit{glutamate of 151.9 mM}; a value significantly higher than the apparent \( K_m \sim 3 \) mM reported by the Horwitz and Bhatnagar groups.\textsuperscript{81} As we noted previously, the adenylylation level is crucial for GS activity. This could be an important variable in the determination of enzyme kinetic parameters. There has been much effort to obtain homogeneous samples of fully adenylylated and fully deadenylylated \textit{MtbGS} for direct comparison in kinetic assays. For this purpose, Atkins’ group recombinantly expressed \textit{MtbGS} in engineered \textit{E.coli} strains. They successfully obtained fully deadenylylated \textit{MtbGS} from \textit{E. coli} YMC21E strain which was engineered to lack the chromosomally encoded GS, \textit{glnA}, and ATase, \textit{glnE}. They also engineered another strain called \textit{E.coli} YMC21D, which lacked chromosomally encoded GS and a uridyl transferase responsible for regulating the adenylylation/deadenylylation rates of ATase. The initial intention was to use \textit{E.coli} YMC21D to obtain fully adenylylated \textit{MtbGS} based on the fact that they acquired adenylylated \textit{E.coli} GS from the same strain. However, they could only achieve 25-35\% adenylylation levels; well short of the desired 100\% adenylylated enzyme. The method they used for measuring enzyme activity was called “\textit{Mg}^{2+} \text{biosynthetic assay}”, which is a coupled enzyme assay that monitors NADPH production at 340 nm to determine the conversion of glutamate to glutamine.\textsuperscript{82} They reported an apparent \( K_m \) for \textit{ATP = 352 \pm 22 \mu M} for fully deadenylylated \textit{MtbGS}.\textsuperscript{65} Mowbray’s group obtained fully deadenylylated \textit{MtbGS} from the \textit{E.coli} GJ4547 strain which lacks ATase activity, and performed activity assays through the detection of phosphate production. The Mowbray study reported an apparent \( K_m \) for \textit{L-glutamate = 21.6 \pm 3.4 mM} and an apparent \( K_m \) for \textit{ATP =}
The range of apparent $K_m$ values reported for $Mtb$GS from these studies is $2.56 - 151.9$ mM for L-Glu and $0.35 - 2.4$ mM for ATP. The relatively higher apparent $K_m$ for L-Glu compared to ATP is consistent with previously reported values for $Ec$GS and makes sense given the high physiological concentrations of L-Glu in bacterial cells and certain host tissues (up to 25 – 100 mM) (see Chapter 2). This large range can be caused by multiple factors. The major reason is that active enzyme samples were obtained in different ways and tested under variable experimental conditions using different analytical methods. Specifically, Horwitz’s group obtained $Mtb$GS directly from $Mtb$ cultures while the other groups obtained active enzymes from heterologous expression in $E. coli$. In addition, the enzyme from Atkins’ and Mowbray’s groups are fully deadenylylated while the adenyllylation levels of enzyme from other groups are unknown and likely variable. Besides the source of enzyme, the difference in analytical methods used to measure GS activity could cause a difference in apparent catalytic parameters. An early study on GS kinetics using human enzyme showed that using two different analytical methods to measure enzyme kinetics for the same batch of enzyme generated apparent kinetic parameters that differed by ~2-fold. Furthermore, specific assay conditions differences such as temperature, buffer components, pH, dicatonic metal species ($Mg^{2+}$ or $Mn^{2+}$), and ionic strength can introduce more variability into the measurements.

To avoid the apparent inconsistencies associated with chemical detection of inorganic phosphate, we decided to use an enzyme-coupled method that is highly selective for ADP production. GS alone in the absence of substrates has no detectable ATPase activity making the detection of ADP strictly associated with the formation of glutamine. We obtained in vitro kinetic parameters for heterologously expressed recombinant $N$-His$_6$-tagged $Mtb$GS reactions under steady state conditions using a coupled PK/LDH spectrophotometric assay (Reaction
As shown in the first line of Reaction 3.3, the byproducts of GS catalytic reaction are Pᵢ and ADP. Consumption of NADH (absorbance decreases at 350nm) caused by this PK/LDH coupled reaction is used as real-time quantification of ADP production with, and thus determines MtbGS activity for glutamine biosynthetic reaction. The same method was used in our earlier study for obtaining kinetic parameters of the reactions catalyzed by E.coli, H.sapiens and S.aureus GS using the substrates ATP and L-Glu described in Chapter 2. The apparent Kₘ values for ATP measured by this method are within the literature reported range for both E. coli GS and H. sapiens GS, while Kₘ for L-Glu are higher than literature values for both GS enzymes. In this study, we obtained the Michaelis–Menten parameters (apparent Kₘ and kₗ for ATP and L-Glu) of the MtbGS catalytic reaction (Table 3.5 and Figure 3.7). We observed an apparent Kₘ for MtbGS for L-Glu = 38.4 ± 6.5 mM, which is within the literature reported range (2.56 – 151.9 mM, Table 3.4). The apparent Kₘ for ATP was 1.6 ± 0.1 mM, which is also within the range of 0.35 – 2.4 mM as literature reported (Table 3.4). Comparing our earlier study on E. coli, S. aureus and H. sapiens GS enzymes, the value of apparent Kₘ for ATP for MtbGS (1.6 ± 0.1 mM) is about the same as that observed for S. aureus (1.8 ± 0.1 mM), but higher than the corresponding values for E. coli and H. sapiens (0.7 ± 0.1 mM for both). The Kₘ value for L-Glu in MtbGS (38.4 ± 6.5 mM) is about the same as human enzyme (37 ± 5mM), but higher than E. coli GS (7.2 ± 0.8 mM), and lower than S. aureus GS (52 ± 7 mM). Although these apparent Kₘ values for L-Glu seem high, they are consistent with reported concentrations of L-Glu in vivo on 25–100 mM order in certain tissues. Given the infection environment of MtbGS in vivo is the human phagosome, the similarity between human and Mtb GS parameters is within expectation although they are two different types of GS with low sequence similarity. The trend for Mtb, E. coli and S. aureus GS can be explained by evolutionary differences between bacterial species; E.
coli is Gram-negative and S. aureus is Gram-positive bacteria. Mtb, however, is a complicated case since its cell envelope has both Gram-positive and Gram-negative characteristics. Mtb is typically classified to be more closely related Gram-positive bacteria based on phylogenetic and 16S ribosomal RNA analysis. Still, others argue Mtb is more closely related to Gram-negative bacteria based on whole-genome comparison. The apparent catalytic efficiency of MtbGS aligns more closely with the values for S. aureus GS, an enzyme from a Gram-positive bacterial strain, under these experimental conditions.

**Inhibition of MtbGS (GlnA1) by Glu Antimetabolites**

As with all drugs used to treat human disease, including antibiotics, toxicity will be a primary concern during the development of MtbGS inhibitors into commercial products. It was shown that the well-known GS inhibitor MetSox has relatively low toxicity in monkeys and rats, but has epileptogenic effect on dogs. Toxicity can arise from off-target effects related to the polypharmacology of antimetabolites. Toxicity can also be caused by the lack of selectivity of inhibitors for a specific GS isoform, MtbGS versus human GS in our case. For this concern, we evaluated inhibition potency for TβL, a 3-hydroxy-β-lactam-based glutamate antimetabolite, along with two well-known GS inhibitors MetSox, a sulfoximine-based glutamate antimetabolite, and Glufos, a phosphinate-based glutamate antimetabolite, against MtbGS using the PK/LDH coupled enzyme assay and compared the inhibition parameters with those for human GS we obtained in our earlier study, in which E. coli GS showed good consistency with the K_I values reported in literature.

Inhibition of MtbGS by all three inhibitors was ATP- and time-dependent, which was consistent with inhibitory activity observed towards GS orthologs from E. coli, S. aureus and
human GS in earlier studies (see Chapter 2). As shown in Figure 3.8a, there was a dramatic inhibition potency increase once ATP is added for all three inhibitors, while no inhibition activity was detected without ATP addition. This confirms the necessity of ATP for inhibition by Tβl, MetSox, and Glufos and is consistent with the requirement for phosphoryl group transfer from ATP to the inhibitors in order to form the tight-binding phosphorylated transition state mimics resembling the GS tetrahedral intermediate. As shown in Figure 3.8b, inhibition of MtbGS activity increases with a longer pre-incubation time of the enzyme and inhibitors, which is consistent with a model of ATP-dependent mechanism-based inhibition. For this mechanism-based enzyme inhibition, we used the Kitz−Wilson model to obtain inhibition parameters and compared the inhibition potency of Tβl, MetSox, and Glufos against MtbGS. For all three inhibitors, plots of 1/k_app versus 1/[inhibitor] showing straight lines with R^2 > 0.98 confirmed a mechanism-based inhibition model (Figure 3.8c). The parameter K_I was used as a metric for apparent binding affinity, k_inact was interpreted as the rate of enzyme inactivation, and the ratio of k_inact/K_I represented inhibitor efficiency. All three inhibitors showed strong inhibition on MtbGS (Table 3.6). Based on our result, the inhibitor efficiency (k_inact/K_I) towards MtbGS decreased following the order of Glufos>Tβl>MetSox, consistent with the relative inhibitor potencies observed against human, E. coli, and S. aureus GS (see Chapter 2). Inhibitor binding affinity (K_I) also followed the same trend, but when comparing inactivation rates (k_inact), Tβl was the slowest among the three inhibitors. TβL inhibited MtbGS with a K_I of 11.5 ± 1.5 μM and a k_inact of 0.3 ± 0.04 min⁻¹, resulting in an inhibitor efficiency (k_inact/K_I) of (30 ± 5) x10⁻³ min⁻¹ μM⁻¹. From our earlier study, TβL against human GS inactivation parameters were as follows: K_I = 130 ± 40 μM, k_inact = 0.3 ± 0.1 min⁻¹, and k_inact/K_I = (2 ± 1) x10⁻³ min⁻¹ μM⁻¹, corresponding to a 15-fold decrease in inhibitor efficiency, indicating a promising therapeutic window between Mtb and
human GSs. This relatively high binding affinity compared to the enzyme’s natural substrate ensures inhibitors outcompeting glutamate. The inactivation parameters for TβL against *Mtb*GS were intermediary compared to values of *E. coli* GS (K_I = 1.7 ± 0.4 μM, k_inact = 0.3 ± 0.1 min^{-1}, and k_{inact}/K_I = (180 ± 70) x 10^{-3} min^{-1} μM^{-1}) and *S. aureus* GS (K_I = 50 ± 10 μM, k_{inact} = 0.4 ± 0.1 min^{-1}, and k_{inact}/K_I = (8 ± 3) x 10^{-3} min^{-1} μM^{-1}). Functioning as l-Glu mimics, TβL has the potency trend same as k_{cat}/K_m parameter for l-Glu amongst the four GS isoforms we tested. The inactivation parameters for Glufos also follow the same trend as TβL toward GS isoforms: *E.coli>*Mtb>*S.aureus>*human, while the order of *Mtb* and *S.aureus* reverses for MetSox. Nevertheless, all three inhibitors give good therapeutic window between bacterial and human GSs. Among these inhibitors, Glufos is the most potent/efficient inhibitor against all four GS enzymes and provides a 10-fold difference in inhibitor efficiency for *Mtb* and human GS. TβL and MetSox have the largest therapeutic window in terms when comparing inhibitor efficiency for *Mtb* and human GS (15-fold difference). Although k_{inact}/K_I values showed good separation, the k_{inact} values are essentially equal across all combinations of enzymes and inhibitors suggesting that the chemical step of inhibition is fast and improvements in binding affinity (K_I) distinguish the overall efficiency of the GS inhibitors.

There have not been many kinetic studies performed on *Mtb*GS inhibitors. The Horwitz’s group reported an apparent K_I of 1.1 μM for MetSox and 0.6 μM for Glufos, which is much lower than we observed (121.5 μM and 10.6 μM respectively). This difference can be caused by multiple factors. The major factor is that they obtained native *Mtb*GS directly from *Mtb* supernatant, while we obtained recombinant His-tagged *Mtb*GS from heterologous expression in *E.coli*. There could be slight variations between the enzymes including post-translational modifications or the presence of some effectors from the expression systems. As Horwitz’s paper
noted, *Mtb* exports abundant quantities of proteins extracellularly,\textsuperscript{10} and as such, it is hard to avoid some other functional proteins that come along with *MtbGS* during purification. It is possible that even trace amounts of unwanted proteins could have an impact on the enzyme function. This might also explain why their $K_m$ of Glu (2.4 mM) is much lower than our $K_m$ (38.4 mM). Other than that, the same reasons as we stated earlier in this paper about $K_m$, such as analytical method and assay conditions including temperature, pH, and reaction matrix can cause apparent differences. One unique factor for the $K_I$ difference is the incubation time of enzyme and inhibitor. As shown in Figure 3.8b, a 10-min incubation duration difference can cause more than 50% activity difference. Given these variations, it is difficult to compare our kinetic parameters with those from other groups unless factors such as inhibitor-enzyme incubation times are accounted for. However, it is clear that data reported in this Chapter 3 for *MtbGS* are consistent with our data from Chapter 2 reported for *E. coli*, *S. aureus*, and human GS obtained under the same assay conditions using the same enzyme-coupled analytical method for measuring real-time ADP production.

**Inhibition of M. tuberculosis whole cell growth by GS inhibitor prodrugs**

Inhibition of *M. tuberculosis* by GS inhibitor prodrugs was assessed using whole cell growth assays conducted by our collaborator, Professor Christina Stallings, at Washington University School of Medicine. **Figure 3.9A** shows growth curves of *M. tuberculosis* after treatment with 50 μM of each prodrug (tabtoxin, Ala-Ala-Glufos, Thr-MetSox) or parent GS inhibitor (Glufos, MetSox). The growth assay was carried out in triplicate cultures over 10 days. Based on the isomerize feature of tabtoxin (**Figure 3.10**) (isomerized product is not active against GS\textsuperscript{94}, tabtoxin was re-dosed at 48, 96, and 144 hours. Only the prodrugs tabtoxin
(compound of interest) and Ala-Ala-Glufos (commercially available herbicide, Glufos is a well-studied GS inhibitor) reduced the growth of *M. tuberculosis*. The parent GS inhibitors Glufos and MetSox did not show inhibition of *M. tuberculosis* growth, which supports the theory that prodrug formulation is required for penetration of *M. tuberculosis* cells through dipeptide permeases followed by dipeptidase cleavage in the cytoplasm to release the GS inhibitor; a so-called Trojan Horse drug delivery strategy (Figure 3.3). Notable, the dipeptide Thr-MetSox, which is also a prodrug, shows no inhibition growth of *M. tuberculosis* growth. We propose that the order and identity of amino acids in the di- or tripeptide prodrug formulation is important for transport and prodrug cleavage. In other words, whether the inhibitor is connected with amino acid on the N-terminal or C-terminal will cause a difference in prodrug efficiency. Further investigation is undergoing in our lab regarding to the influence of inhibitor and amino acid order on prodrug activity.

In addition to investigating planktonic growth of *M. tuberculosis*, which is performed on liquid cultures with active shaking, the Stalling’s lab also observed the influence of compounds on stationary cell cultures to study the biofilm formation. Figure 3.9b shows that Glufos can interrupt the formation of biofilm, which can be beneficial for other drugs, including Ala-Ala-Glufos and tabtoxin, to get access into target cells. This indicates that combinations of GS inhibitors and prodrugs might be effective and required to inhibit both intracellular and extracellular GS. We propose that the combination application of prodrugs and GS inhibitors can be a promising therapy for TB treatment.

**Tabtoxin toxicity study using mice model**


Toxicity is a crucial factor to be tested for a compound to be developed for clinical use. Toxicity of tabtoxin prodrug was tested on female C3H/HeNH mice, conducted by our collaborator: Prof. Jeffrey Henderson’s lab in Medical school of WashU. High dose (10mg/kg) and low dose (1mg/kg) of tabtoxin was injected into mice to test toxicity, along with phosphate-buffered saline (PBS) as control (Figure 3.11). All three groups of mice were alive at 24 and 48 hours after injection. The urine from the mice model were collected after 24 and 48 hours after injection, and only the high dose groups showed dark color. The high dose 24 hours sample also showed some urine sediment, indicating the presence of red blood cells, but there was no urine sediment at 48 hours in high dose sample. Tabtoxin was detected in collected urine via LCMS indicating rapid excretion of the compounds via the kidneys.

One exciting thing was that the mice models did not show any convulsions or seizure symptoms. As mentioned earlier, the limitation for developing MetSox or Glufos into anti-TB drug is that they can cause convulsions or seizure in test animals. However, to our knowledge we are the first to test toxicity of di/tri-peptide prodrugs in mice and the lack of neurological toxicity is promising for future development of GS inhibitor prodrugs as therapeutics. Further studies are needed to optimizing dosing and prodrug formulations to minimize toxicity and maximize efficacy against \textit{Mtb} and other target pathogens.

### 3.5 Outlook and Conclusions

Tβl is an interesting molecule to study since its target is GS instead of transpeptidase for the classical β-lactam antibiotics. This unique target makes Tβl survive the degradation of β-lactamase and a potential anti-TB drug considering GS’s significance in \textit{Mtb} cell wall biosynthesis. For this purpose, we studied Tβl’s potency against active \textit{MtbGS in vitro} as well as
Tβl’s prodrug — tabtoxin’s inhibition on *Mtb* whole cell growth. To the concern of antibiotic development, tabtoxin’s toxicity was tested in mouse model.

We successfully obtained active *Mtb*GS and the test of adenylylation level showed it was fully deadenylylated. Tβl showed an ATP- and time-dependent inhibition against *Mtb*GS. This pattern is also true for two well-known GS inhibitors — MetSox and Glufos, and true for other GS isoforms such as *E. coli, S. aureus*, and human GS. Judging by kinetic parameters of *Mtb*GS alone, Tβl is not the most potent amongst these three inhibitors; however, it has the best therapeutic window when comparing them with human GS parameters. As expected, the trend of Tβl potency is consistent with the trend of $k_{cat}/K_m$ for L-Glu amongst these four GS isoforms, which confirmed that Tβl is competitive with L-Glu by resembling its structure as well as function (glutamate antimitabolite activity). Tβl’s prodrug form, tabtoxin (Thr dipeptide), showed great inhibition against *Mtb* growth with consecutive dosing to compensate for instability via an isomerization reaction. These preliminary studies on tabtoxin and Tβl in vitro and whole cell efficacity make GS inhibitors and prodrugs an attractive prospect as targeted anti-TB antibiotics with a favorable safety profile. The urine from mice injected with low dose of tabtoxin did not contain any red blood cells, and both high and low doses of tabtoxin did not introduce neurological toxicity commonly associated with GS inhibitors. This exciting result made us believe that Tβl could be a promising anti-TB drug where prodrug formulations enable potent and targeted activity towards *Mtb* cells.

Based on our knowledge, there is no anti-TB drug acting on GS as the primary target making Tβl, MetSox, and Glufos the first potential leads. GS inhibitors also have the potential to act synergistically with currently approved anti-TB drugs by destroying the cell wall to facilitate the entry of other drugs into the bacteria, such as RIF that aiming at RNA polymerase. Even
though there already are anti-TB drugs inhibiting cell envelope biogenesis and maintenance, such as INH and EMB, Tβl has different target from them minimizing concern of potential cross resistance. Specifically, INH’s target is fatty acid synthase and EMB’s is arabinosyltransferase. The changing of drug target and potential for synergy are favorable properties to help resolve MDR-TB issue. We also believe that the combination of Tβl and Tβl-thr (tabtoxin) will be a powerful way to fight TB. With Tβl inhibiting the extracellular GS to interrupt cell wall synthesis, Tβl-thr can get into Mtb through dipeptide permeases and inhibit intracellular GS to block bacterial nitrogen metabolism. Other than TB, our study opens up the door for novel β-lactam applications in a broader field that involves the ATP-dependent carboxylate-amine ligase superfamily such as Dihydrofolate Synthetase (DHFS) in the folate biosynthesis pathway.

3.6 Materials and Methods

*MtbGS protein expression and purification*

Construct of GlnA1 in a pCRT7/C7 vector encoding N-His$_6$-tagged GS from Mtb was a kind gift from Professor Mowbray in Uppsala University in Sweden and was transformed into E.coli BL21-Gold (DE3). Overnight transformed BL21 cells were grown at 37°C at 225rpm in 1L of LB media supplemented with 100µg/mL Ampicillin until an OD$_{600nm}$ of 0.6-0.8 was reached. The culture was put on ice for 30min to cool down, followed by addition of 2.38g IPTG (10mM). The temperature was reduced to 18°C for overnight induction with shaking at 225rpm. Cells were harvested by centrifuging at 5000rpm at 4°C for 30min. Cells/protein were kept at 4°C or on ice for all remaining steps. All cell pellet was resuspended in 40mL of cold buffer A (50mM HEPES pH 7.5, 300mM sodium chloride, 10% glycerol) with protease inhibitor, flash frozen with liquid nitrogen, thawed on ice and then lysed by homogenizer. Cell lysates were
clarified by ultracentrifugation at 45000g for 35min at 4°C. The supernatants were loaded onto pre-equilibrated Ni-NTA resin in buffer A, and rocked for 30min at 4°C. Ni-NTA resin was washed twice with wash buffer (50mM HEPES pH 7.5, 300mM sodium chloride, 50mM imidazole) after being drained. Bound protein was eluted with elution buffer (50mM HEPES pH 7.5, 300mM sodium chloride, 10% glycerol, 400mM imidazole) and elution fractions were analyzed by SDS-PAGE with visualization by Coomassie blue staining. Protein was then dialyzed into storage buffer (25mM HEPES pH 7.5, 50mM sodium chloride, 10% glycerol) overnight at 4°C and concentrated by 30kMW filter tube. Final yield is about 31 mg/L. Concentrated protein was flash frozen in liquid nitrogen and stored at -80°C.

**GlnE protein expression and purification, preparation of A-EcGS**

Overnight culture of *E. coli* BL21 grown at 37 °C harboring the appropriate plasmid were grown in LB broth containing 50 μg/mL kanamycin and 200 μL was used to inoculate 1L batches of sterile Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 5 g/L glycerol, 17 mM KH₂PO₄, and 72 mM K₂HPO₄) containing 50 μg/mL kanamycin. The cultures were grown at 37 °C with agitation at 225rpm to an OD₆₀₀ of 0.6~0.8 and then cooled to 20 °C prior to induction with 1 mL of 0.5 M IPTG (0.5 mM final concentration). Cultures were then grown for ~18 h at 20 °C with agitation at 225 rpm. Cells were harvested by centrifugation (5,000 r.p.m. for 20 min at 4 °C). Cells/protein solutions were kept at ~4 °C for all remaining steps. Cell pellets were suspended in 40 mL of lysis buffer (50 mM K₂HPO₄, 500 mM NaCl, 5 mM β-mercaptoethanol, 20 mM imidazole, 10% glycerol, adjusted to pH 8.0) and flash frozen in liquid nitrogen. After thawing, cells were mechanically lysed using an Avestin EmulsiFlex-C5 high-pressure homogenizer. Cell lysate was clarified via ultracentrifugation (45,000 r.p.m. for 35 min) and
incubated for 30 min with Ni-NTA resin preconditioned with lysis buffer. The Ni-NTA resin was washed twice with 40 mL aliquots of lysis buffer, and then eluted in three separate washes with 30 mL of elution buffer (50 mM K$_2$HPO$_4$, 500 mM NaCl, 5 mM β-mercaptoethanol, 300 mM imidazole, 10% glycerol, adjusted to pH 8.0). Fractions containing pure N-His$_6$-GS as judged by SDS-PAGE analysis were combined, dialyzed into size exclusion buffer (50 mM K$_2$HPO$_4$, 500 mM NaCl, 1 mM DDT, pH adjusted to 8.0), concentrated via centrifugal filtration, flash frozen in liquid nitrogen, and stored at −80 °C to give stocks ready for *in vitro* biochemical assays.

The expression and purification of normal *E. coli* GS (EcGS) was described in Chapter 2. Normal ECGS was incubated with GlnE with the addition of ATP, magnesium, and ammonium, incubated at 19°C overnight with shaking at 100 rpm to obtain fully adenylylated ECGS (A-EcGS).

*Determinación de grado de adenilación de GS purificado*

The phosphate content of *Mtb*GS was determined by hydrolysis to release phosphate. Purified *Mtb*GS (8nmol in 1mL) was digested by 1ml 4M HCl, and the mixture was concentrated to dryness via rotary evaporation. The residue was resuspended in 1 mL of 2M HCl plus 20μL of 30% v/v H$_2$O$_2$, and the mixture was concentrated to dryness using rotary evaporation. Five sequential additions of 200 μL 30% v/v H$_2$O$_2$ with evaporation (sample concentrator) to dryness were used for complete hydrolysis. The residue was resuspended in 500 μL Milli-Q water. Same method was used on A-EcGS (8 nmol in 1 mL) as positive control, and HEPES as negative control. The phosphate content was detected using *Bio Vision* Phosphate colorimetric assay kit, and the OD$_{650}$ of treated samples were measured by SpectraMax Plus 384 plate reader from Molecular Devices. Phosphate standard curve was generated in the phosphate range of 0 to 2.5
nmol, which gave a straight line with $R^2 > 0.99$. At least three different volume of treated samples from each of HEPES, MtbGS and A-EcGS was used as parallel triplicate experiments. The amount of detected phosphate was converted from OD$_{650nm}$ based on the phosphate standard curve. Percentage of adenylylation was calculated based on the ratio of detected and theoretical phosphate amount if it is fully adenylylated, which is 12 times of the amount of enzyme.

**GS Michaelis-Menten Kinetics**

All experiments were performed at 37 °C in quartz cuvettes under steady state conditions with continuous monitoring at 350 nm in an Agilent Carey 50 UV–vis spectrophotometer. Glufos was racemic, and reported concentrations reflect only the L-enantiomer. For all experiments, the total reaction volume was 500 μL and contained 100 mM HEPES (pH 7.4), 100 mM KCl, 25 mM MgCl$_2$, 10 mM NH$_4$Cl, 0.5 mM PEP, 0.2 mM NADH, 0.2 unit of PK, 0.3 unit of LDH, and 75 nM MtbGS. For determination of the apparent K$_m$ for ATP, reaction mixtures contained 50 mM L-Glu and variable concentrations of ATP ranging from 0 to 2.4 mM. For determination of the apparent K$_m$ of L-Glu, reaction mixtures contained 10 mM ATP and variable concentrations of L-Glu ranging from 1 to 25 mM. MtbGS was added last to initiate the reaction. Reaction velocities ($k_{obs}$ in absorbance per minute) were determined by calculating the slope of the linear region of the 350 nm absorbance versus time plot with background correction for control reactions lacking GS. Kinetic constants were determined from $k_{obs}$ versus substrate concentration data using a nonlinear, least-squares fitting method with GraphPad Prism, version 7.0a fit to the Michaelis–Menten equation (eq 1)

$$k_{obs} = \frac{k_{cat}[S]}{K_m+[S]}$$

(1)
where $k_{\text{cat}}$ is the maximal velocity, $[S]$ is the substrate concentration, and $K_m$ is the substrate concentration corresponding to $k_{\text{obs}} = 1/2k_{\text{cat}}$. All reactions were performed in triplicate as independent trials.

**MtbGS Kitz–Wilson Kinetics**

All experiments were performed at 37 °C in quartz cuvettes under steady state conditions with continuous monitoring at 350 nm in an Agilent Carey 50 UV–vis spectrophotometer. MetSox was purchased from Sigma-Aldrich as the L-enantiomer. TβL isolated from *P. syringae* cultures was the L-enantiomer. The total reaction volume for experiments with MetSox and Glufos was 500 µL. To conserve sample, the total reaction volume for experiments with TβL was 400 µL. Each reaction mixture contained 100 mM HEPES (pH 7.4), 100 mM KCl, 25 mM MgCl$_2$, 10 mM NH$_4$Cl, 0.5 mM PEP, 0.2 mM NADH, 0.2 unit of PK, 0.3 unit of LDH, 50 mM L-Glu, and enough GS to give a starting rate close to -0.05 absorbance unit/min at 350 nm, yielding a straight line for ~10 min without consuming all NADH (*MtbGS* stock is prepared in advance from frozen concentrated stock in -80 °C the day of inhibition assay). Control reactions with either no L-Glu or no inhibitor were used to correct for nonspecific ATPase activity and NADH degradation. An appropriate preincubation time of the enzyme and inhibitor was established by investigating the time dependence of *MtbGS* inhibition by TβL, MetSox, and racemic Glufos. The percent *MtbGS* activity relative to a no inhibitor control was measured for inhibitor concentrations ranging from 1 to 100 µM using preincubation times of 0–60 min. No ATP controls were included to demonstrate the requirement of ATP for GS inhibition. Kinetic inhibition constants were determined from $k_{\text{app}}$ versus inhibitor concentration data using a linear
fitting method with GraphPad Prism, version 7.0a, fit to the Kitz–Wilson equation for irreversible enzyme inhibition (eq 2)

\[
\frac{1}{k_{app}} = \frac{1}{k_{inact}} + \frac{K_I}{k_{inact} [I]}
\]

(2)

where \(k_{inact}\) is the rate constant for conversion of the reversible enzyme-inhibitor complex to the irreversible complex, \([I]\) is the inhibitor concentration, and \(K_I\) is the dissociation constant for the initial reversible enzyme-inhibitor complex. The parameter \(k_{app}\) is defined by equation 3

\[
k_{app} = \frac{t}{\ln \left( \frac{v_I}{v_o} \right)}
\]

(3)

where \(t\) is the preincubation time, \(v_I\) is the rate with inhibitor, and \(v_o\) is the rate without inhibitor. Preincubation times for Kitz–Wilson kinetic studies were chosen to give linear plots of \(1/k_{app}\) versus \(1/[I]\) with inhibitor concentrations above and below the \(K_I\) value. Inhibitor concentrations were varied between 0.33 and 100 µM, and preincubation times were typically between 2 and 10 min. All reactions were performed in triplicate as independent trials.

### 3.7 Acknowledgements

We thank Dr. Sherry Mowbray (Department of Cell and Molecular Biology, Uppsala University, Sweden) for providing \(MtbGS\) plasmid. We thank Dr. Christina Stallings (Department of Molecular Microbiology, Washington University School of Medicine) for efforts in \(M.~tuberculosis\) whole cell and biofilm formation studies. We thank Dr. Jeffery Henderson (Department of Medicine and Molecular Microbiology, Washington University School of Medicine) for efforts in mice model toxicity study. Funding for this research was provided by the
Children’s Discovery Institute at St. Louis Children’s Hospital through Interdisciplinary Research Initiative grant MI-PD-II-2018-748.
3.8 Figures and Tables

\[
\text{Glutamate} + \text{ATP} + \text{NH}_3 \xrightarrow{\text{Glutamine Synthetase}} \text{Glutamine} + \text{ADP} + \text{P}_i
\]

**Reaction 3.1.** Biosynthetic reaction catalyzed by glutamine synthetase (GS)

**Reaction 3.2.** Mechanism of GS catalytic reaction.
**Reaction 3.3.** Enzyme-coupled spectrophotometric kinetic assay for measuring steady state GS kinetics. Pyruvate kinase (PK) and lactate dehydrogenase (LDH) are used to convert GS-generated ADP to ATP resulting in consumption of NADH as measured by absorbance decrease at 350 nm.

\[
\text{Glutamate} + \text{ATP} + \text{NH}_3 \xrightarrow{\text{GS, Mg}^{2+} \text{ or Mn}^{2+}} \text{Glutamine} + \text{ADP} + P_i
\]

\[
\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{Pyruvate}
\]

\[
\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+
\]
<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th><em>S. aureus</em></th>
<th><em>Mtb</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>24.63</td>
<td>23.77</td>
<td>24.93</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>24.63</td>
<td>36.62</td>
<td>39.95</td>
<td></td>
</tr>
<tr>
<td><em>Mtb</em></td>
<td>23.77</td>
<td>36.62</td>
<td>51.39</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>24.93</td>
<td>39.95</td>
<td>51.39</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: GS isoforms sequence identity, generated from Cluster Omega.

Table 3.2: Nucleotide and protein sequence of recombinant N-His<sub>6</sub>-tagged *Mtb*GS use in this work.
Table 3.3: Results from percent adenylylation determination using total phosphate measurement.

<table>
<thead>
<tr>
<th>Expression host</th>
<th>% Adenylylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>-4.35 ± 1.55</td>
</tr>
<tr>
<td>$MtbGS$</td>
<td>-4.20 ± 1.78</td>
</tr>
<tr>
<td>A-EcGS</td>
<td>131.39 ± 8.78</td>
</tr>
</tbody>
</table>

Table 3.4: Apparent $K_m$ values for $MtbGS$ from published studies (see main text for references).

<table>
<thead>
<tr>
<th>Group</th>
<th>Expression host</th>
<th>Adenylylation %</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glu</td>
</tr>
<tr>
<td>Horwitz</td>
<td>$Mtb$ Erdman (ATCC 35801)</td>
<td>—</td>
<td>2.4mM</td>
</tr>
<tr>
<td>Bhatnagar</td>
<td>E.coli M15</td>
<td>—</td>
<td>2.56mM</td>
</tr>
<tr>
<td>Sriram</td>
<td>E.coli BL21 (DE3)</td>
<td>—</td>
<td>151.9mM</td>
</tr>
<tr>
<td>Atkins</td>
<td>E.coli YMC21E</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>E.coli YMC21D</td>
<td>3.1±0.2</td>
<td>—</td>
</tr>
<tr>
<td>Mowbray</td>
<td>E.coli GJ4547</td>
<td>0</td>
<td>21.6±3.4mM</td>
</tr>
</tbody>
</table>
**Table 3.5:** Apparent Michaelis-Menten parameters for recombinant GS from *Mtb*, *E. coli*, *S. aureus*, and *H. sapiens*.

<table>
<thead>
<tr>
<th>GS</th>
<th>ATP</th>
<th>L-Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (mM)</td>
<td>( k_{cat} ) (x10^3 min(^{-1})mM(^{-1}))</td>
</tr>
<tr>
<td><em>M. tuberculosi</em></td>
<td>1.6 ± 0.1</td>
<td>2900 ± 100</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.7 ± 0.1</td>
<td>7300 ± 300</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>1.8 ± 0.1</td>
<td>720 ± 20</td>
</tr>
<tr>
<td><em>H. sapiens</em></td>
<td>0.7 ± 0.1</td>
<td>2600 ± 100</td>
</tr>
</tbody>
</table>

*All standard deviations were determined for three independent trials.

**Table 3.6:** Apparent inactivation parameters for TβL, MetSox, and Glufos against *Mtb*(TB) *E. coli* (EC), *S. aureus* (SA), and *H. sapiens* (H) GS.

<table>
<thead>
<tr>
<th>GS</th>
<th>TβL</th>
<th>MetSox</th>
<th>Glufos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_I ) (μM)</td>
<td>( k_{inact} ) (min(^{-1}))</td>
<td>( k_{inact}/K_I ) (x10^3 min(^{-1})μM(^{-1}))</td>
</tr>
<tr>
<td>TB</td>
<td>11.5 ± 1.5</td>
<td>0.3 ± 0.4</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>EC</td>
<td>1.7 ± 0.4</td>
<td>0.3 ± 0.1</td>
<td>180 ± 70</td>
</tr>
<tr>
<td>SA</td>
<td>50 ± 10</td>
<td>0.4 ± 0.1</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>H</td>
<td>130 ± 40</td>
<td>0.3 ± 0.1</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

*All standard deviations were determined for three independent trials.
**Figure 3.1:** Active site of GS. Yellow balls: metal ions, green balls: ammonium ion, red stick: glutamate, blue stick: ATP, pink stick: key side chains of GS residues that move to close the active site.

**Figure 3.2:** Structures of GS inhibitors.

a. MetSox  

b. Glufos  

c. Tabtoxine-β-lactam(TBL)  

d. Aminomethylenebisphosphonic acid  

e. 2,4,5-trisubstituted imidazole
Figure 3.3: Trojan Horse strategy for tabtoxin function as a prodrug.

Figure 3.4: SDS-PAGE gel of purified N-His$_6$ EcGS (GlnE). (*E. coli* GlnE: 108kDa)
**Figure 3.5:** SDS-PAGE analysis of purified N-His₆-MtbGS used in this work. SDS-PAGE gels (Any kD, Bio-Rad) were loaded with protein ladder (Precision Plus Protein Dual Xtra Prestained Protein Standards, Bio-Rad) in lane 1 and Ni-NTA elutions #1 from two batches of protein prep in lanes 7,6, elutions #2 and #3 in lanes 5,4 and 3,2 respectively. Gel was stained with Coomassie blue.

**Figure 3.6:** Adenylylation level test of GS variants using total phosphate assay.
Figure 3.7: Steady-state Michaelis-Menten kinetic plots for \( MtbGS \) performed in triplicate.

Figure 3.8: Inhibition kinetics for \( T\beta L \), MetSox, and Glufos against recombinant \( MtbGS \) show (a) ATP and (b) time dependence and (c) fit the Kitz-Wilson model for mechanism-based enzyme inhibition. Error bars represent standard deviations for three independent trials.
**Figure 3.9:** (A) growth curve of *M. tuberculosis* with addition of prodrugs. (B) biofilm formation study of *M. tuberculosis*. (Data and figure provided by Stalling’s lab)

**Figure 3.10:** Tabtoxin (Tβl-Thr) isomerization to form Tδl-Thr.
**Figure 3.11**: Mice information and urine analysis from tabtoxin toxicity study. (Figure provided by Henderson’s lab).

<table>
<thead>
<tr>
<th>ID#</th>
<th>Strain</th>
<th>DOB</th>
<th>Weight (g)</th>
<th>vol of drug</th>
<th>Drugs</th>
<th>concentration</th>
<th>24 hrs Post injection</th>
<th>48 hrs Post Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C3HHeNH</td>
<td>3/1/16</td>
<td>19.42</td>
<td>77.88</td>
<td>T25</td>
<td>2.5 mg/kg</td>
<td>20 Y  Dark  Red blood cells?</td>
<td>1 Y  Dark  N/A</td>
</tr>
<tr>
<td>2</td>
<td>C3HHeNH</td>
<td>3/1/16</td>
<td>20.72</td>
<td>82.88</td>
<td>T25</td>
<td>0.25 mg/kg</td>
<td>20.7 Y  Light  N/A</td>
<td>20.34 Y  Light  N/A</td>
</tr>
<tr>
<td>3</td>
<td>C3HHeNH</td>
<td>3/1/16</td>
<td>19.92</td>
<td>79.88</td>
<td>PBS</td>
<td>N/A</td>
<td>20.3 Y  Light  N/A</td>
<td>22.34 Y  Light  N/A</td>
</tr>
</tbody>
</table>

![Image of urine samples and sediment](image.png)
3.9 References


68. Parish, T.; Lewis, J.; Stoker, N. G., Use of the mycobacteriophage L5 excisionase in Mycobacterium tuberculosis to demonstrate gene essentiality. Tuberculosis (Edinb) 2001, 81 (5-6), 359-64.
Chapter 4: Cl-tabtoxin Isolation and Biosynthetic Gene Cluster Investigation
4.1 Preface

This chapter was written by Luting Fang (LF) and edited by Timothy Adam Wencewicz (TAW). Funding for this research was provided by the Children’s Discovery Institute at St. Louis Children’s Hospital through Interdisciplinary Research Initiative grant MI-PD-II-2018-748, the Sloan Foundation through a Sloan Fellowship Award to TAW (FG201810935), and the Dreyfus Foundation through a Camille Dreyfus Teacher-Scholar Award to TAW (TC-19-079).

4.2 Abstract

Cl-Tabtoxin is a non-proteinogenic dipeptide natural product isolated from a soil-derived strain of *Streptomyces* bacteria. Since its discovery in 1974, there is only one published study about this compound reporting on the structure elucidation and glutamine-dependent antimicrobial activity. Cl-Tabtoxin is a structural derivative of tabtoxin produced by plant pathogenic *Pseudomonas syringae*, which also shows potential as an anti-tuberculosis (anti-TB) antibiotic. Tabtoxin (TβL-Thr) is a dipeptide composed of the non-proteinogenic amino acid tabtoxinine-β-lactam (TβL), a glutamate antimetabolite, and L-threonine as the C-terminal residue. Cl-Tabtoxin (Ala-Cl-TβL) is a dipeptide composed of C4-chlorinated TβL and L-Alanine as the N-terminal residue. Due to structural similarity and shared mechanism-of-action, Cl-tabtoxin might also be a good candidate anti-TB compound. We were able to isolate and purify Cl-tabtoxin from *Streptomyces*, and used recombinant dipeptidase PepA to release the free glutamate antimetabolite Cl-TβL. Using whole genome sequencing and comparative genomics guided by the known biosynthetic gene cluster (BGC) for tabtoxin from *P. syringae*, we were able to locate the biosynthetic gene cluster (BGC) of this compound in the known Cl-tabtoxin producer *Streptomyces* 372A, NRRL 8045. We cloned, heterologously expressed, purified, and
characterized the biosynthetic reactions catalyzed by three enzymes, CabB (N-
succinyltransferase), CblA (SAM-dependent C-methyltransferase), and CabD (PLP-dependent
aminotransferase), from the Cl-tabtoxin BGC. We firmly establish how metabolic flux is
diverted from L-Lysine biosynthesis to Cl-tabtoxin biosynthesis at the level of
tetrahydropicolinate (THDPA). CabB catalyzes both N-succinylation and N-acetylation of
THDPA to generate a mixture of N-acyl-L-2-amino-6-oxopimelate. CblA catalyzes methyl group
transfer from S-adenosyl-methionine (SAM) to C5 of N-acyl-L-2-amino-6-oxopimelate
generating C5-methyl-N-acyl-L-2-amino-6-oxopimelate. This methylation reaction commits
carbon flux towards TβL and away from L-Lys where aminotransferase CabD converts C5-
methyl-N-acyl-L-2-amino-6-oxopimelate to C5-methyl-diaminopimelate. This biosynthetic
strategy is shared between P. syringae and Streptomyces where TβL is presumably a shared
biosynthetic intermediate. In P. syringae TβL is converted to the Thr-TβL dipeptide via ATP-
dependent carboxylate-amine ligase TbfF prior to efflux. In Streptomyces the biosynthetic
pathway deviates to allow for dipeptide formation with L-Ala predicted to occur via a non-
ribosomal peptide synthetase (NRPS) and C4-chlorination presumably via a predicted
halogenase encoded in the Cl-tabtoxin BGC. Genome mining revealed that homologous Cl-
tabtoxin BGCs are widespread amongst Streptomyces paving the way for new compound
discovery and further investigation of monocyclic beta-lactam biosynthesis.

4.3 Introduction

Streptomyces is a family of Gram-positive bacteria that is predominantly found in
decaying vegetation and soil. Over two-thirds of the clinically useful antibiotics with a natural
origin are produced by this type of bacteria. Streptomyces can produce antibacterial, antifungal,
and antiparasitic drugs, as well as a wide range of other bioactive compounds such as immunosuppressants. Cl-Tabtoxin (Figure 4.1a) is one example of a non-proteinogenic dipeptide antibiotics produced by soil Streptomyces. Since the discovery of Cl-tabtoxin discovery little work has been reported beyond structure elucidation in 1974 and a patent (US3956067) reporting antibacterial activity in 1976. This compound is of great interest to our lab based on the fact that it has a similar structure to tabtoxin (Figure 4.1b), which is a potential anti-tuberculosis drug discussed in Chapter 3. Tabtoxin (TβL-Thr) is a dipeptide plant phytotoxin produced by plant pathogenic Pseudomonas syringae. Tabtoxin is composed of the non-proteinogenic amino acid tabtoxinine-β-lactam (TβL), a glutamate antimetabolite, and L-threonine as the C-terminal residue. Cl-Tabtoxin (Ala-Cl-TβL) is a dipeptide composed of C4-chlorinated TβL and L-Alanine as the N-terminal residue.

TβL is presumably converted to Cl-TβL by a halogenase encoded in the genome of producing Streptomyces. Halogenases have evolved by nature to regioselectively halogenate a wide range of biosynthetic precursors, resulting in more than 4,000 natural products containing chlorine, bromine or iodine atoms. Introduction of the halogen atom can have a profound impact on bioactivity and physicochemical properties of the resulting natural products. This impact has been exploited and is beneficial in both agrochemicals and medicinal chemistry. A large proportion of the bestselling herbicides, pesticides, insecticides and drugs on the market or in clinical trials contain halogen atoms. Among the four halogens, chlorine (Cl) is found to be the most frequent in drugs (Cl > F >> Br > I). Based on the limited information regarding Cl-tabtoxin and its high structural similarity to the better-studied tabtoxin, we believe the function and biosynthesis between these two
compounds are related. Therefore, we studied the biosynthesis and mechanism of action of Cl-tabtoxin based on knowledge accumulated from the study of tabtoxin from *P. syringae*. Tabtoxin is a dipeptide comprised of an unusual amino acid tabtoxinine-β-lactam (TβL) (Figure 4.1d) and a C-terminal L-threonine (or L-serine in a minor variant)\(^{16}\). The structure of Cl-tabtoxin is different from tabtoxin in three ways: 1) the presence of Cl in the non-proteinogenic amino acid (Figure 4.1c); 2) the identity of the amino acid used to form the prodrug (L-alanine instead of L-threonine or L-serine); 3) the orientation of the prodrug dipeptide (L-alanine is at the N-term of the prodrug for Cl-tabtoxin while L-threonine is at the C-term position for tabtoxin). In addition to the structural differences, their respective producers are also different. Cl-tabtoxin appears to be produced exclusively by *Streptomyces* while tabtoxin appears to be produced exclusively by Pseudomonads.\(^{17}\)

*Pseudomonas syringae* is a family of Gram-negative bacterium that exists in almost all kinds of terrestrial plant species\(^{18}\). This species of bacteria can produce a wide spectrum of phytotoxic compounds\(^{19}\), many of which are antimetabolite phytotoxins that consist of non-proteinogenic amino acids and small peptides that can disrupt the normal metabolism of host plant cells contributing to the severity of plant disease. Tabtoxin is one of the most notable antimetabolite toxins which causes irreversibly inhibition of plant glutamine synthetase (GS) (EC 6.3.1.2)\(^ {20,21}\). GS is a critical enzyme in nitrogen metabolism because of its role in catalyzing the condensation of ammonia and glutamate into glutamine (Reaction 3.1), which functions as the nitrogen donor for many crucial molecules such as pyrimidines, purines, amino acids, carbamoyl phosphate and glucosamine-6-phosphate\(^ {22}\). This inhibition results in limited availability of glutamine, and interferes with the only efficient pathway of ammonia detoxification in plants, leading to ammonia toxicity and thus characteristic chlorosis.\(^ {23-25}\) In
Chapter 3, we showed that tabtoxin can be developed into an anti-TB drug based on the fact that it can inhibit the growth of *Microbacterium tuberculosis* (*Mtb*). Tabtoxin is a prodrug composed of the free TβL metabolite and L-threonine (or L-serine).\textsuperscript{16, 26} Chapter 2 has shown that TβL inhibits GS in the same mechanism as the other two best-known GS inhibitors MetSox and Glufos where GS-catalyzed phosphorylation of the inhibitors in the enzyme active site leads to formation of a noncovalent but irreversible transition state inhibitor\textsuperscript{27}. The dipeptide tabtoxin, however, cannot be recognized by GS; it is a prodrug. This is an example of a Trojan Horse drug delivery strategy, which is not uncommon in nature. The coupling of amino acids to phosphonic acids occurs in diverse bioactive natural products such as rhizoctin, plumbemycin, bialaphos, and phosalacine and seems to universally impart cell permeability to normally impervious phosphinates\textsuperscript{28, 29}. As for tabtoxin, the dipeptide prodrug is secreted by the producer strain, and neighboring plant cells take it up by dipeptide permeases, and then TβL is released by peptidase cleavage inside of plant cells (Figure 4.2).\textsuperscript{30-32} Because of the structural similarity, free TβL metabolite is misrecognized by the plant GS as its natural substrate glutamate, and causes an irreversible ATP- and time-dependent GS inactivation.\textsuperscript{20, 23, 24, 33}

Phytotoxin utilization by plant pathogenic *P. syringae* involves biosynthesis of the prodrug tabtoxin, efflux of tabtoxin, prodrug activation to release TβL, inhibition of plant GS, buildup of ammonia, and blockage of membrane function resulting in chlorosis. Regulation and efficient control of tabtoxin biosynthesis is essential to ensure useful titers of the phytotoxin are achieved without self-intoxification.\textsuperscript{34} Hence, a better understanding of tabtoxin biosynthesis will aid in understanding *P. syringae* pathogenesis and virulence against specific host plants and will aid in genome mining efforts to identify tabtoxin-producing pathovars and novel tabtoxin analogs such as Cl-tabtoxin. Early investigations of tabtoxin biosynthesis leveraged stable
isotope feeding studies to identify precursor metabolites. A $^{13}\text{C}$-labeled D-glucose feeding study showed that TβL arises from a 4-carbon fragment from aspartic acid, a 2-carbon fragment from pyruvate, and a single carbon from the single-carbon metabolic pool (Figure 4.3). The single carbon was confirmed to be generated from a methyl group of methionine by an incorporation experiment with $^{13}\text{C}$-labelled compounds. The 6-carbon backbone of TβL is formed by the condensation of aspartate and pyruvate, which is identical to that of lysine biosynthesis. This finding formed the basis for the hypothesis that TβL shares the initial steps of lysine biosynthesis pathway. This was later confirmed by additional stable isotope labeling studies and a gene knockout study supporting that TβL biosynthesis diverges from lysine biosynthetic pathway somewhere between the formation of tetrahydropicolinate (THDPA) and diaminopomelate (DAP).

The TβL biosynthetic enzymes of P. syringae were identified as a cluster of 15kb tabtbl genes. Most of the tab genes (tabABDP) have high sequence homology to lysine biosynthetic genes (lysA, dapDHCL). These genes are believed to function in the earlier stages of tabtoxin biosynthetic pathway. The later stages of tabtoxin synthesis require enzymes encoded by the tblSCDEF genes. Upon formation, TβL is coupled with L-Thr to form the tabtoxin dipeptide prodrug by TblF, an ATP-dependent carboxylate amine-ligase, and subsequently is exported by the transporter TblR, a putative member of the major facilitator superfamily (MFS) of membrane transporters. Ttr is a gene outside of tabtoxin biosynthesis gene cluster that causes resistance of tabtoxin in transgenic tobacco plants by acetyl-modification of the compound. Structural studies indicated that Ttr belongs to the GNAT family of acetyltransferases that utilize acetyl coenzyme A as acetyl group donor. A later study confirms
the acetyl transfer activity of Ttr in vitro and reports the substrate of this enzyme is TβL (acetylation at the alpha-amino group) but not the dipeptide tabtoxin.\textsuperscript{45-49}

In this work, we extracted Cl-tabtoxin from \textit{Streptomyces} and activated the prodrug with a recombinant dipeptidase (PepA), showing that Cl-tabtoxin also uses the Trojan horse strategy for entering cells to access the cytoplasmic GS target. As with TβL, Cl-TβL also shows inhibition against glutamine synthetase in the \textit{in vitro} assay. We discovered the biosynthetic gene cluster encoding for Cl-tabtoxin biosynthesis in \textit{Streptomyces} 372A, NRRL 8045, which shows high similarity to the core BGC for tabtoxin in \textit{P.syringae}. This proposed Cl-tabtoxin BGC was confirmed by validating the substrates and catalytic activity of three biosynthetic enzymes encoded by the genes \textit{cabB}, \textit{cblA}, and \textit{cabD} that are located within the BGC. Based on comparative genomics and BLAST analysis of genes flanking the core TβL biosynthesis genes shared between \textit{P. syringae} and \textit{Streptomyces} we propose a complete biosynthesis pathway of Cl-tabtoxin. The unique shunt pathway in \textit{Streptomyces} enables the halogenation of TβL as well as an alternative way for dipeptide prodrug formation via activation by a nonribosomal peptide synthetase. This work reports a novel strain that contains the biosynthesis gene cluster for a tabtoxin-like product and provides us a way to find more strains that can synthesize similar functional compounds via genome mining.

4.4 Results and Discussion

\textit{Cl-tabtoxin produced by Streptomyces is an inhibitor of glutamine synthetase}

Cl-tabtoxin was first isolated from \textit{Streptomyces}, and was structurally elucidated as (S)-alanyl-3-[a-(S)-chloro-3-(S)-hydroxy-2-oxo-3-azetidinylmethyl]-(S)-alanine in 1974 (Figure 4.1a).\textsuperscript{2} It was reported that Cl-tabtoxin had inhibitory effects on the growth of both Gram-
negative and Gram-positive bacteria in minimal medium, but the growth could be recovered with the addition of L-glutamine. This glutamine-dependent antibacterial activity had supporting evidence showing that Cl-tabtoxin was a prodrug GS inhibitor, similar to the structurally related natural product tabtoxin (Figure 4.1b). Furthermore, just as tabtoxin can evade β-lactamase-mediated antibiotic resistance\(^5\), Cl-tabtoxin was not an apparent substrate for penicillinase.

Following the procedure reported in the patent (US3956067) about Cl-tabtoxin, we cultured *Streptomyces* 372A, NRRL 8045 in an aqueous carbohydrate solution with a nitrogen source under aerobic conditions. The culture was harvested and filtered through Celite, followed by ion-exchange column purification. Cl-tabtoxin was eluted by pyridine, concentrated under reduced pressure and resuspended with 50% ethanol in water. The sample was then loaded onto an HPLC with a C18 column. Each peak of the HPLC trace was collected separately (Figure 4.4) and bioactivity was tested using an agar diffusion bioassay in glutamine-free media agar (Figure 4.5). The earliest peak (~5min) fraction is the only one that shows inhibition on *E.coli* 25922 growth. This short retention time is consistent with the polarity of Cl-tabtoxin based on its structure: the NH\(_3^+\) group makes Cl-tabtoxin very polar and less likely to stick on the C18 column. The inhibition of *E.coli* growth was recovered when L-glutamine was added into minimal media agar (Figure 4.5). This result supported that Cl-tabtoxin is an inhibitor of GS since GS is the only pathway to synthesize L-glutamine in *E. coli*. Sterile kanamycin solution was used as a positive control and showed the same level of growth inhibition activity for both sets of conditions. Sterilized water was used as a negative control which showed no inhibition. We attempted to characterize purified Cl-tabtoxin using LC-MS without success. The original study had the same problem, where they submitted several derivatives of Cl-tabtoxin to MS analysis without any interpretable results obtained.\(^2\) This could be due to the presence of the Cl
atom making the compound sensitive to decomposition during the MS ionization process leading to the formation of uncharged molecular fragments or unidentified fragment ions. We were also able to perform a computational study supporting GS inhibition by docking the C3-O-phosphorylationed Cl-Tβl GS product in the active site of GS using AutoDockTools (Figure 4.40). We obtained an excellent overlay of the docked P1-Cl-TβL with the crystal structure of P1-Glufox bound to GS suggesting Cl-Tβl can be the substrate for GS leading to formation of a tight-binding transition state inhibitor after phosphoryl group transfer to the C3-hydroxy group consistent with observations and experimental data collected for closely related TβL (Figure 2.13).

**Cl-tabtoxin is a dipeptide prodrug that can be cleaved by peptidase PepA**

Tabtoxin is known to utilize the Trojan Horse strategy (Figure 4.2) to release GS inhibitor TβL inside of the plant cells. Since Cl-tabtoxin is a modified version of tabtoxin, we believe it also uses this strategy to get into the victim cells. The enzyme that is responsible for converting tabtoxin to TβL was found in the periplasmic space of Pseudomonas syringae pv. tabaci.\(^5^1\) It functions as an aminopeptidase and requires zinc or other divalent cations for activity.\(^3^1\) In our earlier study with tabtoxin in Chapter 2, *P. syringae* lysate containing this dipeptidase and ZnCl₂ was used to release free TβL.\(^2^7\) Initial attempts to cleave the dipeptide bond of Cl-tabtoxin with *P. syringae* cell lysate failed indicating that the dipeptidases are specific for certain dipeptide isoforms (TβL-Thr vs Ala-Cl-TβL). We next attempted to use *E. coli* cell lysate; however, the *E. coli* lysate was not capable of cleaving the Cl-Tabtoxin peptide bond. This limited dipeptidase substrate specificity is reminiscent of reports for two phosphonate tripeptide antibiotics, rhizoticin (Figure 4.1e) and plumbemycin (Figure 4.1f) that share the same active component but contain different amino acid side chains. Plumbemycin is an
antibacterial compound with little activity against fungi while rhizoctin is an antifungal compound with little activity against bacteria. People believed that the difference in bioactivity is caused by substrate specificity in uptake or processing, which includes the cleavage of peptide bond for inhibitor release\textsuperscript{28, 52}. From this information, we decided to turn to Cl-tabtoxin’s producer: \textit{Streptomyces} lysate, which also failed in cleaving the peptide bond. This failure may be caused by instability feature of peptidase. It was reported that \textit{P.syringae} peptidase was unstable during the purification process\textsuperscript{31}, which may also be true for \textit{Streptomyces} peptidase. The failure can also be caused by other reasons such as a loss in function of the peptidase after cell break step, or the peptidase gets inhibited by some component in the cell lysate after cell break.

After realizing the lack of dipeptide bond cleavage by \textit{P. syringae}, \textit{Streptomyces} and \textit{E. coli} lysate, we focused on using a stable recombinant dipeptidase, PepA, to finish this cleavage for Cl-tabtoxin. PepA was reported to be the most contributive to the cleavage of peptide bond of a glycineleucine dipeptide prodrug: dehydrophos (Figure 4.1g), which is also a natural product of \textit{Streptomyces}, to obtain its bioactivity.\textsuperscript{53} For our study, we cloned the \textit{E. coli} pepA gene into a pET28 protein expression vector and transformed this plasmid construct into an \textit{E. coli} BL21 (DE3) heterologous host for over-expression of \textit{N}-His\textsubscript{6}-tagged PepA followed by Ni-NTA affinity purification (Figure 4.6).

Optimization of pH conditions is crucial for the PepA peptide cleavage assay since Tβl/tabtoxin, and presumably Cl-TβL/Cl-tabtoxin, can isomerize under neutral to basic pH environments via intramolecular translactamization to form the thermodynamically more stable δ-lactam isomer, which is inactive against GS (Figure 4.7). It was reported that at 23°C, the half time for tabtoxin isomerization is 630 hr at pH 5.4, 37 hr at pH 7.2 and only 15 hr at pH 8.9.\textsuperscript{49}
other words, tabtoxin is more stable in lower pH. Based on this feature, a lowest possible pH for PepA to function is needed to prevent undesired isomerization. A broad pH screening with buffer pH adjusted from 5 to 8 was conducted to test PepA dipeptide cleavage activity with a model L-Ala-L-Ala dipeptide substrate including control reactions lacking enzyme or substrate (Figure 4.8 a,b). LCMS analysis of PepA reaction mixtures showed an increase in PepA activity with pH increasing as judged by an increase in the formation of free L-Ala. We also tested the effects of metal addition (MgCl₂) on PepA activity under the same pH conditions to see if the presence of Mg²⁺ ions could allow the enzyme to function under a lower pH range. Results in Figure 4.8 c,d showed a great increase in enzyme activity in the pH range that PepA was already active, but the presence of Mg²⁺ did not broaden the pH range that the enzyme was effective. Specifically, PepA remained inactive at pH 6 with Mg²⁺ addition. A finer pH screening study between 6 to 7.5 (Figure 4.9) showed that the most appropriate pH for PepA assay is 7.0 with Mg²⁺ addition.

Under these optimized conditions, we tested the PepA cleavage reaction on Cl-tabtoxin purified from Streptomyces culture supernatant along with several synthetic and natural dipeptide prodrugs on hand: Ala-Ala-GLufs (AAG), Ala-GLufs (AG), Glufs-Thr, Thr-MetSox, and tabtoxin. We screen all substrates in PepA reactions conducted at both room temperature and 37 °C. These prodrugs represent a range of prodrug formulations including different amino acid prodrug components (Ala or Thr), different amino acid number (two or three), and the order of amino acid and active component (N-term or C-term orientations). “No substrate” or “no enzyme” were used as controls under the same reaction conditions. PepA cleavage activity was evaluated by the amount of inhibitor formation as measured by LC-MS analysis and the in vitro GS inhibition assay (more the inhibitor produced = more GS inhibition observed). Figure 4.10 shows the relative inhibition levels of GS caused by the free GS inhibitor released from PepA-
catalyzed dipeptide bond cleavage (higher bar = better GS inhibition observed). The general trend is that cleavage rate increases at 37 °C compared to room temperature (RT) since the relative apparent GS inhibition was higher at 37°C in most cases. The exceptions are Cl-tabtoxin and Thr-MetSox, which have similar GS inhibition under both temperature conditions. The Cl-tabtoxin case may be caused by increased isomerization at high temperature (37°C) stressing the important balance of substrate/product stability vs enzyme activity in reaction optimization.

Figure 4.11 shows the ion counts of released free drug. The LC-MS results support the GS inhibition assay in Ala-Glufos, Glufos-Thr, tabtoxin, and Thr-MetSox cases. Higher ion counts is attributed to greater release of free drugs by dipeptide cleavage, which correlates to greater inhibition of GS enzyme activity. Due to our inability of detecting Cl-TβL and Cl-tabtoxin by LC-MS no LC-MS data is available for Cl-tabtoxin cleavage. Thus, we relied on measuring GS inhibition activity to judge cleavage efficiency of Cl-tabtoxin, which shows a strong inhibition on GS suggesting that Cl-tabtoxin is a good substrate for PepA (Figure 4.10).

As a conclusion, we optimized the conditions for PepA assay, and performed the peptide bond cleavage on the prodrugs that we currently have on hand. Based on GS inhibition assay and LC-MS detection, we are confident with our conclusion that PepA is able to cleave the peptide bond of diverse prodrugs to release the free GS inhibitors. Excitingly, PepA is able to cleave the peptide bond of Cl-tabtoxin providing a potentially useful route for obtaining larger quantities of Cl-TβL that will be needed for complete characterization of GS inhibitory activity.

Genome mining for the Cl-tabtoxin biosynthetic gene cluster

The biosynthetic gene cluster for tabtoxin in P. syringae was reported in 2005 (Table 4.1). This 28 kb-length gene cluster is made up of four blocks. The first block gives evidence that
the whole gene cluster is inserted into the current host’s chromosome by horizontal transfer due to the presence of phage integrase at a juncture with a tRNA gene. The last block’s function remains a mystery while the middle two blocks encode enzymes that are directly involved in the biosynthesis of tabtoxin. The second block was originally referred to as the “left biosynthetic block”, and the third block was called the “right biosynthetic block” based on their relative transcriptional direction. The left biosynthetic block is made up of genes \textit{tabP, D, B, A, C} and \textit{tblA}. TabC is predicted to contain a motif that is homologous to zinc-binding proteins and a most recent BlastP analysis shows it is a homologue of a peptidase. TblA showed no matches in the original study, but our BlastP search shows high identity to class I SAM-dependent methyltransferase from multiple strains. TabA, B and D show homology to three conserved enzymes in the lysine biosynthesis pathway (\textbf{Figure 4.12} and \textbf{Table 4.2a}). TabA is similar to LysA, which functions as diaminopimelate (DAP) decarboxylase, TabB has high similarity to DapD and DapH, a succinyl or acetyl-diaminopimelate (DAP)-transferase, and TabD is an aspartate aminotransferase (aat)-like aminotransferase, which is a homologue of DapC. TabP is believed to be a zinc metallopeptidase that was predicted to hydrolyze tabtoxin to TβL and L-Thr by peptide bond cleavage. However, our attempts to reconstitute the activity of TabP have failed, in part due to the autoproteolytic activity of this enzyme in the presence of ZnCl\textsubscript{2}. The right biosynthetic block is composed of \textit{tblS, C, D, E, F, R}. TblS is homologous to asparagine synthetase and the related enzyme, β-lactam synthase\textsuperscript{54}, which was proven to catalyze the β-lactam ring formation in the β-lactam family molecules such as clavulanic acid (\textbf{Figure 4.1h}) biosynthesis. TblS catalyzes AMP group transfer from ATP to the carboxyl group of a beta-amino acid to form the acyl adenylate that is subsequently cyclized to the corresponding beta-lactam via intramolecular nucleophilic acyl substitution with release of AMP. TblC is a
homologue of clavamine synthase\textsuperscript{55}, which is related to the Fe(II)/alpha-KG-dependent catabolic enzyme taurine dioxygenase. TblD is a fusion of glucose-methanol-choline (GMC) oxidoreductase and a GCN5-related N-acetyltransferase (GNAT) domain on the C-terminal end. TblE is a membrane protein that is hypothesized to form a functional pair with TblF, which was proven to be an ATP-dependent carboxylate-amine ligase, responsible for converting TβL and L-Thr to tabtoxin, the prodrug form that is exported outside of producer cells with the help of major facilitator superfamily (MFS) transporter TblR (Figure 4.13). For now, in vitro reconstitution of enzyme activity and substrate identity have been revealed for two enzymes in the pathway, TabB\textsuperscript{56} and TblF\textsuperscript{49}. Additionally, TabD was shown to have aminotransferase activity in vitro but the relevant substrate in tabtoxin biosynthetic pathway is still unknown.\textsuperscript{56}

Based on the high structural similarity between tabtoxin and Cl-tabtoxin, we hypothesized that the producer BGCs will be similar despite the distal evolutionary relationship of known produces; namely, Pseudomonas and Streptomyces, respectively. We use whole-genome Illumina shotgun sequencing to sequence and assembly the genome of the known Cl-tabtoxin producer Streptomyces 372A, NRRL 804. We used comparative genomics with genes from the known tabtoxin BGC to identify a homologous gene cluster containing most of the genes required for tabtoxin biosynthesis in P. syringae (Figure 4.14 and Table 4.3). After first assembly of the BGC it appeared as though direct orthologs of tblA (methyltransferase; we later found this to be present), tabP (dipeptidase), tblE (membrane protein) and tblR (MFS transporter) genes were missing. The relative transcriptional direction of genes is also slightly different from tabtoxin BGC. All of the genes are transcribed leftward in Cl-tabtoxin BGC instead of the distinct ‘left’ and ‘right’ blocks observed for the tabtoxin BGC. We hypothesized that additional producing strains that harbor similar BGCs encoding for the biosynthesis of
tabtoxin-like compounds were likely to exist in public databases. Since TblS, predicted β-lactam synthetase, is unique for catalyzing β-lactam ring formation and TabB has been characterized, we conducted a targeted BlastP analysis based on the sequence of the analogous proteins, CblS and CabB from *Streptomyces*, respectively, and found a variety of *Streptomyces* strains and *Pseudomonas* harboring homologous genes in the NCBI database.

We utilized the antiSMASH (https://antismash.secondarymetabolites.org/#!/start) software to analyze genomes of the candidate tabtoxin producing *Streptomyces* strains to identify the putative BGCs. The BGCs identified in the top 14 strain hits from the original NCBI BlastP search are shown in Figure 4.15 and Table 4.4. Among these BGCs, nearly all have identical gene composition analogous to the proposed BGC from *Streptomyces* 372A, NRRL 8045 (producer of Cl-tabtoxin). Specifically, all of them contain the same partial “right biosynthetic block” (shown in green), which is in the order of tblS,C,D. The leftover genes from the “right biosynthetic block” (tblE,F,R) are shown in blue; however, *Streptomyces* lack the tblE and tblR genes while all three *P. syringae* strains (#11-13) maintain these genes. Two strains (#5 and #14) are also missing a tblF homolog. Only one *Streptomyces* (#6) strain contains homologs of both tblE and tblF. Since the tblE,F,R are believed to be functional pairs for prodrug formation and transportation, we think that the pair can vary among different bacterial species with different cell envelope compositions. For example, *P. syringae* is a Gram-negative bacteria that has an outer membrane, and will need transporter TblR to transport prodrug from periplasm out of the cell through outer membrane (Figure 4.13). However, *Streptomyces* is Gram-positive bacteria, which only has one layer of membrane. Thus, *Streptomyces* potentially does not require this triad of proteins to achieve antibiotic efflux. As reported, TblF itself is enough for prodrug formation in vitro without the help of membrane protein TblE, and membrane transport was found to be a
non-enzyme regulated in *P. syringae*. The mechanism of prodrug formation and subsequent efflux is likely to be different among bacteria. The BGCs in *Streptomyces* contain flanking genes encoding for a nonribosomal peptide synthetase (NRPS) that might be involved in formation of the dipeptide bond and halogenation during Cl-tabtoxin biosynthesis (*Table 4.5*; discussed in subsequent sections). All of the BGCs share the same partial “left biosynthetic block” (shown in red/orange), which is in the order of *tabC*, *tblA*, and *tabA,B,D*, except one strain (#6) missing *tabD*. Only the three *P. syringae* strains have *tabP* (shown in yellow) (#11-13). The absence of *tabP* in *Streptomyces* 372A, NRRL 8045 is consistent with the failure of peptide cleavage of Cl-tabtoxin with *P. syringae* and *Streptomyces* lysate because *Streptomyces* may use different for dipeptide cleavage and release of free TβL or Cl-TβL. One unique attribute for *Streptomyces* 372A, NRRL 8045 is that it appeared to be missing *tblA*, encoding for a SAM-dependent methyltransferase, which is present in all of the other 14 top strains from this comparison. The absence of *tblA* will be be discussed in a later section of this chapter. Another notable thing is that all of the *Streptomyces* strains maintain a single direction of transcription for the consensus conserved block of genes associated with TβL biosynthesis while the three *P. syringae* strains (#11-13) maintain two distinct blocks of biosynthetic genes with opposing transcriptional directions.

In summary, the consensus genes required for synthesis of tabtoxin-like compounds include: *tblS*, *tblC*, *tblD*, *tabC*, *tblA*, *tabA*, *tabB*, and *tabD*, with *tblF* at the end or in between *tblD* and *tabC*. However, the function of most of the enzymes, the order of these enzymes function, and the mechanism of each step of tabtoxin biosynthesis is still not fully elucidated. Even though we have each piece of the puzzle, much work is still needed to assemble the pieces of the puzzle to see the complete picture of tabtoxin biosynthesis.
CabB as a N-succinyl/acetyl-transferase

Lysine and TβL are structurally homologous sharing a portion of the same biosynthetic pathway in producing strains. A dapB knockout study in P. syringae proved that DapB (dihydrodipicolinate reductase) is required for both lysine and TβL biosynthesis suggesting that L-2,3,4,5-tetrahydrodipicolinate (THDPA), product of DapB, is the last common intermediate shared between the lysine and TβL biosynthesis pathways (Figure 4.12). After the THDPA metabolic branch point, four major pathways have been identified for the formation of meso-diaminopimelate (meso-DAP) from THDPA in bacterial lysine biosynthesis (Figure 4.12). In proteobacteria, firmicutes and actinobacteria, THDPA is succinylated by DapD, which is a THDPA succinyltransferase (THDPA-ST) with succinyl coenzyme A (CoA) as a co-substrate to form N-succinyl-2-amino-6-ketopimelate. DapC catalyzed transamination from L-glutamate followed by DapE catalyzed hydrolysis of the N-succinyl group to yield L,L-DAP, which is converted by an epimerase DapF to meso-DAP. A similar lysine biosynthesis pathway is used by Bacillus subtilis and Bacillus megaterium except that THDPA is acetylated by an acetyltransferase (THDPA-AT) DapH, instead of THDPA-ST, and as a result the subsequent intermediates are acetyl derivatives. The third pathway converts ammonium and THDPA to L,L-DAP directly by DAP aminotransferase DapL without succinylation nor acetylation. This pathway is discovered in cyanobacteria, chlamydia, archaeon Methanothermobacter thermautotrophicus, and plant Arabidopsis thaliana. The fourth pathway is present in Bacillus and Brevibacterium species and Corynebacterium glutamicum which can convert ammonium and THDPA to meso-DAP directly with NAD(P)H catalyzed by enzyme meso-DAP dehydrogenase (DDH). Based on this information, the two strains in our study: P. syringae and
Streptomyces should both use the first lysine biosynthesis pathway (succinylation pathway) since Pseudomonas belongs to proteobacteria and Streptomyces is in actinobacteria family.

A blast study in P. syringae and Streptomycyes did not show any homologues of LysC, Asd, DapA, DapB, DapE, DapF and Ddh in the tabtoxin BGC (Table 4.2). The blast study shows high percentage homologies of DapD and DapH (homologous to TabB), DapC (homologous to TabD), and LysA (homologous to TabA). This result confirmed that tabtoxin used enzymes and intermediates from lysine biosynthesis pathway, and that these two pathways branch off at THDPA point. The result also supports that tabtoxin uses the succinylation/acetylation and transamination actions in its biosynthesis, presumably on an unknown metabolite derived from THDPA which is the common intermediate of lysine biosynthesis. In spite of the parallels with the well-known lysine biosynthetic pathway, many details in the TβL biosynthesis are still a mystery. The comparison between proteins encoded by lysine and tabtoxin BGC led us to target TabB, a predicted THDPA-N-acyltransferase, as our initial study. Since TabB is a homologue of DapD, the first enzyme after branch point in lysine BGC, we proposed TabB would also be involved in a very early step in the TβL biosynthesis and the substrate for this enzyme would be the branching metabolite THDPA.

The first study on TabB was in 1997, when Shaw’s group gave the name of tabB gene and proposed the function of TabB. They confirmed that this enzyme is essential for tabtoxin production by knocking out tabB gene in P. syringae. They also showed that TabB expressed in E. coli showed THDPA-ST activity and was able to replace a defective dapD gene in E. coli. However, TabB played no essential role in lysine biosynthesis in P. syringae. Instead, they found that TabB could function as an acetyltransferase in TβL biosynthesis pathway, which is consistent with the discovery of acetylated intermediates (Figure 4.1k) being involved in
tabtoxin biosynthesis in an earlier study. After a 21-year gap of studying TabB, in 2018, the Calderone group characterized this enzyme and their findings support a slightly different function of TabB. They found that TabB is preferentially a THDPA-N-succinyltransferase in vitro instead of an acetyltransferase. In fact, their study showed the enzyme has 10-fold preference of succinyl-CoA over acetyl-CoA as the acyl donor substrate for THDPA.

We were curious about the function of TabB (acetylation vs succinylation) in controlling THDPA flux through Lys and TβL biosynthesis. We also sought to validate the analogous tabtoxin biosynthetic pathway in Streptomyces and thought CabB (homolog of TabB) would be a good place to start. We cloned out cabB (homologous gene of tabB) from Streptomyces 372A, NRRL 8045 into pET28 and heterologously expressed the protein in E. coli BL21 (DE3) as the N-His<sub>6</sub> construct to allow for purification with Ni-NTA affinity chromatography (Figure 4.16). The predicted substrate for CabB, THDPA, was generated from commercially available meso-DAP by treatment with the recombinant enzyme Ddh. As mentioned earlier, Ddh is the enzyme involved in lysine biosynthesis pathway which can bypass traditional acylated or succinylated intermediates by catalyzing reductive amination of THDPA to meso-DAP directly. The reverse direction of this reaction was utilized in this study to form THDPA from meso-DAP in situ (Figure 4.17). Codon optimized Corynebacterium glutamicum Ddh was cloned into pET28 and expressed in E.coli BL21(DE3) followed by Ni-NTA purification of the recombinant N-His<sub>6</sub>-tagged Ddh (Figure 4.18). Pure Ddh was used to catalyze conversion of meso-DAP and NADP<sup>+</sup> into THDPA and NADPH, respectively. The reaction was monitored via optical absorbance spectroscopy at 340 nm corresponding to the known absorbance of NADPH product at this wavelength. This kinetic analysis showed Ddh is a fast enzyme that rapidly establishes
equilibrium for this reaction (Figure 4.19). LC-MS analysis of the Ddh reaction mixture showed good conversion of *meso*-DAP to THDPA after 2 hrs of reaction time (Figure 4.20).

The CabB reaction (Figure 4.21) was monitored by optical absorbance (UV-Vis) and LC-MS. For UV-Vis detection, 5,5’-dithio-bis-(2-nitrobenzoic acid) (DTNB) was added to the reaction mixture as an indicator of coenzyme A thiol formation with monitoring at 412 nm (Ellman assay) (Figure 4.22). Figure 4.23 showed that CabB has succinyltransferase activity with THDPA as substrate. On the other hand, we observed lower activity when acetyl-CoA was used as the acy donor; consistent with the result reported by Calderone group for TabB from *P. syringae* (Figure 4.24). The succinylated and acetylated THDPA-derived products were detected via LC-MS (Figure 4.25 and Figure 4.26) after an overnight incubation of enzyme and substrate. The retention time for succinyl-THDPA is reasonably longer than acetyl-THDPA since the succinyl group is more hydrophobic than the acetyl group. We used chemical modification of the products by treatment with NH$_2$OH (not shown in figure) and O-benzylhydroxyamine (Figure 4.27 and Figure 4.28) to form the corresponding oxime products after reaction with the ketone group only present in the acylated products. We observed molecular ions corresponding to the oxime products for both reactions using either succinyl-CoA and acetyl-CoA. We observed a double peak pattern in the LC-MS trace corresponding to molecular ions with the same mass. This was attributed to formation of *cis-* and *trans-*isomers (diastereomers) of the oxime when O-benzylhydroxyamine reacts with the succinyl/acetyl-THDPA. Controls lacking enzyme, acyl-CoA substrate, or THDPA substrate were as expected with no product formation observed via LC-MS. The overall percent conversion for CabB-catalyzed succinylation and acetylation of THDPA are about the same based on LC-MS detection, but the reaction rate for succinyl-THDPA formation is much faster than acetyl-THDPA based on Ellman’s assay. We
conclude that CabB can act as both $N$-succinyltransferase and $N$-acetyltransferase using THDPA as substrate to presumably provide a pool of $N$-succinyl-$L$-2-amino-$6$-oxopimelate and $N$-acetyl-$L$-2-amino-$6$-oxopimelate products that can either proceed through Lys or TβL biosynthesis depending on the presence or absence of downstream enzymes.

**CblA is a SAM-dependent C-methyltransferase**

TblA, the predicted methyltransferase from the tabtoxin BGC in *P. syringae*, was first studied by the Willis group. Willis and coworkers reported the nucleotide sequence of *tblA* gene and monitored gene transcription levels during TβL biosynthesis. Their study showed the *tblA* transcription is elevated at all stages of bacterial growth, which correlates to the fact that TβL can be detected at every stage of bacterial growth. They also found that *tblA* is regulated by *lemA* gene (now known as *gacS* gene) at the mRNA level, most likely at the stage of transcription. No follow up studies have been reported for TblA and the biochemical function of this enzyme is still a mystery. However, the most recent BlastP search conducted in our lab revealed it is a homologue to class I SAM-dependent methyltransferase from multiple strains.

Methyltransferases (MTs) are a big group of enzymes that are capable of methylating their substrates. Class I MT is the most common class among this group of enzymes. All members of the Class I MT family have a Rossmann fold required for binding $S$-Adenosyl methionine (SAM) ([Figure 4.1i](#)), which is the classical methyl donor for MTs. Methyl groups can be added onto $S$, $N$, $O$, or $C$ atoms, and are named by which atoms are modified, with $O$-methyltransferases being the most common class. The generally accepted mechanism for methyl transfer is an $S_N2$-like nucleophilic attack on the electrophilic SAM methyl group polarized by the attached sulfur atom of the methionine component of the SAM cosubstrate. After methyl
group transfer to the substrate, SAM is converted to 5'-Adenosyl homocysteine (SAH) which can be recycled via the so-called SAM-e cycle.67

As we noted earlier, a TblA homolog appeared to be missing in the Cl-tabtoxin BGC identified in Streptomyces 372A, NRRL 8045 while all of the other top 14 strains identified via comparative genomics do contain a clear TblA homolog. Upon closer analysis of the Cl-tabtoxin BGC cluster (Figure 4.15 and Table 4.5) we noticed there is a gap in the assignment of open reading frames between tabA (TAW3_06392) and tabC (TAW3_06394), where tblA is expected based on comparison to homologous BGCs. We used LaserGene software to analyze the DNA sequence in this gap region (TAW3_06393) and discovered a homologue of glutamine tRNA that interfered with our assignment of an open reading encoding a TblA homolog. During genome assembly this short (86 bp) Glu tRNA, TAW3_06393, was prioritized in assignment over the overlapping tblA open reading frame (420 bp). Thus, we manually searched the region between TAW3_06392 (end at 63923 position of the contig) and TAW3_06394 (start at 64645 position of the contig). We looked into 6 possible (3 possibilities for leftward and 3 possibilities for rightward) translation results and found one with reasonable intact translated protein with clear start and stop codons (Table 4.6c). We used the predicted coding sequence in Table 4.6c for homology searches in the NCBI database using BlastP and discovered closely related enzymes in the class I SAM-dependent MT protein family sharing 98% sequence identity. Since typical protein translation starts from Methionine, we think the real sequence (Table 5d) is four amino acids shorter than what we described above. The sequence provided in Table 5d has up to 100% identity to class I SAM-dependent MT in the same database from BlastP search results. This gene is translated leftwards, which is consistent with the direction of transcription for all other genes in conserved portion of tabtoxin BGC shared between Pseudomonas and
Streptomyces. Furthermore, structure simulation of this protein using Swiss model (https://swissmodel.expasy.org/) showed a Rossmann fold, which is a tertiary structure in three-layered sandwich shape with the filling being six beta sheet and the two bread slices are connecting alpha helices (Figure 4.29).\(^6\) As mentioned earlier, all of the Class I MTs have a Rossmann fold. We conclude that *cblA* is homologous to *tblA* and encodes for the ‘missing’ methyltransferase, CblA, in the Cl-tabtoxin BGC (Table 5b).

We cloned *cblA* into a pET28 vector and expressed CblA as the N-His\(_6\) tagged protein in *E.coli* BL21 (DE3) to enable affinity purification with Ni-NTA resin (Figure 4.30). In order to prove our proposed function and reaction of CblA as a SAM-dependent C-methyltransferase shown in Figure 4.31, a couple of assays were set up with a series of controls. For the biosynthetic reaction assays, THDPA, succinyl/acetyl-CoA, CabB (these are the same as “CabB assay”), SAM, and CblA are co-incubated at room temperature overnight before analyzing the reaction mixture by LC-MS. Figure 4.32 shows LC-MS extracted ion chromatograms that support the ability for CblA to methylate both succinyl- and acetyl-THDPA (a.k.a. \(N\)-succinyl-L-2-amino-6-oxopimelate and \(N\)-acetyl-L-2-amino-6-oxopimelate) as substrate to form the corresponding C5-methylated-succinyl/acetyl-THDPA (C5-methyl-\(N\)-succinyl-L-2-amino-6-oxopimelate and C5-methyl-\(N\)-acetyl-L-2-amino-6-oxopimelate) with similar percent conversions. In the control reactions where succinyl/acetyl-CoA are omitted, no methylated products were detected, which ruled out the possibility for CblA taking THDPA directly as a substrate. In another word, CblA functions after CabB in the Cl-tabtoxin biosynthetic pathway and accepts both acetylated and succinylated substrates. Figure 4.33 shows the controls of CblA reaction where only acetylated substrates were utilized. The product C5-methyl-\(N\)-acetyl-L-2-amino-6-oxopimelate is only detected in the complete reaction and is absent in all control
reactions (no CblA enzyme, no SAM, and no THDPA (no substrate)). In other words, this methyl-transfer action is indeed catalyzed by CblA enzyme. The “no SAM” control result confirms that CblA is a SAM-dependent methyltransferase, which was predicted from the earlier BlastP result. The increase in retention time for products compared to substrates is reasonable since the additional methyl group makes the molecule more hydrophobic.

It is noteworthy that attempted oxime-formations of the ketone of C5-methyl-N-acetyl-L-2-amino-6-oxopimelate CblA product with hydroxylamine and O-benzylhydroxylamine failed to generate the corresponding oxime products. We attribute this to unfavorable steric due to C5-methylation alpha to the ketone group. Presumably, the C5-position can be readily activated by CblA as the corresponding enolate suitable for nucleophilic attack on SAM. We are currently validating C5-methylation and exploring the stereospecificity of this methylation using preparative scale enzyme reactions with full characterization of the product via NMR. We conclude that CblA is a SAM-dependent methyltransferase which accepts by succinyl- and acetyl-THDPA (products of CabB) as substrate and forms CH$_3$-succinyl/acetyl-THDPA with similar yield. While this methylation commits carbon flux towards TβL biosynthesis and away from Lys biosynthesis it is unclear of both succinylated and acetylated intermediated are carried forward through downstream TβL biosynthetic reactions.

**CabD is a PLP-dependent aminotransferase**

The Calderone group first characterized the TabD enzyme from the *P. syringae* tabtoxin BGC in 2018, along with TabB. They reported that TabD is a pyridoxal phosphate (PLP) -dependent aminotransferase that utilized L-glutamate as the preferred amine donor.$^{56}$ They also reported that TabD was able to function on THDPA analogs as an aminotransferase, with the
substrate preference THDPA > succinyl-THDPA ≥ acetyl-THDPA based on reaction rate. Unfortunately, they did not establish which TabD substrate continues forward through TβL biosynthesis. With the discovery and validation of CblA in our work, we think that the reason for the failure in finding the real substrate for TabD in Calderone’s study is that they didn’t investigate the methylated analogs of THDPA and were unaware of the timing for methylation occurring after TabB/CabB-catalyzed methylation of THDPA. We propose that the product of the CblA reaction, C5-methyl-\(N\)-acetyl-L-2-amino-6-oxopimelate or C5-methyl-\(N\)-succinyl-L-2-amino-6-oxopimelate is the potential preferred substrate for TabD and CabD (Figure 4.34). We hypothesized that this could also be the enzymatic stage where acetylated and succinylated products could be distinguished.

In order to test our hypothesis, \(cabD\) was cloned into a pET28 vector and over-expressed as the \(N\)-terminal hexahistidine fusion in \(E.coli\) BL21 (DE3) followed by purification with Ni-NTA resin (Figure 4.35). For CabD \textit{in vitro} reactions, THDPA, succinyl/acetyl-CoA, CabB, SAM, CblA (these are the same as “CblA assays”), L-glutamate, PLP, and CabD were co-incubated at room temperature overnight before analysis by LC-MS (Figure 4.36a). A series of control reactions were carried out simultaneously under the same reaction conditions including no L-glutamate (amine donor), no THDPA (substrate), no CabD enzyme, and no PLP co-factor control experiments (Figure 4.36b–e). The complete CabD reaction gave good conversion to the expected C5-methyl-\(N\)-acetyl/succinyl-DAP products. Surprisingly, the reaction lacking addition of L-glutamate also gave detectable products (Figure 4.36b). We attributed this to some contamination of L-Glu from copurification in the enzyme preparation of PLP-dependent CabD. This is a common occurrence for PLP enzymes and we have observed this in our lab when
studying L-Thr transaldolase ObiH. The addition of excess L-Glu shifts the equilibrium towards more product compared to the “no-Glu” control reaction.

Now that we confirmed CabD are able to C5-methyl-\textit{N}-acyl-L-2-amino-6-oxopimelates as substrates, we tested to see if there is a preference for succinylated or acetylated substrate. We incubated CabD with different substrates including THDPA, acetyl/succinyl-THDPA (CabB product), and CH$_3$-acetyl/succinyl-THDPA (CabB & CblA product) at room temperature overnight prior to LC-MS analysis to detect the corresponding aminotransferase product (Figure 4.37). As shown in Figure 4.38, CabD can utilize all of these substrates along with L-Glu as the amino donor to produce the corresponding aminotransferase products. Apparent product conversions using methylated THDPA analogs as substrates is relatively higher than non-methylated THDPA analogs as substrate (Figure 4.38f). As shown in Figure 4.38f, there appears to be a slight preference for acetylated substrates compared to the analogous succinylated substrates, which contradicts conclusions from the Calderone group’s study of TabD in 2018. The explanation for this apparent contradiction can be explained by considering that the Calderon group was unaware that the apparent substrates for TabD/CabD are methylated by TblA/CblA prior to the aminotransferase step. Additionally, the Calderone group judged substrate preference by enzyme kinetics while we are judging enzyme preference by end-point equilibrium conversion to product. Considering the CabB step reaction rate for succinyl-CoA is much higher than acetyl-CoA based on kinetic results from the Ellman assay (see section on CabB), we may come into the same conclusion as Calderone group for the CabB/CblA/CabD assay when considering only relative reaction rates.

Another interesting finding came from the “no CabD enzyme” control experiment using THDPA as the substrate. At the very beginning of this project, we generated THDPA from \textit{meso}-
DAP and Ddh enzyme without further purification. As a result, unreacted meso-DAP is inherently carried over throughout subsequent coupled enzyme assays, including the CabD reaction. The “THDPA as substrate” for CabD assay can thus be a false positive result since the unreacted meso-DAP has the same apparent m/z as the product from THDPA and CabD. For this purpose, we conducted a “no CabD enzyme” control for “THDPA as substrate” of CabD assay. By comparing the amount of DAP, we can determine whether or not CabD is able to use THDPA as a substrate. Specifically, if there is more meso-DAP at equilibrium when CabD is added, it means the enzyme takes THDPA as a substrate and produces the additional meso-DAP, but if the amount of meso-DAP is the same between the reactions with and without enzyme, it means the enzyme cannot take THDPA as a substrate. Interestingly, neither of the scenarios happened. Instead, there is less DAP in the enzyme added group than without enzyme group (Figure 4.39). Further discussion and experiments are needed to figure out what is happening here.

As a conclusion, we confirmed that CabD enzyme has the ability to take CH₃-acetyl/succinyl-THDPA as substrates and convert into the corresponding CH₃-acetyl/succinyl-DAP products. We also found that CabD prefers methylated over non-methylated THDPA analogs, and prefers acetyl to succinyl format of THDPA analogs in the aspect of yield. It appears that CabD is not a selection filter for succinylated or acetylated intermediates leaving this to an unidentified downstream enzyme. This enzyme seems to be able to take any kind of THDPA analogs as a substrate and produce a pool of intermediates for various purposes such as the biosynthesis of CI-tabtoxin, tabtoxin, and lysine.

*CblF and chlorinating halogenase enzymes using an NRPS assembly line*
TblF in tabtoxin biosynthesis pathway was well characterized as a dipeptide ligase that conjugates TβL with L-Thr to form tabtoxin (Tβl-Thr) as a prodrug forming self-protection mechanism. The predicted homologue of TblF in Streptomyces is CblF, which we hypothesized would couple TβL with L-Ala to form the dipeptide with orientation corresponding to that observed in Cl-tabtoxin (Ala-TβL). The cblF gene was cloned into a pET28 vector and over-expressed in E.coli BL21 as the N-His$_6$-tagged protein. Recombinant N-His$_6$-CblF was purified by Ni-NTA chromatography. Following conditions from the published TblF assay procedure, CblF was incubated with TβL and L-Ala in order to form dipeptide product Ala-Tβl. However, instead of the formation of Ala-Tβl, we detected only L-Ala-L-Ala dipeptide as the product (Figure 4.41). The dipeptide formation confirmed that CblF has ATP-dependent ligase activity. The reason why CblF is unable to ligate Ala with Tβl may be because this enzyme has a high substrate specificity, and the absence of the Cl atom makes the enzyme unable to function. Or more likely, the Cl-tabtoxin dipeptide bond formation paradigm in Streptomyces differs from that for tabtoxin in P. syringae. We hypothesized that flanking genes encoding a nonribosomal peptide synthetase (NRPS) are involved in amide bond formation during Cl-tabtoxin biosynthesis in Streptomyces. Instead of functioning as an independent enzyme, an NRPS-mediated mechanism could ligate L-Ala with Tβl /Cl-Tβl to form the dipeptide prodrugs. The involvement of NRPS enzymes in the biosynthesis of peptide prodrugs is common in nature. This is the case of phosphinothricin-tripeptide (Figure 4.1j), a well-studied natural product from Streptomyces that is structurally similar to tabtoxin and also has inhibition activity on GS. It is reported that both of the peptide bonds of this tripeptide prodrug are formed using an NRPS assembly line. The introduction of NRPS into Cl-tabtoxin biosynthetic pathway instead of tabtoxin biosynthetic pathway may be because of the order of the amino acid and TβL/Cl-TβL
and the need to chlorinate C4 position of TβL. The NRPS could activate TβL as the corresponding thiolation domain thioester where the free alpha-amino group can undergo amide bond formation with L-Ala, perhaps catalyzed by CblF, and halogenation at C4. The dipeptide orientation in Cl-tabtoxin (Ala-Cl-TβL) is important to avoid the problematic isomerization associated with tabtoxin (TβL-Thr) (Figure 4.7) since the free amine of Cl-Tβl is blocked by amide bond formation with the carboxyl group of L-Ala. As a product of soil bacteria, Cl-tabtoxin needs to be structurally stable to handle the complicated environment in soil where it thrives when it is floating to look for victim cells. The stability for tabtoxin produced by P. syringae since this phytotoxin is produced directly at the site of action in and on plant cells. Another possible reason for the utilization of NRPS is to facilitate chlorination at C4 of TβL. To our knowledge, there is no independent halogenase acting on an amino acid substrate that functions without interfacing to an NRPS module. Our current working hypothesis is that in Streptomyces, TβL is synthesized in the same way as in P. syringae consistent with the conserved core biosynthetic genes and uses additional enzymes including the NRPS assembly line to facilitate chlorination and ligation with L-Ala.

Streptomyces is known to utilize various systems such as polyketide synthases (PKSs) and NRPSs in secondary metabolites biosynthesis, which occur within acyl carrier proteins (ACPs) for PKSs, or peptidyl carrier proteins (PCPs) for NRPSs. In the case of NRPSs, the substrate is specifically recognized by adenylation (A) domain, followed by being activated by ATP. Activated substrate is then transferred to the holo form of peptide carrier protein (PCP) domain (also known as T domain), which contains a 4′-phosphopantetheinyl group at a specific serine residue. The thioester bond is formed as a connection between thiol moiety of T domain and carboxyl group of the substrate. The loaded substrate can be further modified by other
enzymes included in the NRPS (Figure 4.42). The presence of NRPS in *Streptomyces* 372A, NRRL 8045 is confirmed by searching the flanking region of Cl-tabtoxin BGC (Table 4.5). The A domain (TAW3_06407), T domain (TAW3_06406) and acyl-carrier protein transferase (TAW3_06400) are found in this region, along with a potential chlorinating enzyme (TAW3_06404). This unique chlorinating enzyme is also found in several top matches in the 14 strains we discussed earlier (Figure 4.15, shown in purple). A blast analysis was conducted based on TAW3_06404, the potential chlorinating enzyme, and we found it had a high percentage identity to some well-studied NRPS-mediated chlorinase including cmaB72, SyrB273, KtzD74, and CytC375. In those events, substrate is loaded onto the T domain followed by enzyme catalyzed chlorination at the methyl group (Figure 4.43). All four of the well-studied chlorinases belong to non-haem Fe(II)/α-ketoglutarate-dependent enzyme superfamily, which share a common fold in structure: cupin fold—a barrel shape formed by jelly roll motif, which is classically composed of eight beta sheets arranged in two four-stranded antiparallel beta sheets76. The structure of TAW3_06404 showed a partial cupin fold based on the structure simulation (Figure 4.44) predicted by SWISS model. Interestingly, we also found a “cupin domain protein” (TAW3_06387) in the flanking region (Table 4.5). We propose either TAW3_06404 or 06387 is responsible for Cl atom addition.

Cl-Cupin assay was conducted on TAW3_06387 (Figure 4.45) alone without NRPS proteins.77 However, no chlorinated TβL was detected by LC-MS and HPLC analysis. We predict that that NRPS module is needed to stimulate the chlorination activity of the halogenase perhaps via formation of a TβL-T-domain thioester as substrate. In order to study the NRPS assembly line for chlorination and ligation, we cloned out TAW3_06407 (A domain), 06406 (T domain), 06400 (acyl-carrier protein transferase), and 06404 (chlorinating enzyme) into pET28

186
vector and over-expressed in *E.coli* BL21 followed by purification (Figure 4.46–4.49). TAW3_06406 was incubated with CoASH and Sfp (phosphopantetheinyl transferase) at room temperature for 2 hours to activate the T domain with the required phosphopantetheinyl prosthetic group (Figure 4.50). Additional proteins were then added along with the potential substrates including N-formyl-L-lysine, Tβl, TβL-COOH, Ala, Lysine, acetyl-TβL-COOH, or acetyl-lysine (Figure 4.51). A series of controls are conducted including no substrate, no TAW3_06407 (A domain), no TAW3_06407 (A domain) & TAW3_06400 (acyl-carrier transferase), and no proteins. The A-domain activity was measured based on the conversion of ATP to AMP during the loading of substrate to T domain. A PK/LDH/myokinase assay is used to detect the formation of AMP (Figure 4.52). The decreasing of absorbance at OD_{350nm} indicates the progress of the reaction. However, almost all substrate candidates show the same result, which is the same as the controls, except “no any of the three enzymes” control has a slower reaction rate (Table 4.7 and Figure 4.53, only several substrate candidates’ result are shown in the figure, but the results are the same for all candidates).

Because the substrate was unable to load onto the T domain, no further action regarding to the CblF or chlorinating enzyme (TAW3_06404 or TAW3_06387) was taken. For future studies, more substrate candidates will be explored, including synthetic substrates, and screened to investigated A-domain loading of substrates onto the T domain. Once the preferred substrate is identified for the NRPS system, CblF and chlorinating enzyme assays will be conducted in the same way as we did without NRPS, and detect by LC-MS.

*Substrate screen for TblS (beta-lactam synthetase)*
TblS is the homolog of β-lactam synthetase (β-LS). β-LSs are related to asparagine synthetase and are unique from other well-studied β-lactam producing enzymes include isopenicillin N synthase (IPNS), which is a non-heme iron-dependent enzyme cyclizes the linear tripeptide to form the isopenicillin N.\(^{78}\) \(^{\beta}\)-LS by orf3 from *Streptomyces clavuligerus* is a good example of a β-lactam synthetase that catalyzes β-lactam ring formation from a precursor β-amino acid substrate in the clavulanic biosynthesis. Similar to Asn synthetase, β-LS is an ATP/Mg\(^{2+}\)-dependent enzyme.\(^{54}\) There are also examples of β-lactam ring formation with the involvement of NRPS domains such as sulfazecin monobactam\(^{79}\), and nocardicin A\(^{80}\).

We attempted to clone and express *tblS* from *P. syringae* in our lab. Unfortunately, the protein turned out to be insoluble even though a lot of efforts were put in to increase the solubility. The only way that can improve the solubility to some extent is by expressing the MBP (maltose-binding-protein)-fusion TblS. MBP is a 42.5 kDa protein that is involved in transport and regulating maltose/maltodextrin in *E. coli*. MBP-fusion proteins can prevent the aggregation of the target protein in heterologous expression systems. The MBP-TblS fusion was expressed in *E. coli* and purified with amylose resin, followed by elution from the column with a solution of maltose. The amount of protein from purified MBP-TblS was very little, even after filter centrifuged (*Figure 4.54*). Despite this, it is good sign that we see both MBP-TblS (112kDa), and a light band around 42kDa, which indicates the presence of free MBP protein. It is known that the MBP from MBP-fusion protein can be released from the fusioned protein naturally via cleavage by endogenous proteases. Excitingly, the band between MBP-TblS and MBP is TblS (70kDa) which was also released from the fusioned protein. With soluble TblS/MBP-TblS in hand, we are able to study the function of this enzyme using model substrates.
First, we screened TβL-COOH, a beta-amino acid derived from parent TβL via hydrolysis, as a substrate for MBP-TblS (Figure 4.55). LC-MS analysis of the reaction mixture did not show any TβL product formation. We screened other possible substrates for TblS. A paper about Tβl-related metabolites from P.syringae reported an acetylated TβL analog (Figure 4.1k) was likely to be one of the intermediate of the tabtoxin biosynthetic pathway based on a gene disruption study. This is the only intermediate ever reported regarding to the tabtoxin biosynthetic pathway. Based on this information, we considered the possibility that acetyl-TβL-COOH could be the substrate for TblS since the modification of acetyl group on TβlCOOH can protect the host cell from the harm of TβL once the β-lactam ‘warhead’ is formed.

In order to test our hypothesis, we first need to synthesize the acetyl-TβL-COOH from TβL-COOH. Since there are two amine group (-NH₂) in the structure of TβL-COOH, we needed to selectively acetylate the ω-nitrogen. Luckily, there is a paper about selectively acetylate the ω-position of amine group in the diamino acids by optimizing the pH for the reaction. Due to the structural similarity with TβL-COOH, lysine was used as a test model molecule for the selective acylation reaction (Figure 4.56a). Following the published procedure, lysine was dissolved in water, and pH is adjusted to 11.0 before the addition of nitrophenyl-acetate. The reaction solution was stirred for an hour while checking pH frequently. We used a 1:1 ratio of lysine: nitrophenyl acetate in order to get the selectively acetylated product. The product is analyzed by NMR (Figure 4.57a), showing addition of a single acetyl group. A 1:2 ratio of lysine and nitrophenyl acetate was also explored, which resulted in the addition of two acetyl groups (Figure 4.57b). With the success on lysine as a model diamine substrate, we moved on to TβL-COOH (Figure 4.56b). The sample of TβL-COOH in this work was generated by hydrolysis of tabtoxin isolated from P. syringae. Considering the limited amount of sample, MS was used for
Figure 4.5 shows the formation of single addition of acetyl to TβL-COOH. With acetyl-TβL-COOH in hand, we tested the reaction of MBP-TblS catalyzed β-lactam ring formation (Figure 4.59). However, no anticipated product was formed based on LC-MS analysis. Our interpretation is that either MBP-TblS is not active or acetyl-TβL-COOH is not the actual substrate for TblS. Further investigation is needed to establish the structure of the TblS substrate.

4.5 Outlook and Conclusions

We isolated and purified Cl-tabtoxin from Streptomyces 372A, NRRL 8045 which showed glutamine-dependent antibacterial activity. Similar to tabtoxin, Cl-tabtoxin utilizes the “Trojan Horse” drug delivery strategy by entering victim cells through dipeptide permeases followed by peptidase cleavage to release the GS inhibitor. The peptidase PepA from E. coli can be used to cleave the Cl-tabtoxin dipeptide bond and release Cl-TβL, which was shown to have potent inhibition activity towards GS based on our in vitro inhibition study. We sequenced the whole genome of Streptomyces 372A, NRRL 8045 and located the Cl-tabtoxin BGC that shares most of the genes in the tabtoxin BGC with the exclusion of tabP, tblE and tblR which seem to be required only for P. syringae producers. In the past few decades, the literature precedence was that the peptidase found in Pseudomonas periplasm responsible for the release of TβL from tabtoxin is TabP. However, based on attempts to reconstitute TabP catalytic activity it was found incapable of cleaving the tabtoxin dipeptide bond in vitro. We validated that TblA is a class I SAM-dependent MT that converts N-acetyl/succinyl-L-2-amino-6-oxopimelate to C5-methyl-N-acetyl/succinyl-L-2-amino-6-oxopimelate. This simultaneously validated our proposal for the Cl-tabtoxin BGC in Streptomyces. We propose that instead of using a standalone ligase to form the
dipeptide bond as observed for *P. syringae*, Streptomyces producers of Cl-tabtoxin potentially use an NRPS assembly line that facilitates amide bond formation between TβL and L-Ala and facilitates chlorination of TβL by a halogenase encoded in the BGC. This presents two biosynthetic paradigms for tabtoxin biosynthesis in two unique species, Pseudomonads and Streptomyces.

We characterized and validated enzymatic activity of CabB, which functions as THDPA-succinyl/acetyltransferase. The product of CabB can be served as a substrate for CblA to get methylated. CabD was found to be a PLP-dependent aminotransferase capable of acting on diverse THDPA analogs to form a pool of DAP-like intermediates to supply TβL and Lys biosynthesis. We have established three biosynthetic steps for Cl-Tβl formation after the THDPA branch point from lysine biosynthesis pathway. We proposed the remaining steps based on inferred function from comparative genomics of genes in Cl-tabtoxin BGC (Figure 4.60).

Additional producers of tabtoxin-like compounds were identified by genome mining. By analyzing the genes in 14 selected strains we proposed that the minimum genes necessary for making the tabtoxin-like natural products are *tblS, tblC, tblD, tabC, tblA, tabA, tabB, tabD* and *tblF*. This information helps us obtain a faster route for studying the tabtoxin biosynthetic pathway by bypassing unnecessary flanking enzymes. Also, the genome mining method we used in this study showed us a way to discover more strains that can make similar compounds. There is a chance that we can find some molecules with similar structures with the potential for improved antibacterial activity or and stability.

### 4.6 Materials and Methods

*Isolation of Cl-tabtoxin*
A glycerol spore stock of *Streptomyces* 372A, BRRL 8045 was used to inoculate a culture in 150mL sterile *Streptomyces* media A (6g/L bacto-peptone, 4g/L tryptone, 3g/L yeast autolysate, 1.5g/L meat extract, 1g/L D-glucose) in 500 mL baffled flasks, grown for 96 h while being shaken (225 rpm) with 3-5 beads at 28 °C. 9mL culture from media A was transferred to 500mL sterile *Streptomyces* media B (20g/L tomato paste, 20g/L glycerol, 5g/L calcium carbonate, pH 7.0) in 2.8L baffled flasks grown for 72 h while being shaken (200 rpm) with 3-5 beads at 28 °C. Cells were pelleted by centrifugation for 20 min at 5000 rpm and 4 °C. The supernatant was vacuum filtered through Celite, followed by purification via ion exchange chromatography using Dowex 50WX8-200 cation exchange. Product was eluted with 5% pyridine in water (pH = 5.7, adjusted with acetic acid) and concentrated by rotary evaporation. The resulting product was re-dissolved in a 1:1 EtOH/water mixture for purification by HPLC chromatography. Preparative HPLC was performed using a Beckman Coulter SYSTEM GOLD 127P solvent module and 168 diode array detector using a Luna 10 μ C18(2) 100 A column (250 × 21.2 mm) from Phenomenex fit with a guard column (15 × 21.2 mm). Mobile phases for RP-C18 prep-HPLC were 0.1% TFA in (A) water and (B) acetonitrile. Sample was loaded in 0% B for 20min, ramp to 100%B over 20min, hold at 100%B for 10min, go back to 0%B over 3min, hold at 0%B for 2min. Every peak was collected separately for agar diffusion bioassay.

**Agar diffusion bioassay**

Overnight culture of *E.coli* 25922 was grown in Luria Broth (LB) for 18-22h starting from a frozen glycerol stock. 100uL of this culture was added to 40mL of sterile, melted, and tempered (~50°C) Glutamine free media agar (7.0g/L K₂HPO₄, 3.0g/L H₂PO₄, 0.47g/L Na·citrate·2H₂O, 0.1g/L MgSO₄·7H₂O, 1.0g/L (NH₄)₂SO₄, 2.0g/L glucose, 15g/L agar). After
gentle mixing, the inoculated melted agar was poured into a sterile Petri dish (145mm X 20mm, Greiner Bio-One) and allowed to solidify at room temperature (rt). Wells of 9mm diameter were cut from the Petri dish agar and filled with 70uL of the samples from HPLC purification step. Sterile H₂O and kanamycin were used as controls.

**General procedure for protein expression and purification**

Overnight cultures of *E. coli* BL21 grown at 37 °C harboring the appropriate plasmid were grown in LB broth containing 50 μg/mL kanamycin and 200 μL was used to inoculate 1L batches of sterile Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 5 g/L glycerol, 17 mM KH₂PO₄, and 72 mM K₂HPO₄) containing 50 μg/mL kanamycin. The cultures were grown at 37 °C with agitation at 225 rom to an OD₆₀₀ of 0.6~0.8 and then cooled to 15 °C prior to induction with 1 mL of 0.5 M IPTG (0.5 mM final concentration). Cultures were then grown for ~18 h at 15 °C with agitation at 225 rpm. Cells were harvested by centrifugation (5,000 r.p.m. for 20 min at 4 °C). Cells/protein solutions were kept at ~4 °C for all remaining steps. Cell pellets were suspended in 40 mL of lysis buffer (50 mM K₂HPO₄, 500 mM NaCl, 5 mM β-mercaptoethanol, 20 mM imidazole, 10% glycerol, adjusted to pH 8.0) and flash frozen in liquid nitrogen. After thawing, cells were mechanically lysed using an Avestin EmulsiFlex-C5 high-pressure homogenizer. Cell lysate was clarified via ultracentrifugation (45,000 r.p.m. for 35 min) and incubated for 30 min with Ni-NTA resin preconditioned with lysis buffer. The Ni-NTA resin was washed twice with 40 mL aliquots of lysis buffer, and then eluted in three separate washes with 30 mL of elution buffer (50 mM K₂HPO₄, 500 mM NaCl, 5 mM β-mercaptoethanol, 300 mM imidazole, 10% glycerol, adjusted to pH 8.0). Fractions containing pure N-His₆-GS as judged by SDS-PAGE analysis were combined, dialyzed into size exclusion buffer (50 mM K₂HPO₄, 500
mM NaCl, 1 mM DDT, pH adjusted to 8.0), concentrated via centrifugal filtration, flash frozen in liquid nitrogen, and stored at −80 °C to give stocks ready for in vitro biochemical assays.

**Expression and purification of TblS-MBP fusion**

Overnight cultures of *E. coli* BL21 grown at 37 °C harboring the appropriate plasmid were grown in LB broth containing 100 μg/mL ampicillin and 200 μL was used to inoculate 1L batches of sterile LB Broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, and 2 g/L glucose) containing 100 μg/mL ampicillin. The cultures were grown at 37 °C with agitation at 225rpm to an OD₆₀₀ of 0.6~0.8 and then cooled to 16 °C prior to induction with 600 uL of 0.5 M IPTG (0.3 mM final concentration). Cultures were then grown for ~18 h at 16 °C with agitation at 225 rpm. Cells were harvested by centrifugation (5,000 r.p.m. for 20 min at 4 °C). Cells/protein solutions were kept at ~4 °C for all remaining steps. Cell pellets were suspended in 40 mL of lysis buffer (50 mM K₂HPO₄, 500 mM NaCl, 5 mM β-mercaptoethanol, 20 mM imidazole, 10% glycerol, adjusted to pH 8.0) with protease inhibitor, and flash frozen in liquid nitrogen. After thawing, cells were mechanically lysed using an Avestin EmulsiFlex-C5 high-pressure homogenizer. Cell lysate was clarified via ultracentrifugation (45,000 r.p.m. for 35 min) and incubated for 30 min with amylose resin preconditioned with column buffer (20mM Tris-HCl, 200mM NaCl, 1mM EDTA, and 10mM β-mercaptoethanol, pH adjusted to 7.4). The amylose resin was washed three times with 40 mL aliquots of column buffer, and then eluted in five separate washes with 9 mL of elution buffer (column buffer with 10mM maltose). Fractions containing pure TblS-MBP as judged by SDS-PAGE analysis were combined, dialyzed into size exclusion buffer (50 mM K₂HPO₄, 500 mM NaCl, 1 mM DDT, pH adjusted to 8.0), concentrated via centrifugal filtration, flash frozen in liquid nitrogen, and stored at −80 °C to give stocks ready for in vitro biochemical assays.
**Cl-Cupin (potential halogenase, TAW3_06387) assay**

Cl-Cupin enzyme was dialyzed in 100mM HEPES with 5mM EDTA to get rid of metal ions. Two rounds of 100mM HEPES dialysis were followed in order to get rid of EDTA. Cl-Cupin enzyme was now ready for *in vitro* enzyme assay. The reaction was carried out in 100mM HEPES buffer (pH 7.5), with 100uM Cl-Cupin enzyme in a reaction mixture that included 100uM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 2mM alpha-ketoglutarate (a-KG), 1mM Tbl, and 50mM NaCl. Reaction was conducted at room temperature for 3 hours followed by filter centrifuge to remove the enzyme, Fmoc label (75uL sample, 50uL 0.2M sodium borate, pH 8.0, 105uL acetonitrile, 20uL 20mM Fmoc-Cl), and LC-MS analysis.

**Cl-TblF (CblF) assay**

All experiments were performed at room temperature in quartz cuvettes under steady state conditions with continuous monitoring at 350 nm in an Agilent Carey 50 UV–vis spectrophotometer. For all experiments the total reaction volume was 250 μL and contained 100 mM HEPES (pH 7.4), 12 mM MgCl$_2$, 20mM L-Ala, 10mM ATP, 0.5 mM PEP, 0.2 mM NADH, 100uM Tbl, 0.2 units PK, 0.3 units LDH. 1.6 uM CblF enzyme was added as last step to trigger the reaction. OD$_{350nm}$ was monitored for 5-10 min as indication of NADH signal change.

Reacted product was quenched with same volume of ethanol, followed by filter centrifuge to remove protein. Fmoc label was carried out (75uL sample, 50uL 0.2M sodium borate, pH 8.0, 105uL acetonitrile, 20uL 20mM Fmoc-Cl) for LC-MS analysis.

**TblS-MBP assay**
The reaction was prepared in 100mM HEPES buffer (pH 7.5), with 0.0444mg/mL enzyme in a reaction mixture that included 10mM ATP, 4mM MgCl₂, and certain amount of Tbl-COOH (made by Garrett Patrick) or acetylated Tbl-COOH. Reaction was conducted at room temperature for 1 hour, followed by filter centrifuge to remove the enzyme, Fmoc label (75μL sample, 50μL 0.2M sodium borate, pH 8.0, 105μL acetonitrile, 20μL 20mM Fmoc-Cl), and LC-MS analysis. Reaction product was also tested using PK/LDH coupled GS activity assay.

**PK/LDH coupled GS activity assay**

All experiments were performed at 37 °C in quartz cuvettes under steady state conditions with continuous monitoring at 350 nm in an Agilent Carey 50 UV–vis spectrophotometer. Each reaction mixture contained 100 mM HEPES (pH 7.4), 100 mM KCl, 25 mM MgCl₂, 10 mM NH₄Cl, 0.5 mM PEP, 0.2 mM NADH, 0.2 unit of PK, 0.3 unit of LDH, 50 mM L-Glu, and enough GS to give a starting rate close to -0.05 absorbance unit/min at 350 nm, yielding a straight line for ~10 min without consuming all NADH (GS stocks are prepared in advance from frozen concentrated stocks the day of inhibition assay). Inhibitors were added with different concentrations to test inhibition activity against GS.

**PepA cleavage of dipeptide bonds**

Reaction was initially prepared in 0.1M phosphate buffer with various pH values (5.05, 6.07, 7.5, and 8.03) with 5mM L-Ala-L-Ala, and 6.2μM PepA enzyme. “No enzyme” and “no substrate” control were performed as well. Reaction was carried out at 37 °C overnight. Reacted products were Fmoc’d and centrifuged to remove enzyme, followed by LC-MS analysis.
The addition of metal (50mM KCl, 12.5mM MgCl₂, and 5mM NH₄Cl) has shown great improvement on Ala-Ala dipeptide bond cleavage. A finer pHs were tested with the addition of metal: in 0.1M phosphate buffer with various pHs (6.31, 6.59, 6.83, and 7.08), 5mM Ala-Ala, 50mM KCl, 12.5mM MgCl₂, 5mM NH₄Cl and 6.2uM pepA enzyme was mixed and the reaction was carried out at 37°C overnight. Reacted products were Fmoc-tagged by treatment with FmocCl and centrifuged to remove enzyme, followed by LC-MS analysis.

PepA cleavage of prodrugs are performed under RT and 37 °C overnight at pH 7.08, with metal (50mM KCl, 12.5mM MgCl₂, and 5mM NH₄Cl) addition. The release of free drugs are detected by LC-MS and PK/LDH coupled GS activity assay.

**NRPS assay**

The NRPS assay was coupled with myokinase/PK/LDH reaction to monitor the AMP formation. Sfp enzyme and CoASH was used to active the T domain (TAW3_06406, or 6406 in this case) of the NRPS system. The activation of T domain was prepared in phosphate buffer (pH 7.0) with 180uM CoASH, 10mM MgCl₂, 3uM Sfp enzyme, and 3.9mM 6406 enzyme, incubating at room temperature for 2 hours. The reaction was added into a PK/LDH coupled reaction system, which is 10x the volume of the Sfp/CoASH reaction. The PK/LDH coupled reaction was prepared in phosphate buffer (pH 7.0) with 20mM KCl, 5mM MgCl₂, 2mM NH₄Cl, 3mM ATP, 5mM PEP, 0.5mM NADH, 0.2 unit of PK, 0.3 unit of LDH, myokinase, certain amount of potential substrate (Formyl-L-Lysine, or TβL, or TβL-COOH, or L-Ala, or L-Lysine, or acetyl-L-Lysine, or acetyl-TβL-COOH), 0.9uM TAW3_06400 (or 6400) enzyme, and 0.15uM TAW3_06407 (or 6407) enzyme. After the addition of Sfp/CoASH into PK/LDH coupled reaction, OD₃₅₀nm was monitored over 16 hours at room temperature.
4.7 Acknowledgments

Funding for this research was provided by the Children’s Discovery Institute at St. Louis Children’s Hospital through Interdisciplinary Research Initiative grant MI-PD-II-2018-748, the Sloan Foundation through a Sloan Fellowship Award to TAW (FG201810935), and the Dreyfus Foundation through a Camille Dreyfus Teacher-Scholar Award to TAW (TC-19-079). We thank Garrett Patrick for cloning, expressing, and purifying the MBP-TblS protein construct and providing pure samples of TβL-COOH.
### 4.8 Figures and Tables

<table>
<thead>
<tr>
<th>ORF number</th>
<th>Block</th>
<th>Gene name</th>
<th>Protein homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gene transfer block</td>
<td>-</td>
<td>Phage integrase (XerC)</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>Unknown</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>Prophage</td>
</tr>
<tr>
<td>4</td>
<td>Left biosynthetic block</td>
<td>tabP</td>
<td>Metallopeptidase</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>tabD</td>
<td>Aat-like aminotransferase</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>tabB</td>
<td>THDPA N-succinyltransferase</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>tabA</td>
<td>DAP decarboxylate</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>tblA</td>
<td>Class I SAM-dependent methyltransferase</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>tabC</td>
<td>Peptidase</td>
</tr>
<tr>
<td>10</td>
<td>Right biosynthetic block</td>
<td>tblS</td>
<td>b-lactam synthetase</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>tblC</td>
<td>Clavaminic acid synthetase</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>tblD</td>
<td>GMC oxidoreductase-GNAT acetyltransferase fusion</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>tblE</td>
<td>Membrane protein</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>tblF</td>
<td>Ligase</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>tblR</td>
<td>MFS transporter</td>
</tr>
<tr>
<td>16</td>
<td>Gene cluster block</td>
<td>-</td>
<td>Membrane protein</td>
</tr>
<tr>
<td>17</td>
<td>-</td>
<td>-</td>
<td>Membrane protein</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>-</td>
<td>Unknown</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>-</td>
<td>Unknown</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>-</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

*Table 4.1: Tabtoxin BGC from *P. syringae*.  

199
a.

<table>
<thead>
<tr>
<th>Enzyme in lysine pathway</th>
<th>Function</th>
<th>Homologue in Tabtoxin BGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysC</td>
<td>Lysine-sensitive aspartokinase</td>
<td>X</td>
</tr>
<tr>
<td>Asd</td>
<td>Aspartate-semialdehyde dehydrogenase</td>
<td>X</td>
</tr>
<tr>
<td>DapA</td>
<td>4-hydroxy-tetrahydrodipicolinate synthase</td>
<td>X</td>
</tr>
<tr>
<td>DapB</td>
<td>4-hydroxy-tetrahydrodipicolinate reductase</td>
<td>X</td>
</tr>
<tr>
<td>DapD</td>
<td>N-succinyltransferase</td>
<td>√ TabB</td>
</tr>
<tr>
<td>DapH</td>
<td>N-acetyltransferase</td>
<td>√ TabB</td>
</tr>
<tr>
<td>DapC</td>
<td>aminotransferase</td>
<td>√ TabD</td>
</tr>
<tr>
<td>DapE</td>
<td>desuccinylase</td>
<td>X</td>
</tr>
<tr>
<td>DapL</td>
<td>aminotransferase</td>
<td>√ TabP</td>
</tr>
<tr>
<td>DapF</td>
<td>epimerase</td>
<td>X</td>
</tr>
<tr>
<td>Ddh</td>
<td>dehydrogenase</td>
<td>X</td>
</tr>
<tr>
<td>LysA</td>
<td>decarboxylase</td>
<td>√ TabA</td>
</tr>
</tbody>
</table>
### Table 4.2: Comparison of tabtoxin and Cl-tabtoxin BGCs in Pseudomonads and Streptomyces, respectively.

<table>
<thead>
<tr>
<th>Enzyme in lysine pathway</th>
<th>Function</th>
<th>Homologue in Cl-Tabtoxin BGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysC</td>
<td>Lysine-sensitive aspartokinase</td>
<td>X</td>
</tr>
<tr>
<td>Asd</td>
<td>Aspartate-semialdehyde dehydrogenase</td>
<td>X</td>
</tr>
<tr>
<td>DapA</td>
<td>4-hydroxy-tetrahydrodipicolinate synthase</td>
<td>X</td>
</tr>
<tr>
<td>DapB</td>
<td>4-hydroxy-tetrahydrodipicolinate reductase</td>
<td>X</td>
</tr>
<tr>
<td>DapD</td>
<td>N-succinyltransferase</td>
<td>√ CabB</td>
</tr>
<tr>
<td>DapH</td>
<td>N-acetyltransferase</td>
<td>√ CabB</td>
</tr>
<tr>
<td>DapC</td>
<td>aminotransferase</td>
<td>√ CabD</td>
</tr>
<tr>
<td>DapE</td>
<td>desuccinylase</td>
<td>X</td>
</tr>
<tr>
<td>DapL</td>
<td>aminotransferase</td>
<td>X</td>
</tr>
<tr>
<td>DapF</td>
<td>epimerase</td>
<td>X</td>
</tr>
<tr>
<td>Ddh</td>
<td>dehydrogenase</td>
<td>X</td>
</tr>
<tr>
<td>LysA</td>
<td>decarboxylase</td>
<td>√ CabA</td>
</tr>
</tbody>
</table>

a) Lysine biosynthesis pathway enzyme homologue in tabtoxin BGC. (Enzyme function is from Uniprot).

b) Lysine biosynthesis pathway enzyme homologue in Cl-tabtoxin BGC. (Enzyme function is from Uniprot)
<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene name in P. syringae</th>
<th>Function</th>
<th>Homologue in Streptomyces</th>
<th>Gene name in Streptomyces</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAM77365</td>
<td>Phage integrase (XerC)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>AAL99262</td>
<td>Unknown</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>AAL92457</td>
<td>Prophage</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>tabP</td>
<td>Metallopeptidase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>tabD</td>
<td>Aat-like aminotransferase</td>
<td>TAW3_06390</td>
<td>cabD</td>
</tr>
<tr>
<td>6</td>
<td>tabB</td>
<td>THDPA N-succinyltransferase</td>
<td>TAW3_06391</td>
<td>cabB</td>
</tr>
<tr>
<td>7</td>
<td>tabA</td>
<td>DAP decarboxylate</td>
<td>TAW3_06392</td>
<td>cabA</td>
</tr>
<tr>
<td>8</td>
<td>tblA</td>
<td>Class I SAM-dependent methyltransferase</td>
<td>-</td>
<td>cblA</td>
</tr>
<tr>
<td>9</td>
<td>tabC</td>
<td>Peptidase</td>
<td>TAW3_06394</td>
<td>cabC</td>
</tr>
<tr>
<td>10</td>
<td>tblS</td>
<td>b-lactam synthetase</td>
<td>TAW3_06397</td>
<td>cblS</td>
</tr>
<tr>
<td>11</td>
<td>tblC</td>
<td>Clavaminic acid synthetase</td>
<td>TAW3_06396</td>
<td>cblC</td>
</tr>
<tr>
<td>12</td>
<td>tblD</td>
<td>GMC oxidoreductase-GNAT acetyltransferase fusion</td>
<td>TAW3_06395</td>
<td>cblD</td>
</tr>
<tr>
<td>13</td>
<td>tblE</td>
<td>Membrane protein</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>tblF</td>
<td>Ligase</td>
<td>TAW3_06389</td>
<td>cblF</td>
</tr>
<tr>
<td>15</td>
<td>tblR</td>
<td>MFS transporter</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>AAO63154</td>
<td>Membrane protein</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>AAP14349</td>
<td>Membrane protein</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>AAP13069</td>
<td>Unknown</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>AAP14350</td>
<td>Unknown</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>AAP14351</td>
<td>Unknown</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Table 4.3: Comparison of P. syringae and Streptomyces BGCs.*


<p>| Cl | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | Comment |
|----|---|---|---|---|---|---|---|---|---|----|----|----|----|-------|
| tabP | | | | | | | | | | | | | | | Unique left biosynthetic block |
| tabD | | | | | | | | | | | | | | | Common left biosynthetic block |
| tabB | | | | | | | | | | | | | | | |
| tabA | | | | | | | | | | | | | | | |
| tblA | | | | | | | | | | | | | | | |
| tabC | | | | | | | | | | | | | | | |
| tblS | | | | | | | | | | | | | | | Common right biosynthetic block |
| tblC | | | | | | | | | | | | | | | |
| tblD | | | | | | | | | | | | | | | |
| tblE | | | | | | | | | | | | | | | |
| tblF | | | | | | | | | | | | | | | Unique right biosynthetic block |
| tblR | | | | | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th>number</th>
<th>name</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl</td>
<td><em>Streptomyces</em> 372A, NRRL 8045</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Streptomyces</em> sp. CB02460</td>
<td>NZ_LIVY00000000.1</td>
</tr>
<tr>
<td>2</td>
<td><em>Streptomyces</em> olivoreticuli subsp. olivoreticuli</td>
<td>NZ_CP031455.1</td>
</tr>
<tr>
<td>3</td>
<td><em>Streptomyces</em> sp. LUP30</td>
<td>NZ_MJAJ00000000.1</td>
</tr>
<tr>
<td>4</td>
<td><em>Streptomyces</em> sp. MBT76</td>
<td>NZ_LNBE00000000.1</td>
</tr>
<tr>
<td>5</td>
<td><em>Streptomyces</em> sp. NRRL F-4489</td>
<td>NZ_LLZI00000000.1</td>
</tr>
<tr>
<td>6</td>
<td><em>Streptomyces</em> sp. NRRL S-118</td>
<td>GCA_000716335.1_ASM71633v1</td>
</tr>
<tr>
<td>7</td>
<td><em>Micromonospora</em> rosaria strain DSM 803</td>
<td>NZ_LRQV00000000.1</td>
</tr>
<tr>
<td>8</td>
<td><em>Microbispora</em> sp. NEAU-HEGS1-5</td>
<td>NZ_VANP00000000.1</td>
</tr>
<tr>
<td>9</td>
<td><em>Micromonospora</em> yangpuensis strain DSM 45577</td>
<td>NZ_FMIA00000000.1</td>
</tr>
<tr>
<td>10</td>
<td><em>Microbispora</em> triticiradicis strain NEAU-HRDPA2-9</td>
<td>NZ_QFZU00000000.2</td>
</tr>
<tr>
<td>11</td>
<td><em>Pseudomonas</em> sp. CMR12a</td>
<td>CP027706.1</td>
</tr>
<tr>
<td>12</td>
<td><em>Pseudomonas</em> sp. RU47</td>
<td>NZ_CP022411.1</td>
</tr>
<tr>
<td>13</td>
<td><em>Pseudomonas</em> coronafaciens pv. oryzae strain I_6</td>
<td>NZ_RBOG00000000.1</td>
</tr>
<tr>
<td>14</td>
<td><em>Nocardia</em> thailandica NBRC 100428</td>
<td>NZ_BAGK00000000.1</td>
</tr>
</tbody>
</table>

Table 4.4: Comparative genomic analysis of Cl-tabtoxin BGC with NCBI database. a) Top 14 strain hits for BLASTp analysis of *Streptomyces* 372A, NRRL 8045 and Cl-tabtoxin BGC antismash result. (Black: contain this enzyme, white: don’t contain this enzyme). b) Name and accession number for these 14 strains.
<table>
<thead>
<tr>
<th>ORF</th>
<th>ATCC11528</th>
<th>Homology</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAW3_06380</td>
<td></td>
<td>integrase catalytic subunit</td>
<td></td>
</tr>
<tr>
<td>TAW3_06381</td>
<td></td>
<td>hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>TAW3_06382</td>
<td></td>
<td>hydrolase</td>
<td>peptidase</td>
</tr>
<tr>
<td>TAW3_06383</td>
<td></td>
<td>Arabinose efflux permease</td>
<td>resistance</td>
</tr>
<tr>
<td>TAW3_06384</td>
<td></td>
<td>hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>TAW3_06385</td>
<td></td>
<td>ATP-binding biosynthetic protein</td>
<td>Active substrate</td>
</tr>
<tr>
<td>TAW3_06386</td>
<td></td>
<td>major facilitator superfamily protein</td>
<td>Resistance</td>
</tr>
<tr>
<td><strong>TAW3_06387</strong></td>
<td></td>
<td>Cupin domain protein</td>
<td>Chlorinating enzyme</td>
</tr>
<tr>
<td>TAW3_06388</td>
<td></td>
<td>ATP-binding biosynthetic protein</td>
<td></td>
</tr>
<tr>
<td>TAW3_06389</td>
<td>tblF</td>
<td>ATP-binding biosynthetic protein</td>
<td></td>
</tr>
<tr>
<td>TAW3_06390</td>
<td>tabD</td>
<td>N-succinylaminopimelate aminotransferase</td>
<td></td>
</tr>
<tr>
<td>TAW3_06391</td>
<td>tabB</td>
<td>2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase</td>
<td></td>
</tr>
<tr>
<td>TAW3_06392</td>
<td>tabA</td>
<td>diaminopimelate decarboxylase</td>
<td></td>
</tr>
<tr>
<td>TAW3_06394</td>
<td>tabC</td>
<td>hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>TAW3_06395</td>
<td>tblD</td>
<td>choline dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>TAW3_06396</td>
<td>tblC</td>
<td>taurine catabolism dioxygenase tauD/tfdA</td>
<td></td>
</tr>
<tr>
<td>TAW3_06397</td>
<td>tblS</td>
<td>asparagine synthase</td>
<td></td>
</tr>
<tr>
<td>TAW3_06398</td>
<td></td>
<td>Sfp type phosphopantetheinyl transferase</td>
<td></td>
</tr>
<tr>
<td>TAW3_06399</td>
<td></td>
<td>sodium/hydrogen exchanger</td>
<td></td>
</tr>
<tr>
<td>Gene ID</td>
<td>Description</td>
<td>Function</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------------------------------------------</td>
<td>-----------------------------------------</td>
<td></td>
</tr>
<tr>
<td>TAW3_06400</td>
<td>Ribostamycin:4-((\gamma)-L-glutamylamino)-(S)-2-hydroxybutanoyl-[BtrI acyl-carrier protein] 4-((\gamma)-L-glutamylamino)-(S)-2-hydroxybutanoate transferase</td>
<td>Acyl-carrier transferase</td>
<td></td>
</tr>
<tr>
<td>TAW3_06401</td>
<td></td>
<td>N-acetyltransferase GCN5</td>
<td></td>
</tr>
<tr>
<td>TAW3_06402</td>
<td></td>
<td>3-oxoacyl-(acyl carrier protein) synthase III</td>
<td></td>
</tr>
<tr>
<td>TAW3_06403</td>
<td></td>
<td>hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>TAW3_06404</td>
<td>cmaB, SyrB2, KtzD, CytC3</td>
<td>phytanoyl-CoA dioxygenase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chlorination enzyme</td>
<td></td>
</tr>
<tr>
<td>TAW3_06405</td>
<td></td>
<td>amidohydrolase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peptidase?</td>
<td></td>
</tr>
<tr>
<td>TAW3_06406</td>
<td></td>
<td>Phosphopantetheine attachment site</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T domain</td>
<td></td>
</tr>
<tr>
<td>TAW3_06407</td>
<td></td>
<td>AMP-dependent synthetase and ligase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A domain</td>
<td></td>
</tr>
<tr>
<td>TAW3_06408</td>
<td></td>
<td>hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>TAW3_06409</td>
<td></td>
<td>LuxR family transcriptional regulator</td>
<td></td>
</tr>
</tbody>
</table>

*Table 4.5:* Flanking region of Cl-tabtoxin BGC. (red: Cl-tatoxin BGC, blue: interesting flanking region proteins.)
|   | a. Original predicted CblA nuclear acid sequence | 5’\texttt{aagcgagacaccatgacccgagcgcaaccaggacatttgccaggttgeggegactactacacccg}
|   |   | gcagcgcggtcaccgcgcggcgaggaagagacgcgtacgctgcgctgctggcttcgccccacaccagcgcgtcaggacttctgggtacctgtccaaggactacctcgccgaaacgcctggaggaccgcggctatcgcgccgtggagagctactacttcccctacctgcgcccggtcagcggcatgcgcaaccgcaccatctgcatcgcccgcgtgccgtaa3’ |
|   | b. Optimized predicted CblA nuclear acid sequence | 5’\texttt{atgatcaccgagggcgaaccaggacctggcagggtgcgcgactactacaccgcgcagcgctccacccggcggcgaggaggagagcatctacgcgatctgggagaagggcggcgcctacaacgactcggtcacgccgtccacgtacgtgcccgagtaccgctcccacatggccctcaaactcctctccctcaccgaggaggcgcgccagcgtcttctcgctgggttgcggcaacgccgccgtcgagggcgtcgtcgtcggtctcggcaggaccgtgcgcggcatcgacttcaacgaggaggccgtactcctcgcccggcagaagggcgtcgacgccttcgccgccgactactacgccctgcgggccgccgacgtcgcgggcaccgacatcgtctacgcggacggcttcctcggccacctcttcgacgccgagcacgagaccggccccgcgctggaccaactggccaccctcgagactgaagtccggcgcccacctggtcttctccaacgacgcgccgcaggaccgcgcgcttcgccccacaccagcgcgtcgaggacttctggtacctgtccaaggactacctcgccgaaacgcctggaggaccgcggctatcgcgccgtggagagctactacttcccctacctgcgcccggtcagcggcatgcgcaaccgcaccatctgcatcgcccgcgtgccgtaa3’ |
|   | c. Original predicted CblA amino acid sequence | KRDTMITEANQLARVRDYYTAQRSTGGEESIYAIWEKGGAYNDSVTPSTYVPEYRSHMALKLLSLTEEGASVFLGCNGAAVEGVVVGGLGRTVRGIFNEEAVLLARQKVGDFAADYYALRAADVAGTDIVYADGFLGLFDAEHTGPAWLQLATLGKSAHLVFSNDAPOQDREARFAPHRQRVDFWYLSDKYLAERLEDRGYRAVESYYFPYLRPVSVMRNRTCIARVP |
|   | d. Optimized predicted CblA amino acid sequence (start from M) | MITEANQLARVRDYYTAQRSTGGEESIYAIWEKGGAYNDSVTPSTYVPEYRSHMALKLLSLTEEGASVFLGCNGAAVEGVVVGGLGRTVRGIFNEEAVLLARQKVGDFAADYYALRAADVAGTDIVYADGFLGLFDAEHTGPAWLQLATLGKSAHLVFSNDAPOQDREARFAPHRQRVDFWYLSDKYLAERLEDRGYRAVESYYFPYLRPVSVMRNRTCIARVP |

Table 4.6: CblA DNA and protein sequences used in this study (gray is the difference between original and optimized CblA).
a.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction end Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formyl-L-lysine</td>
<td>275</td>
</tr>
<tr>
<td>Tbl</td>
<td>250</td>
</tr>
<tr>
<td>TblCOOH</td>
<td>300</td>
</tr>
<tr>
<td>Ala</td>
<td>200</td>
</tr>
<tr>
<td>Lysine</td>
<td>300</td>
</tr>
<tr>
<td>Acetyl-TblCOOH</td>
<td>200</td>
</tr>
<tr>
<td>Acetyl-lysine</td>
<td>200</td>
</tr>
</tbody>
</table>

b.

<table>
<thead>
<tr>
<th></th>
<th>6400</th>
<th>6406</th>
<th>6407</th>
<th>Substrate (formyl-L-lysine)</th>
<th>Reaction end Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>250</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>275</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>400</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>475</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>&gt;960</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.7: NRPS assay with PK/LDH/myokinase coupled reaction. a) Substrate candidates screened. b) Control reaction conditions (6400: TAW3_06400 protein; 6406: TAW3_06406 protein; 6407: TAW3_06407 protein).
Protein

Primer

Nuclei acid sequence

Amino acid
sequence

CabB
(TAW3
_06391)

Forward:

5‘atgtactcccgaccgctcatcgaggacgccttcgcccgcagggccgagctgaccgaa
gccgaactcgccgcgctcgccccgcacgtcgaggccgggatcgacgcgctcgaccgc
ggcgaactgcgcgccgcgcaccccgtggacggctcctgggccgccgaccccttcgtca
agaagctgatcctgctctccttccccctcggcgagaacgccgtcgccgaggcgggcgca
ggactgcccaagagctacgacaagatcccgctgaagttcgcgcactggcaggaggccg
acttccagaaggccgccatccgggtggtgcccggcgcggtcgtccggcgcggcgcgc
acatcggccccggcgccgtcctcatgccctccttcgtcaacatcggcgcccgcgtcggc
gcgggcacgatgatcgacacctgggcgacggtcggctcctgcgcacaggtgggcgag
cgctgccacatctccggcggcgcgggcctcggcggcgtgctcgaacccatcggcgaca
gccccgtcgtcatcgaggacgacgtcttcgtcggggcccgcagcgagatcgccgaggg
cgtacgggtacggcgcggcgcggtcatcggcatgggcgtctacctgggcgcctccacc
ccgatcgtggaccgcgcgacgggcgaggtgacccgcggcgaggtccccgaggacgc
cgtcgtcgtgcccggcacccgcaccgacccccgcaaccccggcctcgccacgtacgcc
gcggtcatcgtgaagtacgccgacgagcgcacccgcggcaagacggccctgaacgac
ctggtgcgggactga3‘

MYSRPLIEDAFARRAE
LTEAELAALAPHVEAG
IDALDRGELRAAHPVD
GSWAADPFVKKLILLS
FPLGENAVAEAGAGLP
KSYDKIPLKFAHWQEA
DFQKAAIRVVPGAVVR
RGAHIGPGAVLMPSFV
NIGARVGAGTMIDTW
ATVGSCAQVGERCHIS
GGAGLGGVLEPIGDSP
VVIEDDVFVGARSEIA
EGVRVRRGAVIGMGV
YLGASTPIVDRATGEV
TRGEVPEDAVVVPGTR
TDPRNPGLATYAAVIV
KYADERTRGKTALND
LVRD

5‘GTCGTGT
AAGCTTCA
TGCG3‘

5‘atgacccccctcgccagcgccttcgcccggctgcgcgccctgcaccacggcatcgac
gccccggccggactcgcccccgtccacctccacctgggcgagtcccggctcggcgcg
ggcgccgtcgacgccacgctcctcgccgacgccgacggctgggcccgctaccccgtcc
tcggcggcacccccgagctgcgcgtggcgtacacggggtggctgcgccgcaggttcgc
ggcggggcgacttttggaccggcgcaccgtcgcggtcgaacccaccccgggcaccaa
gcaggccgtggccgtggcggtcgcccgcgcggtcgagcgagtgcgcggcgcgggcg
gcggggcggccgtggtcatgcccaacccgttctacccgacctaccacgccgccaccga
ggccgtcggcgcgcggccggtgttctacgacccgcgcggcgggggcgccgcactgcg
cgccgccgtcgccgcggccggggcgccggtcgccgcggtcgtcgtctgcgacccggg
caacccgcgcggtgaactccttgacccgggcttcctgcgcaccgccacccgcgccgcc
gtcgctcgggacgccctgctcctggtcgacgagtgctacaccgacctgtggctcacccac
ccggccgccgggtacctcacgctcgtggagcggggcgtgctggaaccggaccggttcc
tcgtcctgcacaccctgtccaagcgctccggcgcaccggggctgcgcagcggcttcgtc
accggcgaccccgcgaccgtcgccgactacgcccggcacaaccaggcgtgcggcgtc
tccaccccgctgccggtgtgcgccgccgcggccctgctgtgggacgacgacgcccacg
tcgagcgggcccgcgccgcactcgccgccaactggggcctcgccgaccggctgctgg
ccgacgtccccggctaccgccgcgccgacgccgggttcttcctctggctgcccgtcgac
gacgacgaggccgccgcccgccacctctggcgcgaccaggcgctgtccgtcatgccc
ggccgctacctggccgccgaagggcccgacggcaccaacccgggcgccgggcacct
gcgcgtcgccctcgtgcacgacgggccgctgatgcgcagggcactgctgcgcctgcgc
gccggcctgacccgaaccgacccccttctccgcgaggacgtccccgcatga3‘

MTPLASAFARLRALHH
GIDAPAGLAPVHLHLG
ESRLGAGAVDATLLA
DADGWARYPVLGGTP
ELRVAYTGWLRRRFA
AGRLLDRRTVAVEPTP
GTKQAVAVAVARAVE
RVRGAGGGAAVVMPN
PFYPTYHAATEAVGAR
PVFYDPRGGGAALRA
AVAAAGAPVAAVVVC
DPGNPRGELLDPGFLR
TATRAAVARDALLLV
DECYTDLWLTHPAAG
YLTLVERGVLEPDRFL
VLHTLSKRSGAPGLRS
GFVTGDPATVADYAR
HNQACGVSTPLPVCAA
AALLWDDDAHVERAR
AALAANWGLADRLLA
DVPGYRRADAGFFLW
LPVDDDEAAARHLWR
DQALSVMPGRYLAAE
GPDGTNPGAGHLRVA
LVHDGPLMRRALLRL
RAGLTRTDPLLREDVP
A

Forward:
5‘GCGAGAC
CATATGAT

5‘atgatcaccgaggcgaaccaggaccttgccagggtgcgcgactactacaccgcgca
gcgctccaccggcggcgaggaggagagcatctacgcgatctgggagaagggcggcg
cctacaacgactcggtcacgccgtccacgtacgtgcccgagtaccgctcccacatggcc

MITEANQDLARVRDY
YTAQRSTGGEEESIYAI
WEKGGAYNDSVTPST

5‘ACAGGA
GCGCCCAT
ATGTACTC
C3‘

RC:
5‘GCGCGAA
GCTTGGCG
AAG3‘

CabD
(TAW3
_06390)

Forward:
5‘GGACTGA
CATATGAC
CCCC3‘

RC:

CblA

209


CACCGAG
RC: 5'CGGGTTA
AGCTTTAC
GGCAC3'

ChlF
(TAW3 _06389)
Forward:
5'CATGACG
AAGCTTTG
CACGTCAG
3'
RC: 5'GACGTCC
CCATATGA
CGCTGAC3'

Cupin
(TAW3 _06387)
Forward:
5'GAGTCGC
GGATCCTC
AGCTCTCG
CGCT3'
RC: 5'GGCGACG
ACATATGA
CGCTGAC3'

YVPEYRSHMALKLSSL
TEEGASYFSLGCNGAAA
VEGVVVGLRTVRGBD
FNEEAULLARQKGYD
AFAADYYRALADDV
GTDIVYADGFLHLD
AEHETGPLDQLATLGL
LSKAHLVFSNDADPQ
REARFAPHQRVEDFW
YLSKDYALERLEDREGY
RAVESYYFYLPRVPSG
MNRRTICARVP

MTLTPRDPDPLLILITS
GLRRYREYLLASIAGR
YRVHILDATAPTWL
YLHAGHSAVPDTAGP
VLAAREAARQPVQA
GVMWSHEEHHQAAL
VAGELGLPTPPDAVR
RCRDKYARTALAEA
GLPQPEFALGSLLEA
LAAADKLGWPVVKIP
RAAGGSGQVVHLVD
DELADQFAATRVDHV
PHNPDFAEVLLLVEYL
DAPEVS/VAVRGRGR
VTPLFVGRKEVFPPPY
FEETGHRVSADDPLLH
DPQALRLTIGHRALG
VTDGWTHTELRLTAE
GPKLIEVNGVGGDLI
PYLGRATGDPDGLAA
ADVACGAEPTAPRR
DAHAAIRFFYPADD
RIAALDFDRTLPAADD
LPLEVVPGDVVSPPR
KGLIDGRALATAVAD
TAAECAAIADHAARAL
RLTVEGS

MTLTPRIVASARTADG
TSATGDGPGPVPATVA
AWPGSRFHLLAWTDPD
GGARVGGPAAPVTLP
FPFPDGFTRLLFARYPE
QSATPAPAGDPHEEHAA
EVERLPGLEWVEFPG
GTMHMTTDTDVGVC
LEGELHLEDDGEEVL
VTPGCTCVQVLGTRHT
WHNRTGSAALMCFVG
IGAERES
<table>
<thead>
<tr>
<th>Domain</th>
<th>Forward:</th>
<th>RC:</th>
<th>Other Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl-carrier transferase</td>
<td>GAACGGCA TATGAAG ACAC</td>
<td>CTGGAACA AGCTTACG CC</td>
<td>MKDTPYPYDTLYSLRL FLNCYQQRQLVLMAE RGRPVHRLRFLGVLST DEILRQVIREQRPKYDF ESGIAGQDDLAHLG VV KEAAEFESYAEAR DLL LDV VAREYAILVGD VGYFWPHCPEYRYKQHL VHIVTDLGHDATGDH WDVDDNPSALLCSY RYPEDVIAASFNGA L RRLRSYATDLDPRA EQGTRAFAALLLGH RDSEHLTGAADLSCI AWARIERVAVSLHAADF SLYYQGSRVTLR EYLRH AGGDPAADDLLDLRVL RGASEVMNNHLLAQLQV TGDALSARWTAADACLGL LRRDERELPRHLAAA GAGGAGA</td>
</tr>
<tr>
<td>Chlorination enzyme</td>
<td>GGCAGCCA TATGTCTG AAAG</td>
<td>GAGGAAGC TTGCGAAC TTC</td>
<td>MSESSTETEFLGLTD E QVEFQPENGFVGFDPD YGEEEAPLWNQAMIE MVTSONKPHNSTVINY DRHLDCDLSHVGRP EIVHKLRSLIDDLICW KTNIFKAPGADTG WHQVETFVGETTDT ATPSLKYTEEGQAYTQ ELTVWTAFAEPSAGKEN GLRYIPIGSHKKWYDDE SKPLNYDVEKSHDDF KYDASYLQLDKDWNP DEEEVFMEMKPGQF VIFLACIHGSLVPNVD TTRLGYASRYVAPSVK VYEDVDSLEFGDTISL DHYGSLVSGEDAYG HNRDLKLNONGVPEKF VETDER</td>
</tr>
<tr>
<td>T domain</td>
<td>GTGGGCAT ATGAACAC GAG</td>
<td>GATCTTAA GCTTCTCG</td>
<td>MNTSQASDEEMCRIIA GAIPKKAAAAGVNP RMLRRGLDVSLAMS LIVFVLKETGIDAFGR VDAFVAAESADVIDI VR RG</td>
</tr>
</tbody>
</table>
**A** (TAW3 \_06407)

**Forward:**
GGGGATCA

**RC:**
GCTCGCTA

5’gtgtcgaattctgataatgaacagctggagcttggcgagaccccgagaaagcggtcgtggcgttcctgcggtgctcgtggagctgctgcatcgtcgggtcttcgaaccacgtcgcctttctccgattgcagaacagctcgataaaatcagcgatgggtacatcagcgccctgctacgtcggggcgaactggaaggaaaaccggggcagacattgttgctgcaccatgttccgaatgtactttccgagcgaattctccttattggttgcgcaaaagaacgtgagctggatgagcgtcagtacaagcaggttattcagaaaaccattaatacgctgatatgatactggctcaatggaagcggtctgctttctgactgaactgcacgttaaaggccgtaacaaactactggaaagtgcgtcaggctgtcgagacggcaaaagaaacgctctacagtctcga
tcagctgaaaacgaacaagagcgaaccgcgtcgtccgctgcgtaaaatggtgttcaacgtgccgacccgccgtgaactgaccagcggtgagcgcgcgatccagcacggtctggcgattgccgccgggattaaagccgcgaaagatctcggcaatatgccaccgaatatctgtaacgcgccttacctcgcttcacaagcgcgccagctggctgacagctacagcaagaatgttatcacccgcgttatcggcgaacagcagatgaaagagctggggatgcattcctatctggcggtcggtcagggttcgcagaacgaatcgctgatgtcggtgattgagtacaaaggcaacgcgtcggaagatgcacgcccaatcgtgctggtgggtaaaggtttaaccttcgactccggcggtatctcgatcaagccttcagaaggcatggatgagatgaagtacgatatgtgcggtgcggcagcggtttacggtgtgatgcgtatggtcgcggagctacaactgccgattaacgttatcggcgtgtt

**RC:**
GGCAATAA

**FOR:**
CCTGACG

5’atggagtttagtgtaaaaagcggtagcccggagaaacagcggagtgcctgcatcgtcgggtcttcgaaccacgtcgcctttctccgattgcagaacagctcgataaaatcagcgatgggtacatcagcgccctgctacgtcggggcgaactggaaggaaaaccggggcagacattgttgctgcaccatgttccgaatgtactttccgagcgaattctccttattggttgcgcaaaagaacgtgagctggatgagcgtcagtacaagcaggttattcagaaaaccattaatacgctgatatgatactggctcaatggaagcggtctgctttctgactgaactgcacgttaaaggccgtaaacaaactactggaaagtgcgtcaggctgtcgagacggcaaaagaaacgctctacagtctcga
tcagctgaaaacgaacaagagcgaaccgcgtcgtccgctgcgtaaaatggtgttcaacgtgccgacccgccgtgaactgaccagcggtgagcgcgcgatccagcacggtctggcgattgccgccgggattaaagccgcgaaagatctcggcaatatgccaccgaatatctgtaacgcgccttacctcgcttcacaagcgcgccagctggctgacagctacagcaagaatgttatcacccgcgttatcggcgaacagcagatgaaagagctggggatgcattcctatctggcggtcggtcagggttcgcagaacgaatcgctgatgtcggtgattgagtacaaaggcaacgcgtcggaagatgcacgcccaatcgtgctggtgggtaaaggtttaaccttcgactccggcggtatctcgatcaagccttcagaaggcatggatgagatgaagtacgatatgtgcggtgcggcagcggtttacggtgtgatgcgtatggtcgcggagctacaactgccgattaacgttatcggcgtgtt

** pep A**

**Forward:**
GATTCAGG

**RC:**
GGCAATAA

5’atggagtttagtgtaaaaagcggtagcccggagaaacagcggagtgcctgcatcgtcgggtcttcgaaccacgtcgcctttctccgattgcagaacagctcgataaaatcagcgatgggtacatcagcgccctgctacgtcggggcgaactggaaggaaaaccggggcagacattgttgctgcaccatgttccgaatgtactttccgagcgaattctccttattggttgcgcaaaagaacgtgagctggatgagcgtcagtacaagcaggttattcagaaaaccattaatacgctgatatgatactggctcaatggaagcggtctgctttctgactgaactgcacgttaaaggccgtaaacaaactactggaaagtgcgtcaggctgtcgagacggcaaaagaaacgctctacagtctcga
tcagctgaaaacgaacaagagcgaaccgcgtcgtccgctgcgtaaaatggtgttcaacgtgccgacccgccgtgaactgaccagcggtgagcgcgcgatccagcacggtctggcgattgccgccgggattaaagccgcgaaagatctcggcaatatgccaccgaatatctgtaacgcgccttacctcgcttcacaagcgcgccagctggctgacagctacagcaagaatgttatcacccgcgttatcggcgaacagcagatgaaagagctggggatgcattcctatctggcggtcggtcagggttcgcagaacgaatcgctgatgtcggtgattgagtacaaaggcaacgcgtcggaagatgcacgcccaatcgtgctggtgggtaaaggtttaaccttcgactccggcggtatctcgatcaagccttcagaaggcatggatgagatgaagtacgatatgtgcggtgcggcagcggtttacggtgtgatgcgtatggtcgcggagctacaactgccgattaacgttatcggcgtgtt

**RC:**
CCTGACG

5’atggagtttagtgtaaaaagcggtagcccggagaaacagcggagtgcctgcatcgtcgggtcttcgaaccacgtcgcctttctccgattgcagaacagctcgataaaatcagcgatgggtacatcagcgccctgctacgtcggggcgaactggaaggaaaaccggggcagacattgttgctgcaccatgttccgaatgtactttccgagcgaattctccttattggttgcgcaaaagaacgtgagctggatgagcgtcagtacaagcaggttattcagaaaaccattaatacgctgatatgatactggctcaatggaagcggtctgctttctgactgaactgcacgttaaaggccgtaaacaaactactggaaagtgcgtcaggctgtcgagacggcaaaagaaacgctctacagtctcga
tcagctgaaaacgaacaagagcgaaccgcgtcgtccgctgcgtaaaatggtgttcaacgtgccgacccgccgtgaactgaccagcggtgagcgcgcgatccagcacggtctggcgattgccgccgggattaaagccgcgaaagatctcggcaatatgccaccgaatatctgtaacgcgccttacctcgcttcacaagcgcgccagctggctgacagctacagcaagaatgttatcacccgcgttatcggcgaacagcagatgaaagagctggggatgcattcctatctggcggtcggtcagggttcgcagaacgaatcgctgatgtcggtgattgagtacaaaggcaacgcgtcggaagatgcacgcccaatcgtgctggtgggtaaaggtttaaccttcgactccggcggtatctcgatcaagccttcagaaggcatggatgagatgaagtacgatatgtgcggtgcggcagcggtttacggtgtgatgcgtatggtcgcggagctacaactgccgattaacgttatcggcgtgtt

**MSNSINDLYSLAEHP**
**ERRMRFSFAGKLVRTR**
**NFPFEYIKDVEMLDEL**
**RESGCGRAGDLVGTGP**
**NSYEWVLADLLGL**
**GCVPVALAEGQADG**
**RALAALADAYRLAAM**
**LTRRAEDEDDREPARE**
**LPNGELPGDGAALTR**
**PLKLRVRDPAGARPT**
**LPGDAFTIASFSTGAGS**
**KCGKLMSAAGVENTM**
**RISAEAWLRTADDLDL**
**IVMPFSNQFQYLLYT**
**AVRYGTDVTVPVPER**
**MLQKRLERAPTVVLG**
**PSFFELPANVRSGAP**
**RERLPYLLATALHALV**
**PGRSRRRLRDLRRR**
**WTGVYGSRVLMLTG**
**SAPVPPRTVRFQCLG**
**APLFEVYGSSEIWFIAW**
**NLPGGHIRSAGRPVD**
**GVRVDVADDGEITVRT**
**GLPQCLGYVFEGQTO**
**DAVFLGDGIRVTGDIL**
**EFDGRFLRLKGRKKN**
**VIITRSYKINPEELEL**
**ALEKACPGRVAMVA**
**PDGGGLCSVWVVED**
**VADADRAVQVEAYLE**
**EANKRESALRIARTV**
**FRPSAELTVEEGLLTR**
**NFKIDRANAVTRRVFAE**
**RTGVGR**
Table 4.8: Primers, nucleic acid sequences, and amino acid sequences of proteins used in this chapter.
Figure 4. 1: Important compound structures (blue is amino acid part for prodrugs). a) Cl-tabtoxin, b) tabtoxin, c) Cl-TβL, d) Tbl, e) Rhizoctin, f) Plumbemycin, g) Dehydrophos, h) Clavulanic acid, i) SAM, j) Phosphinothricin-tripeptide, k) the only intermediate found in TβL biosynthetic pathway.

Figure 4. 2: Trojan Horse strategy for tabtoxin interrupting the growth of the neighboring cells.
Figure 4.3: Biosynthetic building blocks of TβL. Black part is from aspartic acid, blue part is from the two carbon of pyruvate, red part is from the methyl group of L-methionine, and green part is unknown.
Figure 4.4: Prep-HPLC trace for purification of Cl-tabtoxin (after ion-exchange column) from *Streptomyces* culture supernatant.
Figure 4.5: Cl-tabtoxin agar diffusion assay with the samples from peak #1 in the HPLC purification (see Figure 4.4).
**Figure 4.6**: Analysis of recombinant N-His$_6$-tagged PepA (55kDa) by SDS-PAGE. (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder)
Figure 4.7: Spontaneous isomerization of TβL and tabtoxin (blue is amino acid part of the prodrug).
Figure 4.8: In vitro dipeptide cleavage reactions with recombinant PepA. Conditions were screened to optimize pH and addition of MgCl₂ using L-Ala-L-Ala as a model substrate. a) PepA PH optimization (Starting material), b) PepA PH optimization (Product), c) PepA PH optimization, metal addition (Starting material), d) PepA PH optimization, metal addition (Product). y-axes represent extracted ion counts (EICs).
Figure 4.9: Further optimization of PepA dipeptide cleavage reaction. a) PepA finer PH scan (starting material), b) PepA finer PH scan (product). y-axes represent extracted ion counts (EICs).
Figure 4. 10: Substrate comparison for PepA cleavage of dipeptide prodrugs as measured by GS inhibition from released GS inhibitors.

Figure 4. 11: Substrate comparison for PepA cleavage of dipeptide prodrugs using LC-MS detection of Fmoc-tagged free GS inhibitor extracted ion counts.
Figure 4.12: Lysine Biosynthesis pathways in bacteria.
Figure 4. 13: Functional pairing of TblF, TblE, and TblR enable prodrug formation and efflux.

Figure 4. 14: Minimum BGC for TβL biosynthesis in a. *Streptomyces* Cl-tabtoxin BGC found in this work b. Known *P. syringae* tabtoxin BGC.
Figure 4. 15: Comparison of putative tabtoxin BGCs from top 14 strains that have high match with our strain via BLASTp and antiSMASH analyses.
Figure 4.16: SDS-PAGE analysis of recombinant $N$-His$_6$-tagged CabB (29kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Stained with Coomassie blue.)

Figure 4.17: Ddh catalyzed reaction to produce THDPA, the substrate for CabB (stereochemistry not shown here).
Figure 4. 18: SDS-PAGE analysis of recombinant $N$-His$_6$-tagged Ddh (35kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 4.19: Reaction progress curve for Ddh catalyzed conversion of meso-DAP to THDPA as monitored by formation of NADPH (340 nm).
Figure 4.20: LC-MS analysis of Ddh reaction confirms THDPA product formation. (top: extracted ion counts for product THDPA, bottom: extracted ion counts for starting material meso-DAP). y-axes represent extracted ion counts (EICs).
Figure 4.21: Proposed CabB reactions (stereochemistry not shown here).

Figure 4.22: Ellman assay mechanism.
Figure 4.23: Enzymatic activity of CabB towards succinylation of THDPA as judged by Ellman’s assay.
Figure 4.24: Comparison of enzymatic activity of CabB towards succinylation or acetylation of THDPA as judged by Ellman’s assay.

Figure 4.25: LC-MS analysis of CabB reactions with succinyl-CoA. Succinyl-THDPA is detected as product (m/z=290) (y-axes represent extracted ion counts (EICs)). a) Assay with CabB enzyme, succinyl-CoA, and THDPA substrate. b-d) Controls. e) Structure of product: succinyl-THDPA (stereochemistry not shown here).
Figure 4. LC-MS analysis of CabB reactions with acetyl-CoA. Acetyl-THDPA is detected as product (m/z=232) (y-axes represent extracted ion counts (EICs)). a) Assay with CabB enzyme, acetyl-CoA, and THDPA substrate. b-d) Controls. e) Structure of product: acetyl-THDPA.
Figure 4.27: Chemical tagging of CabB succinylated products using O-benzylhydroxylamine. Labeled succinyl-THDPA is detected as product (m/z=395) (y-axes represent extracted ion counts (EICs)). a) Assay with CabB enzyme, succinyl-CoA, and THDPA substrate. b-d) Controls. e) Structure of O-benzylhydroxylamine. f) Structure of oxime product (stereochemistry not shown here). Note: The two peaks corresponding to the oxime product reflect a diastereomeric mixture of cis and trans oximes.
Figure 4.28: Chemical tagging of CabB acetylated products using $O$-benzylhydroxylamine. Labeled acetyl-THDPA is detected as product (m/z=337) (y-axes represent extracted ion counts (EICs)). a) Assay with CabB enzyme, acetyl-CoA, and THDPA substrate. b-d) Controls. e) Structure of oxime product (stereochemistry not shown here). Note: The two peaks corresponding to the oxime product reflect a diastereomeric mixture of $cis$ and $trans$ oximes.
Figure 4. 29: Predicted CblA methyltransferase structure generated using SWISS model (https://swissmodel.expasy.org/).

Figure 4. 30: SDS-PAGE analysis of recombinant N-His\textsubscript{6}-tagged CblA (25.7kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel stained with Coomassie blue.)
**Figure 4.31:** Proposed CblA-catalyzed methyltransferase reaction (stereochemistry not shown here).

**Figure 4.32:** LC-MS analysis of CblA reactions exploring substrate preference for acetyl-THDPA versus succinyl-THDPA. CH$_3$-acetyl-THDPA ($m/z=246$) or CH$_3$-succinyl-THDPA ($m/z=304$) was detected as product (y-axes represent extracted ion counts (EICs)). a) and e) This is the complete assay where acetyl-CoA is added, b) and f) This is the complete assay where succinyl-CoA is added, c) and g) This is the control assay where neither succinyl-CoA nor acetyl-CoA is added. d) Structure of CH$_3$-acetyl-THDPA product. h) Structure of CH$_3$-succinyl-THDPA product (stereochemistry not shown here).
Figure 4. LC-MS analysis of CblA control reactions. a)-d) Shows the amount of substrate as judged by extracted ion counts: acetyl-THDPA under different conditions; e)-h) Shows the amount of product as judged by extracted ion counts: CH₃-acetyl-THDPA under different conditions. a) and e) This is the complete assay with everything added; b) and f) This is the control assay with no CblA enzyme addition. c) and g) This is the control assay with no SAM added. d) and h) This is the control assay with no THDPA (substrate) added. y-axes represent extracted ion counts (EICs).
Figure 4.34: Proposed CabD PLP-mediated aminotransferase reaction with methylated THDPA analogs as substrate (stereochemistry not shown here).

Figure 4.35: SDS-PAGE analysis of recombinant N-His₆-tagged CabD (42kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 4.36: LC-MS analysis of the CabD reaction using CH₃-acetyl-THDPA as substrate. CH₃-acetyl-DAP (m/z=469) was detected as product. a) Complete assay with addition of everything. b) Control reaction with no L-glutamate. c) Control with no THDPA. d) Control with no CabD enzyme. e) Control with no PLP. y-axes represent extracted ion counts (EICs).
Figure 4.37: Possible CabD reaction products from different THDPA analog substrates (stereochemistry not shown here).
**Figure 4.38:** LC-MS analysis of CabD reactions reveals substrate preference. a-d) Possible THDPA analogs as substrate. y-axes represent extracted ion counts (EICs). f) Summary of the extracted ion counts from a-d).

**Figure 4.39:** LC-MS analysis of CabD reactions using THDPA as substrate reveal *meso*-DAP contamination from DdH preparation. a) THDPA as substrate for CabD assay and b) no CabD enzyme control. y-axes represent extracted ion counts (EICs).
Figure 4.40: Docking of Pi-Cl-Tβl in the active site of GS. Top molecule: ATP, purple spheres (metal ions), bottom molecule: Pi-Cl-Tβl.

Figure 4.41: LC-MS analysis of CblF assay shows a preference for L-Ala-L-Ala production. L-Ala-L-Ala product was Fmoc-tagged using FmocCl to form Fmoc-Ala-Ala (m/z=383) to enable LC-MS detection. y-axes represent extracted ion counts (EICs).
Figure 4.42: Representative NRPS assembly line. A: adenylation domain; C: condensation domain; T: thiolation domain (also known as peptidyl carrier domain, PCP); TE: thioesterase domain (release the product from the NRPS assembly line). E and MT (shown in red) are functional domain. (E: epimersation domain; MT: methyltransferase domain.)
Figure 4.3: Mechanism for amino acid substrate chlorination via halogenase tailoring enzyme activity on the NRPS-tether substrate.

![Diagram of amino acid chlorination mechanism]

Figure 4.44: Predicted three-dimensional structure of TAW3_06404, a putative halogenase in the Cl-tabtoxin BGC. Model was generated using SWISS-MODEL (https://swissmodel.expasy.org/).
Figure 4. 45: SDS-PAGE analysis of recombinant N-His$_6$-tagged Cl-Cupin (TAW3_06387) (18.8kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 4. SDS-PAGE analysis of recombinant N-His<sub>6</sub>-tagged TAW3_06400 (35.5kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained using Coomassie blue.)
Figure 4.47: SDS-PAGE analysis of recombinant $N$-His$_6$-tagged TAW3_06404 (34.9kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained using Coomassie blue.)
Figure 4.48: SDS-PAGE analysis of recombinant N-His$_6$-tagged TAW3_06406 (8.8kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 4.49: SDS-PAGE analysis of recombinant N-His$_6$-tagged TAW3_06407 (58.9kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)

![SDS-PAGE analysis](image)

Figure 4.50: Activation of NRPS T domain with CoASH and Sfp (phosphopantetheinyl transferase) enzyme to install the required phosphopantetheinyl prosthetic group.
Figure 4.51: Possible NRPS substrate candidate structures explored in this work.

Figure 4.52: PK/LDH/Myokinase coupled reaction to detect the AMP production by NRPS A-domain. OD350 nm is used as reaction indicator.
Figure 4.53: Reaction progress curves for NRPS reactions measured using the assay shown in Figure 4.52. a) Substrate screen (black line is Formyl-L-lysine). b) A series of control reactions.
Figure 4. SDS-PAGE analysis of recombinant MBP-TblS. MBP-TblS: 112kDa; MBP: 42kDa; TblS: 70kDa. (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gels were stained with Coomassie blue.)
**Figure 4.55**: Proposed TblS reaction using TβL-COOH as substrate to form TβL product (stereochemistry is not shown here, ATP is a required substrate).

a.

![Chemical structure](image)

**Figure 4.56**: Regioselective acetylation of diamines. a) ω-position selective acylation using lysine model molecule. B) ω-position selective acylation using TβL-COOH. (stereochemistry is not shown here)
a.
Figure 4.57: $^1$H-NMR analysis of the products from nitrophenyl acetate react with lysine. a) lysine : nitrophenyl acetate ratio is 1:1. b) lysine : nitrophenyl acetate ratio is 1:2.
Figure 4.58: LC-MS analysis of nitrophenylacetate-mediated acetylation of TβL-COOH to form acetyl-TβL-COOH. a) and b) Show the consumption of starting material TβL-COOH. c) and d) Show the formation of product acetyl-TβL-COOH. y-axes represent extracted ion counts (EICs).

Figure 4.59: Proposed TblS-catalyzed reaction using acetyl-TβL-COOH as substrate to form acetyl-TβL (stereochemistry not shown here, ATP is required as a cosubstrate).
Figure 4.60: One of our current proposals for tabtoxin biosynthesis in *P. syringae*. The pathway is proposed to be analogous in *Streptomyces* up to TβL formation. (stereochemistry is not shown here)
Figure 4. 61: Predicted three-dimensional structure of CabD generated with SWISS-MODEL (https://swissmodel.expasy.org/).

Figure 4. 62: Predicted three-dimensional structure of CabB generated with SWISS-MODEL (https://swissmodel.expasy.org/).
4.9 References


48. He, H.; Ding, Y.; Bartlam, M.; Sun, F.; Le, Y.; Qin, X.; Tang, H.; Zhang, R.; Joachimiak, A.; Liu, J.; Zhao, N.; Rao, Z., Crystal structure of tabtoxin resistance


Chapter 5: Semisynthetic Analogues of Anhydrotetracycline as Inhibitors of Tetracycline Destructase Enzymes
5.1 Preface

This chapter was adapted from an article of the same title (Markley, J. L.; Fang, L.; Gasparini, A. J.; Symister, C. T.; Kumar, H.; Kong, A.; Tolia, N. H.; Dantas, G.; Wencewicz, T. A. “Semisynthetic analogues of anhydrotetracycline as inhibitors of tetracycline destructase.” ACS Infectious Diseases 2019, 5, 618-633) with permission from ACS Publications. Funding for this work was provided by National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID-NIH R01-123394) and the WM Keck Postdoctoral Program in Molecular Medicine for supporting JLM with a postdoctoral fellowship.

5.2 Abstract

The synthesis and biological evaluation of semisynthetic anhydrotetracycline analogues as small molecule inhibitors of tetracycline-inactivating enzymes are reported. Inhibitor potency was found to vary as a function of enzyme (major) and substrate-inhibitor pair (minor), and anhydrotetracycline analogue stability to enzymatic and nonenzymatic degradation in solution contributes to their ability to rescue tetracycline activity in whole cell Escherichia coli expressing tetracycline destructase enzymes. Taken collectively, these results provide the framework for the rational design of next-generation inhibitor libraries en route to a viable and proactive adjuvant approach to combat the enzymatic degradation of tetracycline antibiotics.

5.3 Introduction

Since the isolation of chlortetracycline (2, aureomycin) from Streptomyces aureofaciens in 1948,¹ the tetracycline family of broad-spectrum antibiotics has served as essential medicines for the treatment of bacterial infections in hospital and agricultural settings
Driven by challenges with stability, toxicity, and rising antibiotic resistance, the development of more effective, semisynthetic tetracycline variants has led to the introduction of next-generation tetracycline antibiotics tailored to overcome emerging resistance mechanisms. In this regard, the majority of current treatment strategies employ the use of second-generation C6-deoxy-tetracyclines (i.e., doxycycline and minocycline), which were developed to overcome efflux and stability issues, and third-generation glycylcyclines (tigecycline, eravacycline, and omadacycline), which were designed to evade efflux and ribosomal protection and are used as last-resort treatments for multidrug resistant infections. While the most common, clinically relevant resistance mechanisms for tetracycline antibiotics include efflux and ribosomal protection, those mechanisms that facilitate intra- and extra-cellular antibiotic clearance—often through the enzymatic, irreversible inactivation of antibiotic scaffolds—frequently pervade resistance landscapes as the most efficient means of achieving resistance. Historically, the enzymatic inactivation of β-lactam antibiotics has been well-studied, and strategies aimed at combatting this resistance using an adjuvant approach—where the antibiotic is coadministered with a small molecule inhibitor of the inactivating enzyme—have emerged as fundamentally useful tools for the rescue of β-lactam antibiotics in the clinic. With the discovery and characterization of 10 tetracycline-inactivating enzymes with varying resistance profiles, the development of small molecule inhibitors of tetracycline destructase enzymes stands at the forefront of strategies aimed at combating the imminent clinical emergence of this resistance mechanism in multidrug resistant infections. We herein report preliminary findings focused on understanding the factors that influence inhibitor potency and stability en route to the development of viable adjuvant approaches to counter tetracycline resistance by enzymatic inactivation.
Tetracycline-inactivating enzymes, including the most studied tetracycline destructase, Tet(X),\textsuperscript{33} and the subsequently identified enzymes Tet(47)–Tet(56),\textsuperscript{34} are Class A flavin-dependent monooxygenase enzymes confirmed to confer tetracycline resistance by the nonreversible functionalization of the tetracycline scaffolds (Figure 5.2A). Gut-derived Tet(X) and soil-derived Tet(47)–Tet(56) possess unique three-dimensional (3D) structures, which directly contribute to the observed variation in phenotypic tetracycline resistance profiles across enzyme clades (Figure 5.2B,C).\textsuperscript{35−37} In general, tetracycline destructase enzymes are composed of at least three functional domains: a substrate-binding domain, a flavin adenine dinucleotide (FAD)-binding domain, and a C-terminal α helix that stabilizes the association of the two. The presence of a second C-terminal α helix, termed the “Gatekeeper” helix, was also observed for the soil-derived tetracycline destructases [Tet(47)–Tet(56)] and is thought to facilitate substrate recognition and binding.\textsuperscript{37}

A variety of substrate binding modes have been observed for Tet(X) and the tetracycline destructases. A search for competitive inhibitors identified anhydrotetracycline (aTC, 5), a tetracycline biosynthetic precursor, as a potential broad-spectrum inhibitor (Figures 5.1, 5.2).\textsuperscript{37} aTC showed dose-dependent and potent inhibition of tetracycline destructases \textit{in vitro} and rescued tetracycline antibiotic activity against \textit{Escherichia coli} overexpressing the resistance enzymes on an inducible plasmid. The crystal structure of aTC bound to Tet50 revealed a novel inhibitor binding mode that pushes the FAD cofactor out of the active site to stabilize an inactive enzyme conformation.\textsuperscript{37} Upon the basis of these preliminary results, we crafted two hypotheses with regard to tetracycline destructase inhibition. Because of the variability observed in phenotypic resistance profiles between tetracycline destructase enzymes and phylogenetic clades, we hypothesized that inhibitor potency would also vary as a function of enzyme and inhibitor-
substrate pairing; thus, a library of inhibitors may be required to preserve the viability and effectiveness of an adjuvant approach. This has proven to be the case with β-lactam adjuvants, where multiple generations of inhibitors are required to cover the diverse families of β-lactamase resistance enzymes (classes A–D) present in the clinic. In addition, we proposed that aTC, in particular, could serve as a privileged scaffold about which to design inhibitor libraries. Thus, we herein report the generation and biological evaluation of four semisynthetic derivatives of anhydrotetracycline as potential inhibitors of tetracycline destructase enzymes. To identify the factors affecting the inhibition of tetracycline-inactivating enzymes, we assessed the inhibitory activity of the aTC analogue library, in reference to aTC, against the degradation of first-generation tetracyclines by three representative tetracycline destructase enzymes (Figure 5.1). Taken collectively, these results highlight the factors that influence inhibitor potency and stability and provide the framework for the rational design of next-generation inhibitor libraries.

5.4 Results and Discussion

*Michaelis–Menten Kinetics Highlight Enzyme Differences.*

Three representative tetracycline destructase enzymes were chosen based upon observed phenotypic resistance profiles and phylogenetic clustering. These enzymes—soil-derived Tet(50) and gut-derived Tet(X) and Tet(X)_3 [GenBank KU547176.1]—were recombinantly expressed and purified from BL21-Start (DE3) competent *E. coli*. For each enzyme, the *in vitro* enzyme-dependent inactivation of first-generation tetracyclines was characterized using an optical absorbance kinetic assay developed in our laboratory. Apparent Michaelis–Menten kinetic parameters were determined from the enzyme- and time-dependent degradation of tetracycline (1), chlortetracycline (2), demeclocycline (3), and
oxytetracycline (4). Representative Michaelis–Menten plots for the enzymatic degradation of oxytetracycline are shown in Figure 5.3A.

Consistent with previous reports, micromolar (μM) apparent binding affinities ($K_m$), ranging from 2 μM to 13 μM, were observed across all enzyme–substrate combinations (Figure 5.3). Apparent rate ($k_{app}$) of tetracycline degradation was highest for Tet(X)$_3$, followed closely by Tet(50), and last by Tet(X); this variation in apparent rate is highlighted in the deviation in shape and slope of the raw plots of enzyme-dependent tetracycline degradation, observed as the change in the absorbance at 400 nm over time (Figure 5.3B). Conversely, apparent catalytic efficiency ($k_{app}/K_m$) was highest for Tet(50) over Tet(X)$_3$—due to a 2–10 fold difference in apparent $K_m$. This trend is consistent with the hypothesis that Gatekeeper helix-facilitated substrate recognition results in an increase in substrate specificity and turnover for the soil-derived tetracycline destructases (Figure 5.2). The second, C-terminal gatekeeper helix is notably absent in X-ray crystal structures of gut-derived, canonical tetracycline-inactivating enzyme, Tet(X) (Figure 5.2), and the presence or absence of a similar helix in Tet(X)$_3$—which clusters closely with Tet(X)—is currently unknown. Similar apparent binding affinities ($K_m$) were observed for phylogenetically clustered Tet(X)$_3$ and Tet(X), though five- to eight-fold differences in apparent rate results in drastically different catalytic efficiencies for the two gut-derived enzymes (Figure 5.3C). The paradoxical functional similarities of Tet(X)$_3$ to both Tet(50) in apparent rate and Tet(X) in binding affinity and resistance phenotype, vide infra, has garnered interest in the unique facets of its three-dimensional structure that allows for accelerated turnover and broad substrate scope; efforts to resolve an X-ray crystal structure of Tet(X)$_3$ are currently ongoing in our laboratories and will be reported in due course. Taken collectively, the variability in binding affinity and catalytic efficiency highlights both enzyme-to-
enzyme and substrate-to-substrate differences across the tetracycline destructase family of enzymes. In this light, we hypothesized this same variability would manifest in inhibitor potency fluctuation as a function of enzyme and inhibitor-substrate pairing.

**aTC Inhibitory Activity Varies as a Function of Enzyme and Antibiotic Pair.**

To evaluate the hypothesis that inhibitor potency will vary as a function of enzyme and antibiotic pair, we assessed the *in vitro* inhibitory activity of aTC against the tetracycline destructase-mediated degradation of first-generation tetracycline antibiotics, the results of which are displayed in Figure 5.4A–D. In general, the apparent half-maximal inhibitory concentrations (IC₅₀s) for the aTC inhibition of Tet(50) were higher than those observed for Tet(X) [5- to 10-fold], with the most potent inhibition observed for Tet(X)₃. Surprisingly, the apparent IC₅₀s observed for the aTC inhibition of tetracycline destructase-mediated degradation of tetracyclines varied modestly as a function of inhibitor-substrate pair within the context of a single enzyme (Figure 5.4D). However, the half-maximal inhibitory concentrations of aTC inhibition of CTc were notably higher than those observed for the enzymatic degradation of the other first-generation tetracyclines. In addition, the IC₅₀ associated with aTC inhibition of the Tet(X) degradation of oxytetracycline was over an order of magnitude lower than other aTC-tetracycline pairs for the gut-derived enzyme. It is unclear what factors contribute to this effect; however, the combination of lower binding affinity (higher $K_m$, Figure 5.3C) and the polyhydroxylated nature of oxytetracycline may allow for more favorable inhibitor competition for the substrate binding pocket—since oxytetracycline may appear more Tet(X) product-like than other tetracycline substrates, *vide infra*.³³
Analysis of Lineweaver–Burk plots of the aTC inhibition of tetracycline destructase-mediated degradation of tetracycline supports the idea of a mixed competitive/noncompetitive inhibition model (Figure 5.5). Taken collectively, these results suggest that aTC inhibition involves more than a contest of competitive binding and catalytic efficiency, which is consistent with multicomponent enzyme processes and substrate/inhibitor binding mode flexibility observed for the tetracycline destructase enzymes.\textsuperscript{36,38–42} Consequently, because inhibition model ambiguity and binding mode flexibility can complicate broad computational docking to direct the rational design of inhibitors, direct modification of the aTC scaffold may be the most efficient way to aid in the generation of larger inhibitor libraries by determining empirical inhibitor structure–activity relationships (SAR).

\textit{Semisynthesis of aTC Analogues.}

Inspired by the seminal work of Nelson and co-workers, as well as Gmeiner and co-workers, involving semisynthetic strategies to tetracycline and anhydrotetracycline analogues,\textsuperscript{43,44} we synthesized four aTC analogues from parent tetracyclines to evaluate the potential for aTC to serve as a privileged scaffold for the development of tetracycline-inactivating enzyme inhibitors (Scheme 5.1). Acid-catalyzed dehydration of C6-hydroxytetracyclines chlortetracycline (CTc, 2) and demeclocycline (Dem, 3) provided the corresponding anhydrotetracycline variants, anhydrochlortetracycline (aCTc, 6) and anhydrodemeclocycline (aDem, 7) in quantitative crude yields and excellent isolated yields (C18-silica gel, reverse-phase preparative high-performance liquid chromatography (HPLC)). Correspondingly, electrophilic aromatic substitution of anhydrotetracycline (aTC, 5) with either N-iodosuccinimide (NIS) or molecular bromine (in acid) afforded C7-iodoanhydrotetracycline (7-I-aTC, 8) and C9–Br-anhydrotetracycline (9-BraTC, 9) in excellent crude and isolated yields. These brightly colored
solids were storable at low temperatures (−20 °C), away from light, with little decomposition observed over six month periods (by high-performance liquid chromatography–mass spectrometry, LCMS), and stability and longevity improved when the compounds were stored at low temperature under argon atmosphere. However, triturations with methyl-tert-butyl ether (MTBE) of analogues with trace impurities, followed by filtration, allowed for reisolation of greater than 90–95% purity material as determined by LCMS and NMR.

**Biological Evaluation of aTC Analogue Library.**

With a scalable synthetic route in hand, we evaluated the ability of the aTC analogues to inhibit tetracycline-destructase enzymes via an *in vitro* optical absorbance kinetic assay and referenced the results to those obtained for known inhibitor, aTC, in parallel scenarios (Figure 5.6). In general, inhibitor potency varied in an enzyme-dependent manner that was consistent with what was previously observed with aTC, vide supra. Apparent half-maximal inhibitory concentrations (IC₅₀s) were highest for the Tet(50)-mediated degradation of tetracycline and chlortetracycline (Figure 5.6A,D), followed by Tet(X) (Figure 5.6B,E) and Tet(X)_3 (Figure 5.6C,F), respectively. Inhibitor potency decreased (2- to 10-fold) when inhibitors were coadministered with CTc (Figure 5.6D–F) over tetracycline (Figure 5.6A–C), and chlorinated aTC analogues aCTc and aDem performed marginally better than aTC when coadministered with tetracycline, suggesting little significant cooperativity/synergism of structurally similar inhibitor-substrate pairs. In general, halogenation of the D-ring improved *in vitro* inhibitory activity, though the enhancement observed for C7-chlorination is more pronounced across all enzyme-antibiotic combinations. Removal of the C6-methyl group was well tolerated for the inhibition of Tet(X) and Tet(X)-homologue, Tet(X)_3; however, aDem performed poorly against tetracycline
deconstructase Tet(50). Proposed reasoning for this phenomenon is discussed later in this report, *vide infra*.

With *in vitro* inhibitor potencies established, we next tested the ability of the aTC analogues to rescue tetracycline activity in whole cell inhibition assays of *E. coli* expressing tetracycline destructase enzymes. By varying concentrations of the tetracycline-inhibitor combinations in a checkboard broth microdilution antibiotic susceptibility assay, we were able to identify the lowest concentration of inhibitor that results in at least a fourfold change in the minimum inhibitory concentration (MIC) of the tetracycline alone; highlights of the checkerboard assay are shown in *Figure 5.7A*. Several—not all—tetracycline-inhibitor pairs showed enhanced antibiotic activity, when tetracycline substrates were coadministered with small doses of various inhibitors (four- to eight-fold enhancement over antibiotic alone, *Figure 5.7A*). Notably, though aTC was, in general, the least potent of the inhibitor library, it performs well—and on par with the chlorinated analogues, aCTc and aDem—in the whole cell rescue of tetracycline activity. It is important to note that, while some of the aTC analogues possess baseline antibiotic activity alone, antibiotic synergistic killing and destructase inhibition may not be mutually exclusive (*Figure 5.7A*), and it is not entirely clear how much of a contribution is made by each mechanism for each combination. The calculated fractional inhibitory concentration index (FICI) is provided for reference.45 However, the enhancements in MIC observed for several anhydrotetracycline-variant/antibiotic combinations are modest yet promising, and potent *in vitro* destructase inhibition by each of the aTC variants—in combination with these results—suggests that whole cell adjuvant activity could be optimized in the future second generation synthesis of larger aTC-like inhibitor libraries.
While 9-Br-aTC and 7-I-aTC possess potent \textit{in vitro} destructase inhibitory activity, the analogues showed limited activity in whole cell inhibition studies, potentially due to increased instability in solution over time and/or increased reactivity toward enzymatic degradation. In particular, 7-I-aTC showed no inhibitory activity with any enzyme–antibiotic combination against whole cell \textit{E. coli} expressing destructase enzymes. We hypothesized that the instability of 7-I-aTC was contributing to this phenomenon, as the nonenzymatic degradation of 7-I-aTc in solution was observed by LCMS (overnight) and during extended $^{13}$C NMR experiments. Because the o/p-substitution of phenols with heavy halogens is known to increase the rate of nonenzymatic photooxidation,\textsuperscript{46–49} the incorporation of D-ring halogens to future inhibitor libraries may be limited to chlorination and fluorination to preserve inhibitor stability. However, the Brand I-substituents present in 7-I-aTC and 9-Br-aTC may serve as useful functional handles to access more structurally diverse and stable inhibitor scaffolds. While we propose that disparities in \textit{in vitro} inhibitor potency and whole cell rescue of tetracycline activity are largely due to problems with inhibitor stability, \textit{vide infra}, the potent \textit{in vitro} inhibition of tetracycline destructase enzymes suggests structural modifications that promote inhibitor stability and maintain potency could improve whole cell performance.

\textit{Working Inhibition Model.}

The tetracycline destructase enzymes are class A flavin-monooxygenase (FMO) enzymes that catalyze nicotinamide adenine dinucleotide phosphate (NADPH)- and oxygen-dependent, multicomponent transformations via a series of complex and dynamic conformational changes involving a mobile flavin cofactor.\textsuperscript{33,34,38,39} While the precise sequence of events is currently unknown, the proposed degradation process involves substrate recognition and binding to FAD-OUT enzyme conformation I (Figure 5.7B), followed by rapid flavin reduction (FAD to
FADH$_2$) by NADPH. Through discrete conformational changes, the reduced flavin cofactor is pushed toward the newly bound substrate and reacts with molecular oxygen to generate the reactive C4a-hydroperoxyflavin in the solvent-protected FAD-IN conformation. The hydroperoxyflavin then reacts with the tetracycline substrate, and another series of conformational changes allows for release of the degradation product and dehydration of the resultant C4a-hydroxyflavin to regenerate FAD-OUT conformer I.

Upon the basis of the findings presented herein, in combination with previous studies, we developed a working model for inhibition of tetracycline destructase enzymes in the context of the proposed degradation process (Figure 5.7B). In the event that inhibition occurs competitively, inhibitor could bind to substrate unbound enzyme and exclude accommodation of substrate into the active site; this inhibited state (III) could be a stalled state (nonproductive), or the inhibitor could react with the enzyme as a sacrificial substrate (moving through inhibited state IV and multiple successive inhibited states en route to the enzymatic degradation of the inhibitor, Figure 5.7B). Alternatively, inhibition could occur noncompetitively, where inhibitor binds to substrate-bound enzyme complex (Intermediate II or successive substrate-bound functional enzyme states) and restrains the conformational flexibility of the enzyme to impede productive turnover. Because degradation is a complex, multicomponent process involving a number of discrete enzyme conformational changes, none of the multiple inhibited states derived from substrate-bound and substrate-unbound functional enzyme states can implicitly be excluded. Therefore, to assess the potential of the generated aTC analogues to undergo enzymatic degradation as sacrificial substrates, we chose to evaluate the in vitro degradation of the inhibitor library by canonical tetracycline-inactivating enzyme, Tet(X), which is known to degrade aTC, albeit slowly.
**aTC Analogues as Substrates for Destructase Enzymes.**

Using Tet(X) as a model system, we assessed the potential for the aTC analogues to serve as sacrificial substrates via an *in vitro*, broad-scan optical absorbance kinetic assay coupled to LCMS, as previously reported for the Tet(X)- mediated degradation of aTC. The results of the Tet(X)- mediated aTC analogue degradation assays are summarized below (Figure 5.8). We confirmed that aTC is a substrate for Tet(X), indicated by the time- and enzyme-dependent decrease in the 440 nm absorption band and aTC extracted mass (LCMS) and used this result as a positive control for the enzymatic degradation of the aTC scaffold. In general, aTC analogue stability increased with D-ring halogenation, and chlorination provided the most “protection” against enzymatic degradation. Moreover, observed stability tracks with degree of electron deficiency and the electron-withdrawing nature of the added substituent (i.e., aCTc is more stable than 9-Br-aTC, which is more stable than 7-I-aTC and aTC, respectively). We hypothesize that this stability is due to decreased nucleophilicity of the C,D-ring aromatic framework, which would be fundamentally important to an enzyme-mediated electrophilic hydroxylation event similar to that determined by Wright and co-workers in 2004 for Tet(X) degradation of oxytetracycline (Figure 5.9).

**Anhydrodemeclocycline, a Special Case.**

As detailed above, chlorinated aTC analogue anhydrodemeclocycline (aDem, 7) possesses potent *in vitro* inhibitory activity against the Tet(X)- and Tet(X)_3-mediated degradation of tetracycline antibiotics (apparent IC₅₀ 1–4 μM, Figure 5.10A–C) and rescues tetracycline activity in whole cell *E. coli* expressing these enzymes (Figure 5.7A); however, 7 was found to possess poor *in vitro* inhibitory activity against the enzymatic degradation of both
tetracycline and demeclocycline by soil-derived tetracycline destructase Tet(50). Moreover, when aDem was exposed to Tet(50) in the presence of NADPH and absence of tetracycline in an optical absorbance kinetic assay, a steady, observable decrease in the absorbance at 400 nm was observed, suggesting that aDem itself was a substrate for Tet(50). To expand upon this observation, we determined kinetic parameters for the aDem dose-dependent response, similar to the Michaelis–Menten parameters previously described (Figure 5.10D); however, LCMS-coupled experiments for the reaction showed no measurable decrease in aDem extracted mass over time (Figure 5.11)—suggesting that the decrease in absorbance at 400 nm was the result of rapid, aDem-dependent Tet(50)-mediated consumption of NADPH (broad absorbance band at 340 nm) and not the enzymatic degradation of aDem 7.

The previously reported X-ray crystal structure of aTC bound to Tet(50) revealed a unique inhibitor binding mode, denoted Mode I_{A,D},\textsuperscript{42} resulting from the noncovalent interaction of aTC with residues in both the substrate binding domain and the FAD binding domains of the enzyme (Figure 5.12A).\textsuperscript{37} In particular, the C6-methyl substituent on aTC occupies a small hydrophobic pocket between lysine 198, lysine 205, and methionine 222 of the substrate binding domain. Because this substituent is notably absent in aDem, we hypothesized that binding mode flexibility previously observed for tetracycline-inactivating enzymes could allow for the accommodation of aDem in a unique, nonreactive binding mode to promote the NADPH-dependent reduction of the mobile flavin element without providing a productive pathway for the degradation of enzyme-bound aDem. Moreover, because the addition of aDem promotes the Tet(50)-mediated consumption of NADPH without resulting in the degradation of aDem itself, we hypothesized that the formation of a reactive hydroperoxyflavin cofactor in the absence of a viable substrate would lead to the release of hydrogen peroxide generated from the nonspecific
oxidation of water. The NADPH-dependent formation of hydrogen peroxide from FMOs has been reported previously.\textsuperscript{50,51} Thus, we characterized the aDem-promoted, Tet(50)-mediated consumption of NADPH using a broad-scan optical absorbance kinetic assay (\textbf{Figure 5.12B}) and confirmed the formation of hydrogen peroxide using a colorimetric detection method (Pierce Quantitative Peroxide Assay, ThermoScientific, \textbf{Figure 5.12C}). The nonspecific formation of hydrogen peroxide was not unique to aDem, as it was also observed in the Tet(50)-mediated degradation of tetracycline (\textbf{Figures 5.13 and 5.14}), confirming that “ligand” binding to the substrate-binding domain promotes the consumption of NADPH and reduction of the mobile flavin cofactor (as is canonical with Class A FMO enzymes).\textsuperscript{38} This mode of inhibition is under further investigation in our lab and might prove to be effective against pathogens expressing tetracycline destructases, as a method of inducing oxidative stress by stimulating flavin reduction and release of hydrogen peroxide inside the cell.

\textit{Inhibiting Tet(X) Degradation of Third Generation Tetracycline, Tigecycline.}

The rise of multidrug-resistant superinfections has cultivated a renaissance for tetracycline antibiotics as last-resort treatments.\textsuperscript{19} In 2005, the first member of the third-generation tetracyclines tigecycline was Food and Drug Administration (FDA)-approved for the treatment of skin and intra-abdominal infections and pneumonia.\textsuperscript{52} Earlier this year, two additional third-generation tetracyclines, eravacycline and omadacycline, were FDA-approved for similar treatment strategies (\textbf{Figure 5.15C}).\textsuperscript{53,54} In this report, we used first-generation tetracyclines as model systems for tetracycline-inactivating enzyme activity; however, with the advent of tigecycline, eravacycline, and omadacycline, the enzymatic degradation of last-generation tetracyclines is fundamentally important to study, since new resistance mechanisms, including antibiotic inactivation, are certain to emerge upon widespread antibiotic deployment.
The third-generation tetracyclines were strategically designed to overcome resistance due to efflux and ribosome protection, making antibiotic inactivation a likely candidate for future clinical resistance. Previous reports have identified that Tet(X) can degrade tigecycline and eravacycline, albeit slowly, to achieve resistance to these last-generation tetracyclines.\textsuperscript{55–57} Resistance to tigecycline was not observed with the soil-derived tetracycline destructase enzymes, including Tet(50), presumably because the presence of the “Gatekeeper helix” excludes productive accommodation of tetracyclines with bulky D-ring substituents (Figure 5.2C).\textsuperscript{34,37} Building upon previous reports, we confirmed and characterized the Tet(X)-mediated degradation of tigecycline and determined Michaelis–Menten kinetic parameters using an optical absorbance kinetic assay (Figure 5.15A). After identifying the most promising inhibitor candidates from previously described \textit{in vitro} and whole cell inhibition assays, we evaluated the \textit{in vitro} inhibitory activity of aTC, aCTc, and aDem against the Tet(X)-mediated degradation of tigecycline and found all to be potently inhibitory (apparent IC\textsubscript{50}s from \(~0.4\) to \(1.5\) \(\mu\)M). Unfortunately, in our hands, whole cell \textit{E. coli} expressing Tet(X) displays minimal resistance to tigecycline (twofold increase over empty vector controls); thus, it was difficult to identify inhibition profiles from variations in the limited resistance response. However, the potent \textit{in vitro} inhibition of aTC, aCTc, and aDem against Tet(X) degradation of tigecycline are promising preliminary results for further development of adjuvant approaches to combat the enzymatic degradation of last-generation tetracyclines. Moreover, because of the functional similarities and phylogenetic clustering of the gut-derived enzymes, we hypothesize that Tet(X)_3 may possess similar abilities to degrade last-generation tetracyclines with velocities more amenable to both \textit{in vitro} and whole cell inhibition assays—though full characterization of Tet(X)_3 was somewhat beyond the scope of this report. Studies focused on the resistance profile and microbial evolution
of tetracycline-inactivating enzyme Tet(X)$_3$ are currently ongoing in our laboratories and will be reported in due course.

### 5.5 Outlook and Conclusions

In conclusion, the synthesis and biological evaluation of aTC-like small molecule inhibitors of tetracycline-inactivating enzymes are reported (Figure 5.16A). The four analogues were screened for inhibitory activity against the enzymatic degradation of tetracycline antibiotics by three representative tetracycline destructase enzymes via both *in vitro* and whole cell-based inhibition assays. All synthesized analogues were found to possess *in vitro* inhibitory activity to some degree, and inhibitor potency was found to vary largely as a function of enzyme and moderately as a function of inhibitor–substrate pairing within the context of a single enzyme. The addition of electron-withdrawing groups to the D-ring of aTC was found to improve both the enzymatic and nonenzymatic stability of the aTC analogues, and potent *in vitro* inhibitory activity of this small library shows promise for the rational design of larger tetracycline-inactivating enzyme inhibitor libraries. Notably, aTC and chlorinated analogues aCTc and aDem were found to inhibit the Tet(X)-mediated degradation of last-generation tetracycline, tigecycline. Further development of small molecule inhibitors of glycylcycline-inactivating enzymes like Tet(X), with open active sites that can accommodate large D-ring substituents on tetracycline substrates (Figure 5.16B),$^{35}$ are fundamentally important to establishing viable adjuvant approaches that combat the imminent emergence of this resistance mechanism in multidrug-resistant infections. Efforts aimed at improving inhibitor stability while maintaining potency are currently ongoing in our laboratories and will be reported in due course.
5.6 Materials and Methods

General Methods.

Unless stated, all synthetic reactions were performed under inert, argon atmosphere, and all in vitro kinetic assays were prepared actively open to air (in nondegassed solvents). All solvents—including deuterated NMR solvents—and reagent chemicals used in preparation or analysis of the αTC analogue library were obtained commercially and used without further purification. IR spectroscopy was performed on a Bruker Alpha FTIR machine with a Pt-ATR diamond, and IR data were analyzed using Bruker OPUS 7.5. Melting points were observed using a Stuart SMP10 digital melting point apparatus. NMR spectra were obtained on a Varian Unity-Plus 300 MHz, Varian Unity-Inova 500 MHz, or Agilent PremiumCompact+ 600 MHz spectrometer. All free induction decay files (FIDs) were processed using Mestrenova version 11.0.4 software. Chemical shifts (δ) are reported in parts per million (ppm) and referenced to residual nondeuterated solvent. Coupling constants (J) are reported in hertz (Hz). High-resolution mass spectrometry data were obtained at the Danforth Plant Science Center (DPSC) in St. Louis, MO, by direct infusion using an Advion Nanomate Triversa robot into a Thermo- Fisher Scientific Q-Exactive mass spectrometer, and mass spectra were recorded in positive ion mode from m/z 150–500 and a resolution setting of 140 000 (at m/z 200). In vitro degradation experiments monitored by optical absorbance spectroscopy were performed on an Agilent Cary 50 UV–visible spectrophotometer. In vitro degradation experiments monitored by LCMS were performed using an Agilent 6130 single quadrupole instrument with G1313 autosampler, G1315 diode array detector, and 1200 series solvent module and separated using a Phenomenex Gemini C18 column, 50 × 2 mm (5 μm) with guard column cassette and a linear gradient of 0%
acetonitrile and 0.1% formic acid to 95% acetonitrile and 0.1% formic acid over 20 min at a flow rate of 0.5 mL/min before analysis by electrospray ionization (ESI+). Whole cell assays were performed using Difco BBL Mueller-Hinton broth in Costar 96-well plates at 37 °C. End-point growth was assayed at OD$_{600}$ using a Synergy H1 plate reader (BioTek, Inc.).

**Cloning, Expression, and Purification of Tetracycline-Destructase Enzymes.**

All genes corresponding to the tetracycline destructases$^{34,37}$ used in this report (for Tet(X)$_3$, see Table 5.1) were cloned into pET28b(+) vectors (Novagen) as previously described (BamHI and NdeI restriction sites)$^{34,37}$ and transformed into BL21-Star (DE3) competent cells (Life Technologies). Cells were cultured at 37 °C in lysogeny broth (LB) containing kanamycin (0.03 mg/mL); once the uninduced culture reached an OD$_{600}$ of 0.6, the cells were cooled to 0 °C, induced with 1 mM IPTG, and allowed to grow at 15 °C for 12−15 h (harvest OD$_{600}$ varied by tetracycline destructase expressed, but on average, harvest OD$_{600}$ < 4.5 resulted in greater isolated enzyme yield). To harvest, the induced cells were pelleted by centrifugation at 4000 rpm for 15 min (4 °C) and resuspended in cold 40 mL of lysis buffer (50 mM K$_2$HPO$_4$, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 5 mM 2-mercaptoethanol, pH 8.0) containing SIGMAFAST protease inhibitor. The cells were transferred to falcon tubes, flash frozen in liquid nitrogen, and stored at −80 °C. To harvest, the cells were thawed and mechanically lysed using an Avestin EmulsiFlex-C5 cell disruptor, and the resultant lysate was centrifuged at 45 000 rpm for 35 min. The supernatant was transferred to a column containing prewashed Ni-NTA resin and incubated for 30−45 min; at which point, the resin was washed with lysis buffer (2 × 40 mL), and the protein was eluted from the resin with fractions of elution buffer (5 × 10 mL, 50 mM K$_2$HPO$_4$, 500 mM NaCl, 5 mM β-mercaptoethanol, 300 mM imidazole, 10% glycerol, pH 8.0). The fractions were combined in 10 000 molecular weight cutoff (MWCO) Snakeskin
dialysis tubing (ThermoScientific) and soaked in buffer (50 mM K$_2$HPO$_4$ pH 8.0, 150 mM NaCl, 1 mM dithiothreitol (DTT)) overnight to minimize imidazole concentration. To isolate the desired protein, the dialyzed solution was concentrated using a 30 000 MWCO Amicon centrifugal filter (Millipore-Sigma), and concentrated protein solution was flash frozen in liquid nitrogen (50 μL portions) and stored at −80 °C.

**Kinetic Characterization of Tetracycline Inactivation.**

Kinetic characterization of tetracycline inactivation was achieved in a manner similar to previously reported procedures. In brief, reaction samples were prepared in [tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS) buffer (100 mM, pH 8.5) with 504 μM NADPH, 5.04 mM MgCl$_2$, varying concentrations of tetracycline substrate (typically, 0–40 μM), and 0.4 μM enzyme. After the addition of enzyme, the reactions (in duplicate or triplicate) were mixed, manually by pipet, and the reaction was monitored continuously in a single frame by optical absorbance spectroscopy (absorbance at 380 nm, Carey UV–visible spectrophotometer) for 3–4 min. Initial enzyme velocities were determined by linear regression using Agilent Cary WinUV Software over the linear range of the reaction, and the velocities were fitted to the Michaelis–Menten equation using GraphPad Prism 6.

**Synthesis and Characterization of aTC Analogues 6–9.**

(4S,4aS,12aS)-7-Chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide Hydrochloride (aCTc, 6).

To a clean, dry round-bottom flask, equipped with stirbar and reflux condenser, was added chlortetracycline hydrochloride (50 mg, 0.10 mmol) and 6 N HCl in methanol (5 mL)
under argon atmosphere. The reaction was heated to 60 °C and allowed to stir at 60 °C for 1.5 h (monitored by LCMS). When the reaction was complete, the reaction was concentrated under reduced pressure to provide crude product (50 mg, 0.10 mmol, 100% crude yield) as an orange solid [clean by NMR]. Purification by preparative HPLC (Si–C18 reverse phase column, gradient 0–95% CH₃CN/H₂O with 0.1% formic acid, tᵣ = 16 min) provided (4S,4aS,12aS)-7-chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydro-tetracene-2-carboxamide, which was reconstituted as the hydrochloride salt to provide the title compound as an orange solid (44 mg, 0.089 mmol, 91% yield). FTIR (neat) 3306, 3042, 1621, 1583, 1565, 1531, 1464, 1375, 1217, 1137, 1058, 820, 560 cm⁻¹; mp 211–212 °C (decomposed); ¹H NMR (500 MHz, deuterated dimethyl sulfoxide (DMSO-d₆)) δ 9.68 (s, 1H), 9.22 (s, 1H), 7.66 (d, J = 8.5 Hz, 1H), 6.88 (d, J = 8.5 Hz, 1H), 4.44 (d, J = 4.0 Hz, 1H), 3.46 (ddt, J = 18.0, 14.2, 4.9 Hz, 2H), 3.15 (dd, J = 16.8, 8.9 Hz, 1H), 2.93 (s, 6H), 2.63 (s, 3H); ¹³C NMR (126 MHz, DMSO-d₆) δ 199.7, 192.7, 187.4, 172.1, 163.2, 157.6, 136.2, 135.9, 134.1, 121.5, 119.3, 114.6, 111.8, 109.2, 97.6, 76.2, 66.9, 42.9 (2C), 35.8, 29.6, 19.2; high-resolution mass spectrometry (HRMS) (time-of-flight (TOF) MS ES+) calcd for C₂₂H₂₂ClN₂O₇ [M + H]⁺ 461.1116; found 461.1115.

(4S,4aS,12aS)-7-Chloro-4-(dimethylamino)-3,10,11,12atetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride (aDem, 7).

To a clean, dry round-bottom flask, equipped with stirbar and reflux condenser, was added demeclocycline hydrochloride (50 mg, 0.10 mmol) and 6 N HCl in methanol (5 mL) under argon atmosphere. The reaction was heated to 70 °C and allowed to stir at 60 °C for 3 h (monitored by LCMS). When the reaction was complete, the reaction was concentrated under reduced pressure to provide crude product (54 mg, 0.11 mmol, quant. yield) as a yellow-orange
solid (clean by NMR). Purification by preparative HPLC (Si–C18 reverse phase column, gradient 0–95% CH₃CN/H₂O with 0.1% formic acid, tᵣ = 15 min) to provide (4S,4aS,12aS)-7-chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide, which was reconstituted as the hydrochloride salt to provide the title compound as a light orange solid (37 mg, 0.077 mmol, 77% yield). FTIR (neat) 3301, 3076, 1619, 1567, 1533, 1444, 1375, 1353, 1202, 1188, 1071, 993, 820, 685 cm⁻¹; mp 213–214 °C (decomposed); ¹H NMR (500 MHz, DMSO-d₆) δ 9.69 (s, 1H), 9.29 (s, 1H), 7.68 (d, J = 8.4 Hz, 1H), 7.39 (s, 1H), 6.92 (d, J = 8.5 Hz, 1H), 4.46 (d, J = 6.5 Hz, 1H), 3.58 (dd, J = 17.3, 4.0 Hz, 1H), 3.53–3.39 (m, 2H), 2.89 (s, 6H); ¹³C NMR (126 MHz, DMSO-d₆) δ 198.3, 193.1, 187.2, 172.2, 165.2, 157.2, 135.9, 135.2, 132.7, 119.2, 113.9, 113.5, 111.3, 109.7, 97.5, 76.8, 66.4, 42.3, 41.6, 37.2, 29.5; HRMS (TOF MS ES+) calcd for C₂₁H₂₀ClN₂O₇ [M + H]⁺ 447.0959; found 447.0958.

(4S,4aS,12aS)-4-(Dimethylamino)-3,10,11,12a-tetrahydroxy-7-iodo-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride (7-I-aTC, 8).

Procedure A—To a clean, dry round-bottom flask, equipped with stirbar, was added anhydrotetracycline hydrochloride (100 mg, 0.216 mmol) and methanol (2.2 mL) under argon atmosphere. The flask was cooled to −10 °C, and N-iodosuccinimide (58.3 mg, 0.259 mmol) was added in one portion. The reaction was allowed to warm to room 0 °C over 2 h, then stirred at 0 °C for 1 h (monitored by LCMS). When the reaction was complete, the reaction was diluted with methanol (to 10 mL total volume) and immediately purified by preparative HPLC (Si–C18 reverse phase column, gradient 0–95% CH₃CN/H₂O with 0.1% formic acid, tᵣ = 16 min) to provide (4S,4aS,12aS)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-7-iodo-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide, which was reconstituted as the
hydrochloride salt to provide the title compound as an orange solid (70.0 mg, 0.119 mmol, 55% yield).

**Procedure B**—To a clean, dry round-bottom flask, equipped with stirbar, was added anhydrotetracycline hydrochloride (250 mg, 0.540 mmol) and methanol (25 mL) under argon atmosphere. The flask was cooled to 0 °C, and solid N-iodosuccinimide (0.134 g) was added, in one portion. The reaction stirred at 0 °C for 1 h (monitored by LCMS), and the reaction was concentrated under reduced pressure (no heating) to yield a crude brown solid. The solid was triturated with tert-butylmethyl ether (TBME) for 30 min protected from light; filtration provided the title product (0.3178 g, 0.540 mmol, quantitative crude yield) as a green-brown solid. FTIR (neat) 3307, 3078, 1660, 1615, 1556, 1396, 1377, 1321, 1228, 1131, 1075, 1058, 810, 702, 618 cm⁻¹; mp 188–189 °C; ¹H NMR (500 MHz, DMSO-d₆) δ 9.62 (s, 1H), 9.23 (s, 1H), 8.01 (d, J = 8.9 Hz, 1H), 7.31 (d, J = 8.9 Hz, 1H), 4.38 (s, 1H), 3.57–3.36 (m, 3H), 3.16 (s, 1H), 2.89 (s, 6H), 2.56 (s, 1H), 2.40 (s, 3H); ¹³C NMR (126 MHz, DMSO-d₆) δ 199.5, 192.9, 187.2, 179.3, 163.7, 156.2, 141.4, 138.5, 130.5, 121.6, 117.2, 112.2, 108.7, 97.4, 79.6, 76.3, 67.0, 42.1 (2C), 35.8, 29.5, 14.1; HRMS (TOF MS ES+) calcd for C₂₂H₂₂IN₂O₇ [M + H]⁺ 553.0472; found 553.0469.

(4S,4aS,12aS)-9-Bromo-4-(dimethylamino)-3,10,11,12atetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride (9-Br-aTC, 9).

To a clean, dry round-bottom flask, equipped with a stirbar and argon inlet, was added liquid bromine (13 μL, 0.2592 mmol, 1.2 equiv), acetic acid (10 mL), and trifluoroacetic acid (2 μL, 0.0216 mmol, 0.1 equiv). The mixture was heated to 50 °C (over 20 min), at which point anhydrotetracycline hydrochloride (100 mg, 0.216 mmol) was added in one portion. The reaction mixture stirred at 50 °C for 30 min; then, the reaction was cooled to room temperature and stirred
at room temperature for 4 h (monitored by LCMS). After the reaction was complete, the crude mixture was concentrated under reduced pressure to provide the crude product, which was reconstituted with aqueous HCl to provide the title compound (0.1165 g, 0.215 mmol, quantitative crude yield) as an orange solid HCl salt. FTIR (neat) 3295, 3078, 1618, 1553, 1393, 1373, 1317, 1224, 1124, 1058, 699, 620 cm⁻¹; mp 203–204 °C (decomposed); ¹H NMR (500 MHz, DMSO-d₆) δ 9.64 (s, 1H), 9.22 (s, 1H), 7.84 (d, J = 9.0 Hz, 1H), 7.42 (d, J = 9.1 Hz, 1H), 4.44 (d, J = 4.0 Hz, 1H), 3.56 (dd, J = 17.7, 4.9 Hz, 1H), 3.46 (dd, J = 9.9, 4.7 Hz, 1H), 3.07 (d, J = 14.5 Hz, 1H), 2.91 (s, 6H), 2.39 (s, 3H); ¹³C NMR (126 MHz, DMSO-d₆) δ 200.6, 192.7, 187.0, 172.1, 161.8, 153.4, 137.7, 135.8, 131.6, 122.3, 116.7, 112.6, 109.3, 104.5, 97.3, 76.4, 67.0, 42.1 (2C), 35.3, 21.1, 14.0; HRMS (TOF MS ES⁺) calcd for C₂₂H₂₂BrN₂O₇ [M + H]⁺ 505.0610; found 505.0610.

**In Vitro Characterization of aTC and aTC Analogue Inhibition.**

Half-maximal inhibitory concentrations (IC₅₀) for the aTC and aTC analogue inhibition of Tet(50), Tet(X), and Tet(X)₃ were determined from the nonlinear regression analysis of initial velocities of tetracycline degradation in the presence of varying concentrations of chosen inhibitor. Reaction samples were prepared in 100 mM TAPS buffer (pH 8.5) with 504 μM NADPH, 5.04 mM MgCl₂, 25.3 μM tetracycline substrate, varying concentrations of inhibitor (μM), and 0.4 μM enzyme. After the addition of enzyme, the reactions (in triplicate) were mixed manually by pipet, and the reaction was monitored, continuously in a single frame, by optical absorbance spectroscopy (absorbance at 380 or 400 nm, Carey UV–visible spectrophotometer) for 4 min. Initial enzyme velocities were determined by linear regression using Agilent Cary WinUV Software over the linear range of the reaction. The velocities were plotted against the logarithm of inhibitor concentration, and IC₅₀ values were determined using
nonlinear regression analysis in Graphpad Prism 6. Plus/minus error values were determined using linear regression analysis of initial velocities versus concentrations of inhibitor in Graphpad Prism 6. Each set of experiments was accompanied by a variety of controls, including a no-enzyme control (NADPH + Tet + inhibitor)—which was used to simulate full enzyme inhibition and assigned to inhibitor concentration of $1 \times 10^{15}$, and a no-inhibitor control (NADPH + Tet + enzyme)—which was assigned an inhibitor concentration of $1 \times 10^{-15}$. A no-substrate control (NADPH + inhibitor + Tet) was also performed to identify competitive background signals from the enzymatic degradation of the inhibitor itself. For all inhibitor–enzyme combinations (except for Tet(50)–aDem), the initial velocities of the no-substrate controls were negligible.

**Checkerboard Whole Cell Inhibition Assay.**

Substrates and inhibitors were dissolved DMSO before being diluted to working concentrations in cation-adjusted Mueller–Hinton broth supplemented with 50 μg/mL kanamycin. A twofold dilution series of each drug was made independently across 8 rows of a 96-well master plate before 100 μL of each drug dilution series was combined into a 96-well culture plate (Costar), with rows included for no-drug and no-inocula controls. The plates were inoculated with $\sim 1$ μL of tetracycline deconstructase expressing *E. coli* MegaX (Invitrogen) diluted to OD$_{600}$ 0.1 using a sterile 96-pin replicator (Scinomix). Plates were sealed with Breathe-Easy membranes (Sigma-Aldrich) and incubated at 37 °C with shaking at 220 rpm. End-point growth was assayed at OD$_{600}$ at 20 and 36 h of growth using a Synergy H1 plate reader (BioTek, Inc.). Three independent replicates were performed for each strain/drug combination. Highlighted MIC data were refined from a complete raw data set to identify mixtures resulting in the largest MIC fold change (at least fourfold) with the least amount of inhibitor (fold change/inhibitor dose; see
Figure 5.7A and Table 5.2a–c). Synergy of inhibitor and tetracycline combinations was determined using the fractional inhibitory concentration index (FICI) method\textsuperscript{45}

\[
\text{FICI} = \frac{\text{MIC}_{\text{combo}}}{\text{MIC}_{\text{alone}}} + \frac{\text{MICP}_{\text{combo}}}{\text{MICP}_{\text{alone}}}
\] (1)

where FICI > 1 indicates antagonism, FICI = 1 indicates additivity, and FICI < 1 indicates synergy.

**Kinetic Characterization of aTC and aTC Analogue Degradation by Tet(X).**

The kinetic characterization of the degradation of aTC and aTC analogues by Tet(X) was monitored by optical absorbance spectroscopy (Carey UV-visible spectrophotometer) coupled to LCMS detection (Agilent 6130 single quadrupole instrument with G1313 autosampler, G1315 diode array detector, and 1200 series solvent module and separated using a Phenomenex Gemini C18 column, 50 × 2 mm (5 μm) with guard column cassette and a linear gradient of 0% acetonitrile and 0.1% formic acid to 95% acetonitrile and 0.1% formic acid over 20 min at a flow rate of 0.5 mL/min before analysis by electrospray ionization (ESI\textsuperscript{+})). Reactions (in duplicate) were prepared in 100 mM TAPS buffer (pH 8.5) with an NADPH regenerating system (40 mM glucose-6-phosphate, 4 mM NADP\textsuperscript{+}, 1 mM MgCl\textsubscript{2}, 4 U/mL glucose-6-phosphate dehydrogenase), 28.0 μM substrate (aTC or corresponding analogue), and 0.24 μM enzyme. Reaction progress was monitored by optical absorbance spectroscopy (280–550 nm, 1 nm and 30 min intervals) over 3.5 h, where 150 μL of reaction sample was removed at 30 min intervals and quenched with 600 μL volumes of quench solution (1:1 acetonitrile/0.25 M aqueous HCl). The quenched samples were centrifuged (5000 rpm, room temperature) for 5 min, and 600 μL of supernatant was transferred to an LCMS-compatible vial.
containing Fmoc-alanine internal standard (2.21 μM final concentration) and analyzed by LCMS (reverse-phase HPLC, C18-silica, gradient 0–95% CH₃CN/H₂O, 0.5 mL/min flow rate). Substrate masses [M + H]⁺ and hydroxylated product masses [M−OH +H]⁺ were extracted from the crude mass chromatogram and normalized to the internal standard [M + H]⁺ counts. No enzyme controls were performed for each aTC analogue screened and showed no significant nonenzymatic degradation over the course of the observable reaction. Degradation of 7-I-aTC and 9-Br-aTC at extended solution times (overnight) showed a decrease in LCMS extracted ion counts for both analogues, suggesting some nonenzymatic degradation over longer reaction times.

**Qualitative Detection of aDem-promoted Hydrogen Peroxide Formation by Tet(50).**

Qualitative colorimetric detection of aDem-promoted hydrogen peroxide formation by Tet(50) was performed using an aqueous Pierce Quantitative Peroxide Assay kit (ThermoScientific). Reaction samples were prepared in 100 mM TAPS buffer (pH 8.5) with 252 μM NADPH, 2.52 mM MgCl₂, 25 μM substrate (either aDem or Tet), and 0.4 μM enzyme. After the addition of enzyme, the reaction was mixed manually by pipet, and the reaction was monitored by optical absorbance spectroscopy (280–550 nm, 1 nm and 0.1 min scan intervals) over 8 min. At 8 min, 100 μL of reaction solution was added to a detection Eppendorf containing 1000 μL of working reagent (prepared according to specifications for Pierce Quantitative Peroxide Assay kit). The detection Eppendorf was incubated for 20 min at room temperature to result in the observed color changes reported in the main text (see Figures 5.12C and 5.14).
5.7 Acknowledgements

The authors would like to thank Washington Univ. in St. Louis (WUSTL), Washington Univ. School of Medicine, and the National Institutes of Health for their support of this research and our programs. In particular, N.T. would like to thank the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, for the support of his program. We would also like to acknowledge J. Kao and M. Singh (WUSTL, Dept. of Chemistry) for their assistance with NMR experiments and B. Evans (Danforth Plant Science Center, St. Louis, MO) and his team for their assistance in acquiring high-resolution mass spectra for all synthesized compounds. In addition, J.L.M. would like to acknowledge the WM Keck Postdoctoral Program in Molecular Medicine for funding support of her postdoctoral fellowship.
5.8 Figures and Tables

Scheme 5.1. Semisynthetic Strategies toward aTC Analogues 6–9.
<table>
<thead>
<tr>
<th><strong>Sequences, Strains, Plasmids, Primers</strong></th>
<th><strong>Reference or source</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
</tr>
<tr>
<td>MegaX DH10B</td>
<td>Invitrogen BioSciences, ThermoScientific</td>
</tr>
<tr>
<td><strong>Sequence</strong></td>
<td></td>
</tr>
<tr>
<td>TE_7F_Contig_3 [Tet(X)_3]</td>
<td>NCBI GenBank Accession Number: KU547176.1:452..1588</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pET28b(+)</td>
<td>Novagen, Merck Biosciences</td>
</tr>
<tr>
<td>pZE21</td>
<td>(Lutz and Bujard, 1997)</td>
</tr>
<tr>
<td><strong>Primers (5’ → 3’)</strong></td>
<td></td>
</tr>
<tr>
<td>TE_7F_Contig_3 (452_1588) – ATGACTTTTGCTAAAAAATAGAAAAATTA</td>
<td>Integrated DNA Technologies, Inc. (IDT)</td>
</tr>
<tr>
<td>TE_7F_Contig_3 (452_1588) – TTATAGATTGATTAGTTTTGGAATGA</td>
<td>Integrated DNA Technologies, Inc. (IDT)</td>
</tr>
</tbody>
</table>


**Table 5.1:** Relevant Sequences, Strains, Plasmids, and Primers for Tet(X)_3 [TE_7F_Contig_3].
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>$IC_{50}$ (μM)</th>
<th>Substrate MIC (μg/mL)</th>
<th>Inhibitor MIC (μg/mL)</th>
<th>Substrate + Inhibitor MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>aTc</td>
<td>210 ± 25</td>
<td>128</td>
<td>8</td>
<td>64 [1]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aCTc</td>
<td>37 ± 5</td>
<td>128</td>
<td>32</td>
<td>64 [4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aDem</td>
<td>300 ± 100</td>
<td>128</td>
<td>16</td>
<td>64 [4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-IA-Tc</td>
<td>54 ± 5</td>
<td>128</td>
<td>128</td>
<td>64 [32]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9-Br-aTc</td>
<td>12 ± 1</td>
<td>128</td>
<td>16</td>
<td>32 [8]</td>
</tr>
<tr>
<td>Tet(50)</td>
<td>CTc</td>
<td>aTc</td>
<td>210 ± 52</td>
<td>128</td>
<td>8</td>
<td>64 [4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aCTc</td>
<td>86 ± 17</td>
<td>128</td>
<td>32</td>
<td>64 [4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-IA-Tc</td>
<td>94 ± 16</td>
<td>128</td>
<td>128</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9-Br-aTc</td>
<td>60 ± 16</td>
<td>128</td>
<td>16</td>
<td>32 [8]</td>
</tr>
<tr>
<td></td>
<td>Demecycline</td>
<td>aTc</td>
<td>120 ± 25</td>
<td>64</td>
<td>8</td>
<td>16 [2]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aDem</td>
<td>190 ± 47</td>
<td>64</td>
<td>16</td>
<td>32 [4]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>$IC_{50}$ (μM)</th>
<th>Substrate MIC (μg/mL)</th>
<th>Inhibitor MIC (μg/mL)</th>
<th>Substrate + Inhibitor MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>aTc</td>
<td>41 ± 5</td>
<td>32</td>
<td>16</td>
<td>16 [2]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aCTc</td>
<td>1.4 ± 0.2</td>
<td>32</td>
<td>32</td>
<td>16 [4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aDem</td>
<td>1.8 ± 0.4</td>
<td>32</td>
<td>16</td>
<td>16 [2]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-IA-Tc</td>
<td>16 ± 3</td>
<td>32</td>
<td>256</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9-Br-aTc</td>
<td>5.6 ± 0.8</td>
<td>32</td>
<td>128</td>
<td>--</td>
</tr>
<tr>
<td>Tet(X)</td>
<td>CTc</td>
<td>aTc</td>
<td>75 ± 21</td>
<td>32</td>
<td>16</td>
<td>16 [8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aCTc</td>
<td>13 ± 3</td>
<td>32</td>
<td>32</td>
<td>8 [4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-IA-Tc</td>
<td>19 ± 4</td>
<td>32</td>
<td>256</td>
<td>8 [128]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9-Br-aTc</td>
<td>11 ± 3</td>
<td>32</td>
<td>128</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Demecycline</td>
<td>aTc</td>
<td>41 ± 9</td>
<td>8</td>
<td>16</td>
<td>4 [8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aDem</td>
<td>4 ± 1</td>
<td>8</td>
<td>16</td>
<td>4 [1]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>$IC_{50}$ (μM)</th>
<th>Substrate MIC (μg/mL)</th>
<th>Inhibitor MIC (μg/mL)</th>
<th>Substrate + Inhibitor MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>aTc</td>
<td>2.7 ± 0.5</td>
<td>512</td>
<td>64</td>
<td>64 [16]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aCTc</td>
<td>1 ± 0.1</td>
<td>512</td>
<td>128</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aDem</td>
<td>1.4 ± 0.2</td>
<td>512</td>
<td>16</td>
<td>64 [4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-IA-Tc</td>
<td>16 ± 3</td>
<td>512</td>
<td>256</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9-Br-aTc</td>
<td>5 ± 0.8</td>
<td>512</td>
<td>128</td>
<td>--</td>
</tr>
<tr>
<td>Tet(X)_3</td>
<td>CTc</td>
<td>aTc</td>
<td>26 ± 3</td>
<td>256</td>
<td>64</td>
<td>64 [16]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aCTc</td>
<td>2.6 ± 0.4</td>
<td>256</td>
<td>128</td>
<td>64 [16]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-IA-Tc</td>
<td>67 ± 11</td>
<td>256</td>
<td>256</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9-Br-aTc</td>
<td>9 ± 2</td>
<td>256</td>
<td>128</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Demecycline</td>
<td>aTc</td>
<td>7 ± 1</td>
<td>64</td>
<td>64</td>
<td>32 [16]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aDem</td>
<td>2.4 ± 0.3</td>
<td>64</td>
<td>16</td>
<td>32 [4]</td>
</tr>
</tbody>
</table>

**Table 5.2:** Unabridged aTc analog inhibition of whole cell *E. coli* expressing tetracycline deconstructase enzymes. a, aTc analog inhibition of whole cell *E. coli* expressing Tet(50). b, aTc analog inhibition of whole cell *E. coli* expressing Tet(X). c, aTc analog inhibition of whole cell *E. coli* expressing Tet(X)_3
Figure 5.1: Tetracycline development and parallel emergence of resistance mechanisms.
Figure 5. 2: Introduction to the tetracycline destructase family of FMO enzymes and structure of the first inhibitor, anhydrotetracycline (5). (A) Phylogenetic tree [aligned with Clustal Omega and viewed using iTOL software]. (B) X-ray crystal structure of chlortetracycline bound to Tet(X) (PDB ID 2y6r). (C) X-ray crystal structure of chlortetracycline bound to Tet(50) (PDB ID 5tui).

Figure 5. 3: Michaelis–Menten kinetics of tetracycline destructase degradation of first generation tetracyclines. (A) Representative Michaelis–Menten plot of tetracycline destructase degradation of oxytetracycline. (B) Representative optical absorbance kinetic plots for the degradation of oxytetracycline by tetracycline destructase enzymes [as observed at 400 nm for Tet(50) and Tet(X)_3; 380 nm for Tet(X)]. (C) Apparent $K_m$, $k_{app}$, and catalytic efficiencies for the tetracycline destructase-mediated degradation of tetracycline, chlortetracycline, demeclocycline, and oxytetracycline. Error bars represent standard deviation for two independent trials.
Figure 5.4: *In vitro* aTC inhibition of tetracycline destructase degradation of first-generation tetracycline antibiotics as observed via an optical absorbance kinetic assay. (A) aTc inhibition of Tet(50) degradation of tetracyclines. (B) aTc inhibition of Tet(X) degradation of tetracyclines. (C) aTc inhibition of Tet(X)_3 degradation of tetracyclines. (D) Apparent IC$_{50}$ for aTC inhibition (denoted for each substrate and enzyme). Error bars represent standard deviation for three independent trials. All data points possess error bars, though some are not visible at the plotted scale.
Figure 5.5: Lineweaver-Burk Plots for the *in vitro* inhibition of the tetracycline deconstructase-mediated degradation of tetracycline by anhydrotetracycline

Reaction samples were prepared in TAPS buffer (100 mM, pH 8.5) with 504 μM NADPH, varying concentrations of tetracycline substrate (typically, 0–40 μM), a set amount of inhibitor (varied by enzyme, usually 0 μM to twice IC50), and 0.4 μM enzyme. After the addition of enzyme, the reactions (in duplicate or triplicate) were mixed, manually by pipette, and the reaction was monitored continuously in a single frame by UV-Visible spectroscopy (absorbance at 380 nm, Carey UV-Visible spectrophotometer) for 3–4 minutes. Initial enzyme velocities were determined by linear regression using Agilent Cary WinUV Software over the linear range of the reaction, and the inverse of the velocities were plotted against the inverse of the substrate concentration and fitted to a line (linear regression) using GraphPad Prism 6. Extrapolation of each individual line (for set inhibitor concentrations) allowed for the visualization of line convergence.

a, Lineweaver-Burk plot for αTc inhibition of Tet(50) degradation of tetracycline.

b, Lineweaver-Burk plot for αTc inhibition of Tet(X) degradation of tetracycline.

c, Lineweaver-Burk plot for αTc inhibition of Tet(X)_3 degradation of tetracycline.
Figure 5.6: In vitro inhibition of tetracycline destructase degradation of first-generation tetracycline antibiotics as observed via optical absorbance kinetic assay. Inhibitory activity of aTC library against (A) Tet(50) degradation of tetracycline, (B) Tet(X) degradation of tetracycline, (C) Tet(X)_3 degradation of tetracycline, (D) Tet(50) degradation of chlortetracycline, (E) Tet(X) degradation of chlortetracycline, and (F) Tet(X)_3 degradation of chlortetracycline. Error bars represent standard deviation for three independent trials. All data points possess error bars, though some are not visible at the plotted scale.
Figure 5.7: (A) Whole cell inhibition of *E. coli* expressing tetracycline destructase enzymes including calculated FICI and observed fold change enhancements. (B) Working model of the inhibition of tetracycline destructase enzymes by aTC-like small molecules (competitive inhibitor vs sacrificial substrate).
Figure 5.8: Tet(X)-mediated degradation of aTC and aTC analogues. (A–F) Each panel depicts the degradation of the denoted aTC as observed via optical absorbance spectroscopy and plots of monitored extracted mass counts from LCMS. Each represents a reaction containing purified Tet(X) enzyme (or none, in the case of the control), aTc analogue, and an NADPH regenerating system [including MgCl₂]. The plots represent extracted ion counts normalized to an internal standard (Fmoc-alanine) and depicted as a percent of the total ion count [aTC+aTC−OH].
Figure 5.9: Tet(X)-mediated degradation of oxytetracycline as a model for the degradation of aTC-like sacrificial substrates.
Figure 5. 10: aDem inhibition of tetracycline deuctase-mediated degradation of first generation tetracyclines. (A) aDem inhibition of tetracycline deuctase degradation of tetracycline. (B) aDem inhibition of tetracycline deuctase degradation of demeclocycline. (C) Apparent IC$_{50}$ for the in vitro inhibition of tetracycline and demeclocycline degradation by tetracycline deuctase enzymes with aTC and aDem. (D) Michaelis–Menten plot of dose-dependent aDem acceleration of Tet(50) consumption of NADPH, apparent $K_m$, and calculated catalytic efficiency. Error bars represent standard deviation for two to three independent trials. All data points possess error bars, though some are not visible at the plotted scale.
Reactions (in duplicate) were prepared in 100 mM TAPS buffer (pH 8.5) with an NADPH regenerating system (40 mM glucose-6-phosphate, 4 mM NADP, 1 mM MgCl₂, 4 U/mL glucose-6-phosphate dehydrogenase), 28.0 μM aDem, and 0.24 μM enzyme. Reaction progress was monitored by UV-Visible spectroscopy (280–550 nm, 1 nm and 30 min intervals) over 3.0 hours, where 150 μL of reaction sample were removed at 30-minute intervals and quenched with 600 μL volumes of quench solution (1:1 acetonitrile:0.25M aqueous HCl). The quenched samples were centrifuged (5000 rpm, room temperature) for 5 minutes, and 600 μL of supernatant was transferred to an LCMS-compatible vial containing Fmoc-alanine internal standard (2.21 μM final concentration) and analyzed by LC-MS (reverse-phase HPLC, C18-silica, gradient 0–95% CH₃CN/H₂O, 0.5 mL/min flow rate). Substrate masses [M+H]+ and hydroxylated product masses [M–OH+H]+ were extracted from the crude mass chromatogram and plotted as a function of time.

a, NADPH-aDem, No enzyme control
b, Tet(50)-NADPH, No aDem control
c, Tet(50)-NADPH-aDem reaction
d, Tet(50)-NADPH-aDem Reaction, Plot of extracted masses over time
Figure 5.12: (A) X-ray crystal structure of aTC bound to Tet(50) [PDB ID: 5TUF] and corresponding binding mode identifier; (B) time- and aDem-dependent degradation of NADPH by Tet(50) observed using a broad-scan optical absorbance kinetic assay; (C) hydrogen peroxide colorimetric detection experiments, from left to right: NADPH (no enzyme) control, no enzyme control (NADPH + aDem), Tet(50) + NADPH [no aDem] reaction mixture, and Tet(50) + NADPH + aDem reaction mixture.
Figure 5.13: Broad-scan detection of aDem- and Tet-promoted consumption of NADPH by Tet(50). a, NADPH control: no enzyme, no substrate. b, aDem-NADPH, no enzyme control. c, Tet-NADPH, no enzyme control. d, Tet(50)-NADPH, no substrate control. e, Tet(50)-Tet-NADPH reaction. f, Tet(50)-aDem-NADPH reaction.
Figure 5. 14: Qualitative detection of aDem- and Tet-promoted hydrogen peroxide formation by Tet(50).

As described in the Experimental Section of the manuscript, qualitative colorimetric detection of aDem- and Tet-promoted hydrogen peroxide formation by Tet(50) was performed using an aqueous Pierce Quantitative Peroxide Assay kit (ThermoScientific). Reaction samples were prepared in 100 mM TAPS buffer (pH 8.5) with 252 μM NADPH, 25 μM substrate (either aDem or Tet), and 0.4 μM enzyme. After the addition of enzyme, the reaction was mixed manually by pipette, and the reaction was monitored by UV-Visible spectroscopy (280–550 nm, 1nm and 0.1 min scan intervals) over 8 minutes. At 8 minutes, 100 μL of reaction solution was added to a detection Eppendorf containing 1000 μL of Working Reagent (prepared according to specifications for Pierce Quantitative Peroxide Assay kit). The detection Eppendorf was incubated for 20 minutes at room temperature to result in the observed color changes, shown in the image below.

a, NADPH control: no enzyme, no substrate  
b, aDem-NADPH, no enzyme control

c, Tet-NADPH, no enzyme control  
d, Tet(50)-NADPH, no substrate control

e, Tet(50)-Tet-NADPH reaction  
f, Tet(50)-aDem-NADPH reaction
Figure 5.15: (A) Michaelis–Menten plot and apparent kinetic parameters ($K_m$, $k_{app}$, and catalytic efficiencies) for the Tet(X)-mediated degradation of tigecycline. (B) Inhibitory activity of aTC and chlorinated aTC analogs against Tet(X) degradation of tigecycline. (C) Last generation tetracycline antibiotics tigecycline and eravacycline. Error bars represent standard deviation for three independent trials. All data points possess error bars, though some are not visible at the plotted scale.

Figure 5.16: Toward extended library synthesis of aTC-like inhibitors of tetracycline destructase enzymes. (A) An adjuvant approach to combat enzymatic inactivation of tetracycline antibiotics and summary of preliminary structure–activity information. (B) Surface view of X-ray crystal structure of Tet(X) bound to tigecycline (PDB ID 4a6n) highlights importance of open active site to accommodate bulky D-ring substituents, suggesting further use of glycylcycline antibiotics may drive selective pressure of tetracycline destructase-involved resistance mechanisms.
Figure 5. 17: SDS-Page Gel Image – Purified Tetracycline Destructase Enzymes.
Figure 5.18: Applicable NMR Spectra of (4S,4aS,12aS)-7-chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride (aCTc, 6)
Figure 5.19: Applicable NMR Spectra of (4S,4aS,12aS)-7-chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride (aDem, 7)
Figure 5. 20: Applicable NMR Spectra of (4S,4aS,12aS)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-7-iodo-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride (7-I-aTc, 8)
Figure 5. 21: Applicable NMR Spectra of (4S,4aS,12aS)-9-bromo-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride (9-Br-aTc, 9)
Figure 5.22: LCMS traces for crude and preparative HPLC purified 7-I-aTc, 8.
5.9 References


Chapter 6: The Study of Tetracycline Destructase Evolution and the Application of (Anhydro)tetracycline Analogs as Inhibitors and Diagnostic Agents
6.1 Preface

This chapter was written by Luting Fang (LF) and edited by Timothy Adam Wencewicz (TAW). Dr. Jana L. Markley (JLM) is credited for chemical synthesis of all anhydrotetracycline analogs in this chapter. Funding for this work was provided by National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID-NIH R01-123394) and the WM Keck Postdoctoral Program in Molecular Medicine for supporting JLM with a postdoctoral fellowship.

6.2 Abstract

Tetracycline family antibiotics have been a story of clinical success in the field of antibiotic drugs since the original discovery of the parent tetracycline antibiotic in 1948. However, the emergence of tetracycline destructases, a class of enzymes capable of degrading all known classes of tetracycline antibiotics brings the challenges of resistance to the future application of new tetracycline drug candidates. This chapter reports mechanistic studies related to the FAD cofactor of tetracycline destructases, the evolution of this class of enzymes, and the development of chromogenic tetracycline destructase substrates used in rapid point-of-care diagnostic assays for detecting antibiotic resistance in clinical pathogens. These findings provide a better understanding of tetracycline destructase biochemical mechanisms and provides new chemical probes to accelerate the discovery of new tetracycline antibiotics and tetracycline destructase inhibitors to manage emerging resistance mechanisms.

6.3 Introduction

In the past 70 years, tetracyclines have been one of the most successful classes of antibiotics in agriculture, aquaculture, and the clinic.\textsuperscript{1, 2} In 1948, as the first member in the
tetracycline natural product family, chlortetracycline was isolated from a soil *Streptomyces*. Since then, natural or semisynthetic analogues of chlortetracycline have been widely used for treating bacterial infections due to broad spectrum of antimicrobial activity, low cost, and oral availability. Tetracyclines inhibit bacterial growth in a bacteriostatic manner by interrupting protein synthesis. Specifically, tetracyclines bind to the 30S subunit of ribosome, preventing accommodation of incoming aminoacyl-tRNAs to the acceptor site (A-site), and halting protein biosynthesis (Figure 6.1).4, 5

The intensive use of tetracyclines caused selective expansion of antibiotic resistant strains, resulting in a significant problem of tetracycline resistance.6-8 As a natural secondary metabolite, tetracyclines have existed in nature for millions of years9, and tetracycline resistance is an ancient feature of environmental bacteria10. As for human pathogens, it is typical for tetracycline resistance to be acquired by horizontal gene transfer.11-13 Primary resistance mechanisms include antibiotic efflux, ribosomal protection, or enzymatic degradation3. 8. Tetracyclines can be exported outside of the cells, against the concentration gradient by efflux proteins (Figure 6.2a). First generation tetracyclines such as tetracycline, chlortetracycline, demeclocycline, and oxytetracycline (Figure 6.3, in blue color) are known to be the victims of this efflux mechanism. Second generation tetracyclines such as doxycycline and minocycline (Figure 6.3, in black color) were developed to overcome efflux issue by removal of the hydroxyl group at C6 position, but this class of tetracyclines encountered a new resistance challenge, ribosomal protection proteins (RPPs). RPPs are homologues of elongation factors that bind to the ribosome in a similar manner as native elongation factors to destabilize the interaction between ribosome and second generation of tetracyclines (Figure 6.2b).14. 15 Third generation tetracycline—tigecycline, and fourth generation tetracycline—eravacycline (Figure 6.3, in red
color) were designed to avoid both efflux and ribosome protection resistance due to the presence of a bulky D-ring substituent\textsuperscript{15-17}. However, tigecycline and eravacycline are vulnerable to resistance via enzymatic inactivation (\textbf{Figure 6.2c})\textsuperscript{18-20}. Unlike efflux and ribosomal protection that cannot affect the working concentration of tetracycline antibiotic, enzymatic degradation can permanently inactivate the antibiotic and thus decrease the effective antibiotic concentrations both intracellularly and extracellularly\textsuperscript{21, 22}.

Bacteria can express tetracycline-inactivating enzymes (Tet destructases, TDases) that need to only act once on a given tetracycline molecule to eliminate antibiotic activity\textsuperscript{23}. Studies have demonstrated that TDases are class A flavoprotein monooxygenases (FMOs) that inactive tetracyclines by hydroxyl group transfer\textsuperscript{18, 24, 25} (\textbf{Figure 6.4}). Prior to 2015 only few examples of functional TDases had been reported: Tet34\textsuperscript{26}, Tet37\textsuperscript{27}, and Tet(X)\textsuperscript{28, 29}. This led to the hypothesis that TDase-mediated tetracycline inactivate was an uncommon resistance mechanism. In 2015, 11 more TDases were discovered by functional metagenomic selections from soils screening for tetracycline resistance\textsuperscript{30, 31}. Today, there are as many as 108 TDases that have been discovered from environmental bacteria and human pathogens suggesting this resistance mechanism is more widespread than previously appreciated. The amino acid sequences of these enzymes cluster into two major clades that correspond well with the origin; namely, soil environments or the human gut. We classify TDases from human gut metagenomes as “Type 1” and those isolated from soil are called “Type 2”. A distinct difference between the two types of TDases is that Type 1 TDases only take first and second generation tetracyclines as substrate while Type 2 TDases are able to inactive all known tetracyclines classes including third and fourth generation tetracyclines. TetX was the first discovered Type 1 TDase and has been the representative model enzyme for this type of TDase. The first report of TetX found in human
pathogens occurred in 2013 from urinary tract infections.\textsuperscript{32} As for now, three plasmid-mediated Type 1 TDases are found in multi-drug resistant (MDR) human pathogens that are identified by the CDC as “Urgent Threats”\textsuperscript{33-38} The TDase issue is now brought into people’s attention as an upcoming global crisis and refocused efforts in tetracycline drug development to get ahead of this emerging resistance mechanism threatening recent progress in this antibiotic space.

In order to effectively and rationally overcome antibiotic resistance, we need to conceptually predict where, when, and how resistance will develop. Experimental evolution was used to force selective pressure towards gain-of-function resistance mutations against tigecycline\textsuperscript{39} One particular mutant from this published study caught our attention due to a single-site surface mutation in TetX that caused 5-fold increase of MIC for TetX against tigecycline. Besides tigecycline, this mutant also has increased tolerance against earlier generation of tetracyclines such as tetracycline, doxycycline, and minocycline. This study revealed to us a possibility that existing genes can acquire expanded tolerance to cause antibiotic resistance. We are inspired by this finding and desired to understand the evolutionary process of the TDases to predict the next generation of TDase and come up with a solution before it become a global health issue. In this chapter, we investigate the biochemical properties imparted by point mutations in TDases using \textit{in vitro} enzyme kinetic experiments. We also report our attempts to develop chromogenic substrates for rapid and selective diagnostic applications.

\section*{6.4 Results and Discussion}

\textit{TDases are FAD dependent enzymes}

The TDases are class A Flavin-containing monooxygenases (FMOs), which are a diverse class of flavoenzymes that perform a variety of oxygen-transfer and oxygen-insertion reactions\textsuperscript{40-44}.\textsuperscript{337}
This reaction requires molecular oxygen, an NADPH cosubstrate, and a flavin adenine dinucleotide (FAD) cofactor (Figure 6.5). The fully oxidized form of FAD (FAD$^{\text{ox}}$) can accept as many as two electrons to form the fully reduced form of FAD (FADH$_2$ or FAD$^{\text{red}}$) (Figure 6.6). The proposed TDase-catalyzed reaction is shown in Figure 6.7 for the case of tetracycline substrates. All known TDases, represented by the prototypical TetX Type 1 enzymes, which were first discovered independently by Saylers, Levy, and Guiney from 1986–1988, and later characterized biochemically by Gerry Wright’s lab, possess the FAD-binding and substrate-binding domains.

We tested the TDases dependence on FAD by monitoring the absorbance at 450 nm, which is the signature absorbance wavelength for the FAD$^{\text{ox}}$ cofactor. TetX was used as a representative of TDases for this study. Optical absorbance was monitored at 450 nm immediately after TetX was mixed with substrate (Figure 6.8). The dramatic decrease of the absorbance at 450 nm, especially between 1min and 2.5min is shown in Figure 6.8a. No dramatic decrease was observed in the no-enzyme control (Figure 6.8b), confirming that the reduction of FAD was caused by TetX enzyme in the Figure 6.8a assay. Similarly, no dramatic decrease was observed in the no-NADPH control (Figure 6.8c), indicating the reduction of FAD was caused by NADPH in the Figure 6.8a assay. This result also confirmed that TetX is a FAD-containing enzyme. Noticeably, a decrease in absorbance at 450 nm can only be observed if the buffer is pre-saturated with argon to remove dissolved oxygen. There is no obvious signal change if the buffer is not pre-treated (Figure 6.8d). We believe that under non-argon-pretreated conditions, NADPH reduces FAD$^{\text{ox}}$, but the product FAD$^{\text{red}}$ gets oxidized back into FAD$^{\text{ox}}$ by oxygen that was dissolved in solution. The absorbance signal at 450 nm remains constant.
(Figure 6.6) indicating rapid equilibration between $\text{FAD}^\text{ox}$ and $\text{FAD}^\text{red}$. A rapid quench experimental set-up would be required to measure kinetics of this equilibration event.

**Oxygen is required for TDases degradation of anhydrotetracyclines**

As mentioned in Chapter 5, anhydrotetracyclines (aTcs) have the ability to inhibit TDases and rescue tetracyclines from being degraded. However, type 1 TDases are able to slowly oxidize aTcs (Figure 6.9), which causes resistance towards combinations of tetracyclines and aTcs. In Chapter 5, we reported that halogen modified aTcs have a higher stability against type 1 TDases such as TetX (Figure 5.8). Among these analogs, aDem shows the lowest degradation level against TetX with only 1.4% oxidized product being detected. However, we found an increase in oxidation of aDem when extra oxygen was introduced into the system, where 14 times more product (19%) was detected than in the normal assay (1.4%) (Figure 6.10e). Pure oxygen was bubbled into the TAPS buffer solution for 30 min to make the solution oxygen saturated before the addition of TetX and aDem. The TetX degradation of aDem reaction was conducted at room temperature for 2 hours, followed by LC-MS analysis. The peak at m/z 463 indicated the oxidized product (Figure 6.10c) while no corresponding peak was detected for no TetX enzyme control (Figure 6.10d). The absence of oxidized product in the no-enzyme control ruled out the possibility that aDem gets oxidized by external oxygen gas and confirmed that the oxidation reaction is catalyzed by TetX enzyme.

Similarly, IaTc showed a dramatic increase in oxidized product formation when extra oxygen was introduced into the system (Figure 6.11), which confirmed a universal phenomenon that the external addition of oxygen gas is the factor for increasing the degradation of aTcs by TDases. The possibility that IaTc gets oxidized by $\text{H}_2\text{O}_2$ generated from uncoupled TetX reductase.
chemistry was ruled out by the addition of IaTc into filtered TetX, NADPH, and oxygen reaction product (TetX enzyme was filtered out), where oxidized IaTc product was not detected. Noticeably, the stage of oxygen introduction into the system is crucial. Specifically, when oxygen is bubbled into the solution after addition of enzyme TetX, the oxidized product was not detected. We believed that the oxidized products were not generated since the pure oxygen poisoned the enzyme, making it inactive.

The impact of oxygen in the oxidized product formation is within expectation. It is well known that oxygen is one of the substrates for TetX\textsuperscript{24}. However, the degree of the impact is beyond our expectations. This finding provides a way to obtain oxidized aTcs products with high yield, and simplify the process of purification, and thus benefits the characterization of oxidized products. Further studies such as testing the kinetic parameters of oxygen as substrate for TetX, or oxygen impact across different types of TDases can be crucial for the understanding of TDases reaction mechanism and can help new aTcs development that increases stability against TDases oxidation.

**Allosteric effects on the active site of TDases**

The major structural difference between Type 1 and Type 2 TDases is the presence of an additional C-terminal helix that we call the ‘gate-keeper helix’ in Type 2 TDases\textsuperscript{31, 50} (see Chapter 5, **Figure 5.2**). First and second generation tetracyclines can easily enter and fit into the active site of both TDases. For third and fourth generation tetracyclines, the bulky side chain that attached to the C-9 position in the D ring gets blocked by the gate keeper helix of Type 2 TDases\textsuperscript{51, 52}, and thus cannot be degraded by TDases. However, they can still enter the active site of Type 1 TDases subjecting themselves to degradation. Tigecycline is one of the third
generation tetracyclines. As one of the very few antibiotics that is still effective against rapidly emerging multidrug-resistant pathogens, including Gram-positive and Gram-negative, tigecycline becomes increasingly crucial in infectious disease treatment.\textsuperscript{53} The understanding of Type 1 TDases degradation of tigecycline is thus important to prevent the global tigecycline resistance before it becomes an issue.

We are inspired by a single-site mutation study on TetX that causes 5-fold increase in MIC against tigecycline from Andersson’s work in 2016\textsuperscript{39}. We constructed the same mutant enzyme TetX\textsubscript{T280A} as the paper used by directed single-site mutagenesis and conducted \textit{in vitro} assay to understand the mechanism of this mutant’s impact on the enzyme’s behavior. The absorbance at 380 nm was used to monitor the rate of the reaction based on consumption of tetracyclines. Mutated TetX (TetX\textsubscript{T280A}) showed a faster reaction rate than wild type TetX (Figure 6.12). Michaelis-Menten steady state kinetic studies were used to evaluate the apparent catalytic efficiency of the enzymes. For both tetracycline and tigecycline substrates (Figure 6.14 and Table 6.1), relative to wild-type TetX the TetX\textsubscript{T280A} mutant has a lower \(K_m\), indicating a higher affinity to substrate, and a higher \(k_{cat}\), indicating faster reaction rate. Overall, TetX\textsubscript{T280A} has a 5-6 fold increase in \(k_{cat}/K_m\), a parameter used to evaluate enzyme catalytic efficiency, for the tetracycline substrates while the catalytic efficiencies for NADPH do not change much compared to wild type. This result supports the idea that the single-site mutation of T280A on the surface of TetX has an allosteric effect on the tetracyclines binding active site based on the fact that the binding affinity for tetracyclines is affected but not NADPH. IC\textsubscript{50} was also used for the study of the mechanism of the mutation influence on TetX. The IC\textsubscript{50} of tetracycline and aTc combo is 6-times lower for TetX\textsubscript{T280A} than wild type TetX (Figure 6.16 and Table 6.2), indicating the aTc has better inhibition on TetX\textsubscript{T280A}. This result showed that
as a trade-off of high rate of degradation of tetracyclines, the TetX_T280A is more vulnerable to aTc. Based on the fact that aTc also binds in the tetracycline binding site of TetX, this result confirmed the allosteric effect on the tetracyclines binding active site caused by the mutation on the surface of TetX. On the other hand, the IC₅₀ of tigecycline and aTc combo is comparable with very few differences between both of the enzymes.

TetX7 is a newly found Type 1 TDase identified in a clinical isolate of human pathogenic P. aeruginosa. TetX7 can degrade third and fourth generation tetracyclines significantly faster than TetX. We did the same site mutation on TetX7, named TetX7_V280A (sequence alignment with TetX at mutation site see Figure 6.17), and studied the in vitro properties of this mutated enzyme compared with the wild type. Mutated TetX7 (TetX7_V280A) showed a similar improvement in efficiency for tetracycline—2.8-fold over wild type enzyme, but has the same efficiency for tigecycline compared to wild type enzyme (Figure 6.19 and Table 6.3). Similar to our observations for TetX, the mutant variant of TetX7 has the same efficiency level towards NADPH as the wild type enzyme. TetX7_V280A has the same IC₅₀ for tigecycline and aTc combo as the wild type enzyme (Figure 6.21 and Table 6.4), which is within expectation since the mutation does not have an impact on tigecycline binding and reacting efficiency. However, TetX7_V280A has a higher IC₅₀ for tetracycline and aTc combo, indicating the mutated enzyme is more tolerant against aTc, which also confirmed the surface mutation has an allosteric effect on the tetracyclines binding active site.

Tet 50 was used as a model enzyme for the Type 2 TDases. The analogous surface-site-mutation made for TetX_T280A was introduced to Tet50 in a construct named Tet50_R280A (Figure 6.22). In vitro enzyme kinetic properties were studied on this mutated enzyme under steady-state Michaelis-Menten conditions. The mutated Tet50 (Tet50_R280A) was found to
have 4-fold higher efficiency for tetracycline relative to wild type Tet50 (Figure 6.24 and Table 6.5), while the efficiency for NADPH is about the same. Same as wild type Tet50, Tet50_R280A doesn’t take Tig as substrate consistent with the known substrate scope of all Type 2 TDases. The IC\textsubscript{50} was slightly lower for the mutant enzyme compared to the wild type (less than 2-fold) (Figure 6.36 and Table 6.6).

Taking all three TDases (TetX, TetX7, and Tet50) into comparison, the overall trend is that the enzyme performance is improved for tetracycline by the single-site surface mutation. However, for tigecycline, although mutated TetX has increased potency, mutated TetX7 does not change. The mutation has different impacts on enzyme efficiency in different enzyme or substrate cases, but it increases the enzyme performance in general. On the other hand, the impact of the mutation on NADPH binding or reaction rate is negligible. This led to our hypothesis that the surface mutation has an allosteric effect on tetracycline substrates binding to the active site. The rescue of tigecycline by aTc keeps the same as wild type for both TetX and TetX7, even though TetX showed an increase in degrading tigecycline. Interestingly, the rescue of tetracycline shows the opposite impact for TetX and TetX7. Mutated TetX is more sensitive to aTc, which makes aTc a better adjuvant for tetracycline against TetX, but mutated TetX7 is more tolerant to aTc, making aTc a less effective adjuvant for TetX7.

The X-ray crystal structure is crucial for confirming the allosteric effect by comparing the active site side chain difference between wild type and mutated enzyme. The crystal structure of TetX\_T280A (PDB: 3V3N) and wild type TetX (PDB: 4A99) using minocycline as a substrate are available in protein data bank (PDB). An alignment of the two structures was performed using PyMOL (Figure 6.25a). The structures overlay nicely, but the apparent binding mode of the substrate is a little off between these two proteins. More investigation is needed to look into
the side chain difference between the active site of the two structures. Follow up mechanistic studies are required to untangle the complex nature of TDase evolution.

**New members of the TDase family**

Based on the sequence similarity, our collaborators in the Dantas lab at Washington University School of Medicine identified a new crop of putative TDases from an in-house comparative genomics database (**Figure 6.26**). Specifically, the genes 4_WP_094089728.1 (Tet#4) and 5_WP_044012519.1 (Tet#5) show high similarity to well-known Tet56, gene 13_WP_064970078.1 (Tet#13) is a homologue of validated TDase TE_0402_1789, and gene 1_WP_07516833.1 (Tet#1) is a homologue of validated TDase TE_6F_7. Michaelis-Menten steady-state kinetics were used to evaluate the apparent catalytic efficiency of these new enzymes (**Figure 6.27** and **Table 6.7**). The catalytic efficiency of Tet#1 and Tet#13 falls into the range of other known TDases, especially Type 1 TDases. However, the efficiency of Tet#5 and Tet#4 are much higher than known TDases, about 40-fold and 15-fold better than TetX, respectively. Tet#4 also showed 40-fold more efficient to degrade Cl-tetracycline than TetX, but not much difference in NADPH kinetics (**Figure 6.28** and **Table 6.8**). We think the high efficiency for Tet#4 comes from the evolution/mutation from the already known TDases, instead of being a totally new enzyme species based on the high percentage sequence similarity to the known TDases. The study of more sequence diverse TDases will provide a way for better understanding the evolution and sequence-structure-function space of TDases, and thus predict and possibly prevent potential super TDases from emerging and propagating through the proactive development of adjuvants and inhibitors.

**Tetracycline analogues as diagnostic coloring agents**
Based on the innate color changing property of tetracyclines triggered by enzymatic oxidation by TDases we aimed to leverage this chromogenic effect to develop color-changing diagnostic agents for TDase expression. Depending on the substrate identity, TDase homolog, and product mixture, the oxidation of tetracycline antibiotics by TDases generates a range of different colors (Figure 6.29). We hypothesize that tetracycline analogues can be developed as diagnostic color-changing agents to functionally identify TDase expression in clinical isolates grown in hospital microbiology laboratories. With enough information the color result could provide finer details about the TDase including subfamily (Type 1 or 2), substrate scope, and effectiveness of inhibitors. This could aid in informing the proper prescription of tetracycline antibiotics and adjuvants. However, the range of colors and extinction coefficients of colored TDase products is limited. We hypothesized that introducing more aromatic conjugation via D-ring substitutions might improve the color range and extinction coefficients of TDase degradation products. Dr. Jana Markley (postdoc, Wencewicz lab) designed 3 initial compounds (Figure 6.30), derivatives of doxycycline, that have the potential of showing novel color for products based on the conjugated structure between the doxycycline conjugated pi-system and the amide-linked aromatic side-chain introduced to the D ring. The choice of making modifications on the D ring is because earlier studies have shown that the D ring has the most potential for modifications which allow for the synthesis of new tetracycline analogues. Michaelis-Menten steady-state kinetics were used to evaluate these D-ring substituted doxycyclines as substrates feature for these three compounds. With the exception of compound 6, which is not a very good substrate for TetX within our detection time window, all three compounds are substrates for both TetX and TetX7 (Figure 6.31 and Table 6.9). No obvious color changes are detected within an hour for all substrate and enzyme combinations. However,
the color changes are obvious after letting the reaction go overnight (Figure 6.32), and the product has a novel color compared to known tetracyclines such as tetracycline (Figure 6.33). UV-Vis scan also shows a different maximum absorbance wavelength (~570 nm for compound 2, ~575 nm for compound 5 and 6) compared to tetracycline (~530 nm) (Figure 6.34). We cannot distinguish the color difference of products derived from TetX and TetX7, which might indicated that TetX and TetX7 produce the same oxidation product. A better understanding of TetX and TetX7 reaction mechanism and the feature of degradation products are needed for an optimized design of new tetracycline analogues in this diagnostic color detection application.

6.5 Outlook and Conclusions

Since the first case of enzymatic degradation of tetracyclines was found in the clinic, people have been working on fighting against tetracyclines resistance. As adjuvants, aTcs are developed to rescue tetracyclines from TDases, but Type 1 TDases are tolerant to aTcs by slowly oxidizing the adjuvant. We found that external addition of oxygen gas can accelerate the progress. This finding provides us a way to obtain more oxidized product for the mechanistic studies for TDase oxidation aTcs. This leads us to a direction for the design of stable aTc against TDases by blocking the site on the structure of the molecule that are oxidized by the enzyme. Another approach people are focusing on to overcome tetracyclines resistance is by studying the evolution of TDases. As we known, evolution always comes from mutation. We found that a single-site mutation on the surface of TetX caused an increase in the enzyme activity. In vitro kinetic studies provided hints that this improvement was caused by an allosteric effect on the tetracycline substrate binding site. Along with directed evolution, sequence analysis in comparative genomic databases provided us four new interesting TDase homologs, one of which
showed an outstanding catalytic efficiency for oxidizing tetracyclines. Based on the high sequence similarity to Tet56, we believe this new TDase is actually an evolutionary progeny of Tet56. We are currently undergoing experiments to determine the reason for the high activity of this enzyme. The study of TDase evolution provides a way to understand how resistance was initiated and where the resistance might be headed in the future. With this information we can prepare and devise solutions before the resistance becomes an irreversible global health problem.

Besides overcoming the resistance of tetracyclines, people are also focusing on developing new methods for rapid, accurate and low-cost detection of resistance, including TDase-mediated resistance. Diagnostic coloring agents are common in clinical detection of beta-lactamases, but are nonexistent for tetracyclines. As a first trial, we designed three compounds that showed novel color-changing properties compared to known tetracyclines. Further optimizations are needed to improve color-changing agents to expand the color palette and extinction coefficient of the TDase degradation products in the visible spectrum.

This chapter contains topics related to the evolution, detection, and mechanistic study of TDase-mediated tetracycline inactivation. We demonstrated that flavin is an essential cofactor for all TDases. We reported that addition of external oxygen gas can accelerate the TDase reaction. We investigated the evolution/mutation of TDases at the biochemical level. We also widened the application of tetracycline analogs into diagnostic coloring agents. As a whole, this chapter opens up our mind and provides us with various possible directions to go for tetracyclines research.

### 6.6 Materials and Methods

*Oxygen influence on TDases degradation of anhydrotetracyclines*
The degradation level of aTC and aTC analogues by Tet(X) was monitored by LC-MS detection (Agilent 6130 single quadrupole instrument with G1313 autosampler, G1315 diode array detector, and 1200 series solvent module and separated using a Phenomenex Gemini C18 column, 50 × 2 mm (5 μm) with guard column cassette and a linear gradient of 0% acetonitrile and 0.1% formic acid to 95% acetonitrile and 0.1% formic acid over 20 min at a flow rate of 0.5 mL/min before analysis by electrospray ionization (ESI+)). Reactions were prepared in 100 mM TAPS buffer (pH 8.5) (degassed with oxygen) with an NADPH regenerating system (40 mM glucose-6-phosphate, 4 mM NADP+, 1 mM MgCl₂, 4 U/mL glucose-6-phosphate dehydrogenase), 28.0 μM substrate (aTC or corresponding analogue), 0.26 μM FAD, and 0.24 μM enzyme. Reaction was carried out at room temperature for 1.5 h, followed by being quenched with quench solution (1:1 acetonitrile/0.25 M aqueous HCl). The quenched samples were centrifuged (5000 rpm, room temperature) for 5 min, and 600 μL of supernatant was transferred to an LCMS-compatible vial containing Fmoc-alanine internal standard (2.21 μM final concentration) and analyzed by LCMS (reverse-phase HPLC, C18-silica, gradient 0−95% CH₃CN/H₂O, 0.5 mL/min flow rate). Substrate masses [M + H]⁺ and hydroxylated product masses [M−OH +H]⁺ were extracted from the crude mass chromatogram. “No enzyme” controls were performed for each aTC analogue screened and showed no significant nonenzymatic degradation over the course of the observable reaction.

**FAD reduction by TetX**

The reduction of FAD by Tet(X) was monitored by optical absorbance spectroscopy (Carey UV–visible spectrophotometer). Reactions were prepared in 100 mM TAPS buffer (pH 8.5) (degassed with argon) with 33 μM enzyme and 2.2mM NADPH. Reaction progress was monitored by optical absorbance spectroscopy (400–550 nm, 1 nm and 0.5 min intervals) over 5
min. “No enzyme” controls and “no NADPH” control were performed under the same condition over the course of the observable reaction. The signal change at 450 nm was used as an indicator for FAD reduction.

**Cloning, Expression, and Purification of Tetracycline-Destructase Enzymes.**

All genes corresponding to the tetracycline destructases used in this report were cloned into pET28b(+) vectors (Novagen) as previously described (BamHI and NdeI restriction sites) and transformed into *E.coli* BL21-Star (DE3) competent cells (Life Technologies). Cells were cultured at 37 °C in lysogeny broth (LB) containing kanamycin (0.03 mg/mL); once the uninduced culture reached an OD<sub>600</sub> of 0.6, the cells were cooled to 0 °C, induced with 1 mM IPTG, and allowed to grow at 15 °C for 12–15 h (harvest OD<sub>600</sub> varied by tetracycline destructase expressed, but on average, harvest OD<sub>600</sub> < 4.5 resulted in greater isolated enzyme yield). To harvest, the induced cells were pelleted by centrifugation at 4000 rpm for 15 min (4 °C) and resuspended in cold 40 mL of lysis buffer (50 mM K<sub>2</sub>HPO<sub>4</sub>, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 5 mM 2-mercaptoethanol, pH 8.0) containing SIGMAFAST protease inhibitor. The cells were transferred to falcon tubes, flash frozen in liquid nitrogen, and stored at −80 °C. To harvest, the cells were thawed and mechanically lysed using an Avestin EmulsiFlex-C5 cell disruptor, and the resultant lysate was centrifuged at 45 000 rpm for 35 min. The supernatant was transferred to a column containing prewashed Ni-NTA resin and incubated for 30–45 min; at which point, the resin was washed with lysis buffer (2 × 40 mL), and the protein was eluted from the resin with fractions of elution buffer (5 × 10 mL, 50 mM K<sub>2</sub>HPO<sub>4</sub>, 500 mM NaCl, 5 mM β-mercaptoethanol, 300 mM imidazole, 10% glycerol, pH 8.0). The fractions were combined in 10 000 molecular weight cutoff (MWCO) Snakeskin dialysis tubing (ThermoScientific) and soaked in buffer (50 mM K<sub>2</sub>HPO<sub>4</sub> pH 8.0, 150 mM NaCl, 1 mM
dithiothreitol (DTT)) overnight to minimize imidazole concentration. To isolate the desired protein, the dialyzed solution was concentrated using a 30 000 MWCO Amicon centrifugal filter (Millipore-Sigma), and concentrated protein solution was flash frozen in liquid nitrogen (50 μL portions) and stored at −80 °C.

**Michaelis-Menten kinetics**

Kinetic characterization of tetracyclines inactivation were achieved in a manner similar to previously reported procedures in Chapter 5. In brief, reaction samples were prepared in [tris(hydroxymethyl)methylamino] propanesulfonic acid (TAPS) buffer (100 mM, pH 8.5) with 504 μM NADPH, 5.04 mM MgCl₂, varying concentrations of tetracycline substrate (typically, 0–40 μM), and 0.4 μM enzyme. After the addition of enzyme, the reactions (in duplicate or triplicate) were mixed, manually by pipet, and the reaction was monitored continuously in a single frame by optical absorbance spectroscopy (absorbance at 380 nm, Carey UV–visible spectrophotometer) for 3–4 min. Initial enzyme velocities were determined by linear regression using Agilent Cary WinUV Software over the linear range of the reaction, and the velocities were fitted to the Michaelis–Menten equation using GraphPad Prism 6.

**IC₅₀**

Half-maximal inhibitory concentrations (IC₅₀) were determined from the nonlinear regression analysis of initial velocities of tetracycline degradation in the presence of varying concentrations of chosen inhibitor. Reaction samples were prepared in 100 mM TAPS buffer (pH 8.5) with 504 μM NADPH, 5.04 mM MgCl₂, 25.3 μM tetracycline substrate, varying concentrations of inhibitor (μM), and 0.4 μM enzyme. After the addition of enzyme, the reactions (in triplicate) were mixed manually by pipet, and the reaction was monitored,
continuously in a single frame, by optical absorbance spectroscopy (absorbance at 380 or 400 nm, Carey UV–visible spectrophotometer) for 4 min. Initial enzyme velocities were determined by linear regression using Agilent Cary WinUV Software over the linear range of the reaction. The velocities were plotted against the logarithm of inhibitor concentration, and IC$_{50}$ values were determined using nonlinear regression analysis in GraphPad Prism 6. Plus/minus error values were determined using linear regression analysis of initial velocities versus concentrations of inhibitor in GraphPad Prism 6. Each set of experiments was accompanied by a variety of controls, including a no-enzyme control (NADPH + Tet + inhibitor)—which was used to simulate full enzyme inhibition and assigned to inhibitor concentration of $1 \times 10^{15}$, and a no-inhibitor control (NADPH + Tet + enzyme)—which was assigned an inhibitor concentration of $1 \times 10^{-15}$. A no-substrate control (NADPH + inhibitor + Tet) was also performed to identify competitive background signals from the enzymatic degradation of the inhibitor itself. For all inhibitor–enzyme combinations, the initial velocities of the no-substrate controls were negligible.

**Full scan of diagnostic coloring agents**

The reaction was prepared in 100mM TAPS buffer (pH 8.5) with 5mM MgCl$_2$, 0.5mM NADPH, 65μM tetracycline analogue compound, and 0.4μM TDase enzyme. Same condition but no enzyme addition was performed as control. Reaction was conducted at room temperature overnight. Full scan of reacted product was measured by optical absorbance spectroscopy (Carey UV–visible spectrophotometer), 280–700 nm, 1 nm intervals.
6.7 Acknowledgments

We thank Dr. John-Stephen Taylor lab for allowing us to use his tank of pure oxygen gas, Dr. Gautam Dantas lab for providing Tet#1, Tet#4, Tet#5, and Tet#13 plasmids, and Dr. Jana Markley for synthesize the aTc analogs. Research was supported by NIH funding.
### 6.8 Figures and Tables

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetX T280A</td>
<td>Tet</td>
<td>$2.202 \pm 0.1983$</td>
<td>$0.3157 \pm 0.009719$</td>
<td>$0.14 \pm 0.01$</td>
</tr>
<tr>
<td></td>
<td>Tig</td>
<td>$1.93 \pm 0.2714$</td>
<td>$0.09053 \pm 0.004047$</td>
<td>$0.047 \pm 0.007$</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>$11.44 \pm 0.9647$</td>
<td>$0.3062 \pm 0.009651$</td>
<td>$0.027 \pm 0.002$</td>
</tr>
<tr>
<td>TetX WT</td>
<td>Tet</td>
<td>$6.363 \pm 0.464$</td>
<td>$0.1442 \pm 0.00345$</td>
<td>$0.023 \pm 0.002$</td>
</tr>
<tr>
<td></td>
<td>Tig</td>
<td>$7.026 \pm 0.4947$</td>
<td>$0.02455 \pm 0.001444$</td>
<td>$0.0035 \pm 0.0003$</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>$8.49 \pm 0.6834$</td>
<td>$0.142 \pm 0.003126$</td>
<td>$0.017 \pm 0.001$</td>
</tr>
</tbody>
</table>

**Table 6.1:** Tabulated Michaelis-Menten parameters for TetX T280A and TetX.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetX T280A</td>
<td>Tet</td>
<td>$7.06 \pm 1.701$</td>
</tr>
<tr>
<td></td>
<td>Tig</td>
<td>$2.269 \pm 0.6336$</td>
</tr>
<tr>
<td>TetX WT</td>
<td>Tet</td>
<td>$40.77 \pm 4.869$</td>
</tr>
<tr>
<td></td>
<td>Tig</td>
<td>$1.451 \pm 0.2562$</td>
</tr>
</tbody>
</table>

**Table 6.2:** Tabulated comparison of inhibition parameters for aTC against TetX T280A and TetX.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetX7_V280A</td>
<td>Tet</td>
<td>2.24±0.2424</td>
<td>0.469±0.01209</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td></td>
<td>Tig</td>
<td>3.54±0.4156</td>
<td>0.2097±0.007358</td>
<td>0.059±0.007</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>6.12±0.5069</td>
<td>0.49±0.01145</td>
<td>0.08±0.007</td>
</tr>
<tr>
<td>TetX7_WT</td>
<td>Tet</td>
<td>11±2.49</td>
<td>0.834±0.08488</td>
<td>0.08±0.02</td>
</tr>
<tr>
<td></td>
<td>Tig</td>
<td>4.64±0.4253</td>
<td>0.3355±0.009909</td>
<td>0.072±0.007</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>6.32±0.7337</td>
<td>0.72±0.02123</td>
<td>0.11±0.01</td>
</tr>
</tbody>
</table>

**Table 6.3**: Tabulated Michaelis-Menten parameters for TetX7_V280A and TetX7.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetX7_V280A</td>
<td>Tet</td>
<td>11.56±1.471</td>
</tr>
<tr>
<td></td>
<td>Tig</td>
<td>1.04±0.1208</td>
</tr>
<tr>
<td>TetX_WT</td>
<td>Tet</td>
<td>2.73±0.4917</td>
</tr>
<tr>
<td></td>
<td>Tig</td>
<td>1.06±0.1078</td>
</tr>
</tbody>
</table>

**Table 6.4**: Tabulated comparison of cTC inhibition parameters for TetX7_V280A and TetX7.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tet50_R280A</td>
<td>Tet</td>
<td>0.7242±0.1211</td>
<td>0.3469±0.02242</td>
<td>0.48±0.09</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>47.22±5.569</td>
<td>0.3284±0.01146</td>
<td>0.0070±0.0009</td>
</tr>
<tr>
<td>Tet50_WT</td>
<td>Tet</td>
<td>4.541±1.28</td>
<td>0.5604±0.05722</td>
<td>0.12±0.04</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>79.27±3.717</td>
<td>0.794±0.01238</td>
<td>0.0100±0.0005</td>
</tr>
</tbody>
</table>

*Table 6.5:* Tabulated comparison of Michaelis-Menten parameters for Tet50_R280A and Tet50.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tet50_R280A</td>
<td>Tet</td>
<td>135.4±44.18</td>
</tr>
<tr>
<td>Tet50_WT</td>
<td></td>
<td>214.1±25.16</td>
</tr>
</tbody>
</table>

*Table 6.6:* Tabulated comparison of aTC inhibition parameters for Tet50_R280A and Tet50.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tet#1</td>
<td>7.567±0.5635</td>
<td>0.2088±0.006008</td>
<td>0.028±0.002</td>
</tr>
<tr>
<td>Tet#4</td>
<td>0.7637±0.04616</td>
<td>0.572±0.01205</td>
<td>0.75±0.05</td>
</tr>
<tr>
<td>Tet#5</td>
<td>3.555±0.2529</td>
<td>1.05±0.02516</td>
<td>0.30±0.02</td>
</tr>
<tr>
<td>Tet#13</td>
<td>5.453±0.2535</td>
<td>0.234±0.003616</td>
<td>0.043±0.002</td>
</tr>
</tbody>
</table>

*Table 6.7:* Michaelis-Menten parameters (Tet substrate) for the 4 enzymes from the tree in Figure 6.26.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (μM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tet</td>
<td>0.7637±0.04616</td>
<td>0.572±0.01205</td>
<td>0.75±0.05</td>
</tr>
<tr>
<td>Cl-Tet</td>
<td>0.6755±0.08442</td>
<td>0.51±0.02072</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>NADPH</td>
<td>17.42±1.115</td>
<td>0.4181±0.007231</td>
<td>0.024±0.002</td>
</tr>
</tbody>
</table>

Table 6.8: Michaelis-Menten parameters for Tet#4.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Compounds</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (μM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetX</td>
<td>#2</td>
<td>2.277±0.5056</td>
<td>0.02282±0.001401</td>
<td>0.010±0.002</td>
</tr>
<tr>
<td></td>
<td>#5</td>
<td>5.661±1.783</td>
<td>0.02353±0.001856</td>
<td>0.004±0.001</td>
</tr>
<tr>
<td>TetX7</td>
<td>#2</td>
<td>7.739±2.428</td>
<td>0.2458±0.03376</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td></td>
<td>#5</td>
<td>2.194±0.9736</td>
<td>0.1216±0.01051</td>
<td>0.06±0.03</td>
</tr>
<tr>
<td></td>
<td>#6</td>
<td>6.089±1.327</td>
<td>0.2964±0.02267</td>
<td>0.05±0.01</td>
</tr>
</tbody>
</table>

Table 6.9: Michaelis-Menten parameters for compounds 2, 5, and 6 (see Figure 6.30) as substrates.
Figure 6.1: Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal subunit to block accommodation of incoming tRNAs.
Figure 6.2: An overview of tetracycline resistance mechanisms. a) Efflux b) ribosomal protection c) enzymatical inactivation.
Figure 6. 4: TDase-catalyzed inactivation tetracycline mechanism via C11as hydroxylation with the involvement of a flavin cofactor activated as the C4a-flavin peroxide.

Figure 6. 5: Chemical structure of flavin adenine dinucleotide (FAD).
Figure 6. 6: Reaction scheme for two-electron reduction of FAD.

Figure 6. 7: Proposed catalytic cycle for TDase-catalyzed oxidation of substrate (tetracycline) highlighting the FAD oxidation states.
Figure 6. 8: Optical absorbance assay monitoring for FAD oxidation state in TetX at 450 nm. a) normal assay with Ar pre-treatment. b) “no enzyme” control with Ar pre-treatment. c) “no NADPH” control with Ar pre-treatment. d) no Ar pre-treatment.

Figure 6. 9: Proposed TDase inactivation of aTcs via C11a hydroxylation.
Addition of external oxygen gas enhances TDase-catalyzed oxidation rates. Chromatograms show LC-MS analysis of the aDem oxidized product from TetX with or without external addition of oxygen gas (2hr). a) normal assay, starting material. b) “no enzyme” control, starting material. c) normal assay, product. d) “no enzyme” control, product. e) summary of a)-d).

<table>
<thead>
<tr>
<th></th>
<th>No extra O_2 gas</th>
<th>Add extra O_2 gas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion count (aDem)</td>
<td>62,000</td>
<td>290,000</td>
</tr>
<tr>
<td>Ion count (aDem +16)</td>
<td>900</td>
<td>70,000</td>
</tr>
<tr>
<td>(aDem +16)%</td>
<td>1.4%</td>
<td>19%</td>
</tr>
</tbody>
</table>

**Figure 6. 10:** Addition of external oxygen gas enhances TDase-catalyzed oxidation rates. Chromatograms show LC-MS analysis of the aDem oxidized product from TetX with or without external addition of oxygen gas (2hr). a) normal assay, starting material. b) “no enzyme” control, starting material. c) normal assay, product. d) “no enzyme” control, product. e) summary of a)-d.)
Figure 6.11: Addition of external oxygen gas enhances TDase-catalyzed oxidation rates. Chromatograms in panels a-d) show LC-MS analysis of the IaTc oxidized product from TetX with or without external addition of oxygen gas (1.5 hr). Panel i) is a summary of the extracted ion counts for indicated conditions.
Figure 6.12: Reaction progress curves for wild-type TetX and mutant TetX_T280A. The UV-Vis scan was taken with continuous monitoring at 380 nm absorbance to measure enzyme reaction speed. (31uM Tetracycline was as substrate for both enzymes.)
Figure 6.13: Michaelis-Menten steady-state kinetic plots for TetX_T280A mutant.

Figure 6.14: Michaelis-Menten steady-state kinetic plots for TetX_T280A mutant compared with TetX wild-type.
**Figure 6. 15:** IC$_{50}$ curves for inhibition of TetX T280A mutant by aTC using tetracycline (left) or tigecycline (right) as substrate.

**Figure 6. 16:** Comparison of aTC IC$_{50}$ plots for TetX T280A and wild-type TetX using tetracycline (left) or tigecycline (right) as substrate.
Figure 6.17: Partial sequence alignment of TetX7 (top) and TetX (bottom).

Figure 6.18: Michaelis-Menten plots for TetX7_V280A using variable tetracycline (left), tigecycline (middle), or NADPH (right, with tetracycline as substrate).

Figure 6.19: Comparison of Michaelis-Menten plots for TetX7_V280A and TetX.
**Figure 6. 20:** IC\textsubscript{50} curves for aTC inhibition of TetX7\_V280A using tetracycline (left) or tigecycline (right) as substrate.

**Figure 6. 21:** Comparison of aTC IC\textsubscript{50} curves for mutant TetX7\_V280A and wild-type TetX7 using tetracycline (left) or tigecycline (right) as substrate.
Figure 6.22: Structural alignment of TetX with Tet50. Green: Tet50 (PDB: 5TUF); Magenta: TetX(2XDO); Yellow: mutation site. Image created using PyMOL ‘align’ function.
Figure 6. 23: Michaelis-Menten plots for Tet50_R280A using variable tetracycline (left) and variable NADPH (right).

Figure 6. 24: Comparison of Michaelis-Menten plots for Tet50_R280A and wild-type Tet50 using variable tetracycline (left) and variable NADPH (right).
Figure 6.25: Structural aligned of wild-type TetX with mutant TetX_T280A. a) Complete cartoon structures (green: TetX, pink: TetX_T280A). b) Zoom in on active sites. Images were created using the PyMOL ‘align’ function. PDB Accession Codes: 3V3N for TetX_T280A; 4A99 for wild-type TetX.
Figure 6. 26: Phylogenetic tree figure showing sequence relationships of potentially new TDases compared to known TDases. (Credit: Dantas lab at Washington University School of Medicine)
Figure 6.27: Michaelis-Menten plots (Tet substrate) for the 4 enzymes picked from the tree in Figure 6.26.
Figure 6. 28: Michaelis-Menten plots for Tet#4 using variable tetracycline (left), chlortetracycline (middle), and NADPH (right; with Tet as substrate).

Figure 6. 29: Color changes observed in a 96-well plate with different combinations of tetracyclines and recombinant TDases enzymes. (Image provided by Alex Kong, summer intern in the Wencewicz lab.)
Figure 6.30: Chromogenic doxycycline derivatives as potential TDase color-changing diagnostic agents. a) compound #2, b) compound #5, c) compound #6.
**Figure 6.31:** Michaelis-Menten plots for compounds 2, 5, and 6 (see Figure 6.30) as substrates for TetX (top row) and TetX7 (bottom row) using tetracycline as substrate.
**Figure 6.32:** Images of cuvettes containing chromogenic doxycycline analogs in the presence of Tdases. Left top corner number: compound. Right top corner: enzyme (X: TetX, 3: TetX7, -: no enz)

**Figure 6.33:** Color change observed using tetracycline as substrate for TDases. Right corner: enzyme (X: TetX, 3: TetX7, -: no enz)
Figure 6.34: Optical absorbance spectra of compounds 2, 5, and 6 (Figure 6.30) before and after reaction with TDases. UV-Vis scans taken from 400-700 nm. Note, Tet3 is the same as TetX7.
Figure 6. 35: IC₅₀ curve for aTC inhibition of Tet50_R280A using tetracycline as substrate.

Figure 6. 36: Comparison of aTC IC₅₀ curves for Tet50_R280A and Tet50 using tetracycline as substrate.
Figure 6. SDS-PAGE analysis of recombinant N-His$_6$-tagged Tet #1 (44kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 6. 38: SDS-PAGE analysis of recombinant N-His$_6$-tagged Tet #4 (44kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
**Figure 6.39**: SDS-PAGE analysis of recombinant N-His$_6$-tagged Tet #5 (44kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 6.40: SDS-PAGE analysis of recombinant N-His$_6$-tagged Tet #13 (44kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
**Figure 6.41:** SDS-PAGE analysis of recombinant N-His$_6$-tagged TetX$_{T280A}$(44kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 6.42: SDS-PAGE analysis of recombinant N-His$_6$-tagged TetX7_V280A (45kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 6.43: SDS-PAGE analysis of recombinant N-His$_6$-tagged Tet50_R280A (46kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
6.9 References


Chapter 7: Fimsbactin and Acinetobactin Compete for the Periplasmic Siderophore Binding Protein BauB in Pathogenic Acinetobacter baumannii
7.1 Preface

This chapter was adapted with permission from an article of the same title published in ACS Chemical Biology by Tabetha Bohac, Luting Fang, Daryl Giblin, and Timothy Wencewicz. This chapter was adapted from an article of the same title (Bohac, T. J.; Fang, L.; Giblin, D. E.; Wencewicz, T. A. “Fimsbactin and acinetobactin compete for the periplasmic siderophore binding protein BauB in pathogenic Acinetobacter baumannii.” ACS Chemical Biology 2019, 14, 674-687) with permission from ACS Publications. Funding for this work was provided by the National Science Foundation through a CAREER Award to TAW (CHE-1654611).

7.2 Abstract

Environmental and pathogenic microbes produce siderophores as small iron-binding molecules to scavenge iron from natural environments. It is common for microbes to produce multiple siderophores to gain a competitive edge in mixed microbial environments. Strains of human pathogenic Acinetobacter baumannii produce up to three siderophores: acinetobactin, baumannoferrin, and fimsbactin. Production of acinetobactin and baumannoferrin is highly conserved among clinical isolates while fimsbactin production appears to be less common. Fimsbactin is structurally related to acinetobactin through the presence of catecholate and phenolate oxazoline metal-binding motifs, and both are derived from nonribosomal peptide assembly lines with similar catalytic domain orientations and identities. Here we report on the chemical, biochemical, and microbiological investigation of fimsbactin and acinetobactin alone and in combination. We show that fimsbactin forms a 1:1 complex with iron(III) that is thermodynamically more stable than the 2:1 acinetobactin ferric complex. Alone, both
acinetobactin and fimsbactin stimulate *A. baumannii* growth, but in combination the two siderophores appear to compete and collectively inhibit bacterial growth. We show that fimsbactin directly competes with acinetobactin for binding the periplasmic siderophore-binding protein BauB suggesting a possible biochemical mechanism for the phenomenon where the buildup of apo-siderophores in the periplasm leads to iron starvation. We propose an updated model for siderophore utilization and competition in *A. baumannii* that frames the molecular, biochemical, and cellular interplay of multiple iron acquisition systems in a multidrug resistant Gram-negative human pathogen.

### 7.3 Introduction

The rise of infections caused by multidrug resistant (MDR) Gram-negative pathogens, including MDR *Acinetobacter baumannii*, is driving the exploration of nontraditional therapeutic strategies including antivirulence therapies.\(^1\)\(^2\) Blocking virulence pathways such as cellular adhesion, protein secretion, biofilm formation, motility, and nutrient acquisition are presumed to apply less selective pressure for resistance and might work synergistically with traditional antibiotics.\(^3\) Targeting nutrient acquisition pathways is an attractive option since a disproportionate percentage of the pathogen’s conditionally essential genome is comprised of genes associated with nutrient scavenging.\(^4\)\(^5\) However, concern over the presence of multiple, seemingly redundant pathways has raised concern as to whether inhibiting one nutrient scavenging pathway is enough to slow pathogen growth *in vivo*.\(^6\) For example, pathogenic strains of *A. baumannii* express several types of protein machinery that contribute to iron acquisition including dedicated pathways for the transport of ferrous (*feoABC*),\(^7\)\(^8\) heme (*hemO* and *hemT* gene clusters),\(^9\) and siderophore (*basA−J, bauA−F, barAB, fbsA−Q, bfnA−L, fhuE, piuA, pirA,*
tonB, and exbBD\textsuperscript{10–14} iron sources.\textsuperscript{15} It remains to be determined if blocking one of these iron acquisition pathways alone will be effective \textit{in vivo} as an antivirulence approach. A more comprehensive understanding of the interplay and conditional dependence between the available iron scavenging pathways is needed to determine the best approach for developing effective antivirulence agents that disrupt said pathways. At least three unique structural families of siderophores (acinetobactin,\textsuperscript{16} fimsbactin,\textsuperscript{11} and baumannoferrin/acinetoferrin\textsuperscript{12,17}) have been detected from clinical isolates of \textit{A. baumannii} and \textit{A. hemolyticus} (\textbf{Figure 7.1a}). Acinetobactin (Acb) is the most studied \textit{A. baumannii} siderophore and is highly conserved in all clinical isolates.\textsuperscript{15} Acinetobactin is produced via a nonribosomal peptide synthetase (NRPS) biosynthetic assembly line (\textbf{Figure 7.2}).\textsuperscript{18,19} Preacinetobactin is the kinetic product released from the NRPS, and Acb is the thermodynamic product resulting from spontaneous isomerization of PreAcb.\textsuperscript{20} Both PreAcb and Acb form stable 2:1 ferric complexes, [Fe(PreAcb)\textsubscript{2}] and [Fe(Acb)\textsubscript{2}], respectively, and stimulate \textit{A. baumannii} growth under iron limiting conditions.\textsuperscript{21,22} Isomerization of PreAcb is slow under acidic conditions (t\textsubscript{1/2} ≈ 16 h, pH 5.5), and chelation of iron(III) prevents isomerization, which has led to speculation that both PreAcb and Acb have a natural role in iron scavenging under acidic infection conditions.\textsuperscript{20}

The fimsbactin siderophores, fimsbactin A–F, were discovered in 2013 and are also derived from an NRPS assembly line resembling the Acb NRPS system (\textbf{Figure 7.2}).\textsuperscript{11,23} Fimsbactin A (Fim) was the primary siderophore in the mixture isolated from \textit{A. baylyi} ADP1 accounting for \textgreater85\% of the total mass (\textbf{Figure 7.1a}). The remaining fimsbactin metabolites, B–F, appear to be shunt biosynthetic products, biosynthetic intermediates, or analogs resulting from the incorporation of \textit{L}-Thr instead of \textit{L}-Ser into the siderophore backbone (\textbf{Figure 7.3}). PreAcb/Acb and Fim are structurally related by the catecholate, phenolate oxazoline, and
hydroxamate metal-binding motifs found in common siderophores such as enterobactin,\textsuperscript{24} vibriobactin,\textsuperscript{25} and heterobactin,\textsuperscript{26} respectively.

Baumannoferrin is the most recently characterized \textit{A. baumannii} siderophore, discovery of which was reported in 2015, and is derived from an NRPS-independent siderophore synthetase (NIS) biosynthetic process.\textsuperscript{12} A related NIS siderophore, acinetoferrin, has been reported from environmental strains and the opportunistic human pathogen \textit{Acinetobacter haemolyticus}.\textsuperscript{17,27} Baumannoferrin is structurally distinct from the NRPS siderophores PreAcb, Acb, and Fim. The structure is composed of citrate, 1,3-diaminopropane, 2,4-diaminobutyrate, decenoic acid, and α-ketoglutarate.\textsuperscript{12} The lipophilic nature of the baumannoferrin decenoic acid side chain has led to the proposal that the siderophore might be membrane associated.\textsuperscript{28} The structure contains one hydroxamate and two α-hydroxy carboxylate metal-binding motifs similar to chelating groups found in other NIS-derived citrate-based siderophores such as aerobactin produced by human pathogens including \textit{Klebsiella pneumonia} and uropathogenic \textit{E. coli} (UPEC).\textsuperscript{29,30}

Biosynthetic operons (\textbf{Figure 7.4}) for baumannoferrin and PreAcb/Acb are present in all genome-sequenced clinical isolates of \textit{A. baumannii} deposited in the NCBI database (taxid: 470).\textsuperscript{15} In contrast, only 4 unique strains (<10\% of sequenced \textit{A. baumannii} strains) contain genes associated with fimsbactin biosynthesis and utilization (\textbf{Figure 7.5}). It appears as though human pathogenic strains of \textit{A. baumannii} always retain the capacity for producing at least two siderophores, PreAcb/Acb and baumannoferrin, or occasionally three siderophores if the Fim biosynthetic operon is present and functional. Since Fim production is optional for pathogenicity, this raises the question as to whether the structural similarity of PreAcb/Acb and Fim is
functionally redundant or if the siderophore pathways contribute cooperatively to the acquisition of iron and other metal ions leading to increased pathogen virulence.

It is common for pathogenic and environmental microbes to produce multiple siderophores either via the presence of multiple biosynthetic gene clusters (as for PreAcb/Acb, Fim, and baumannoferrin in *A. baumannii*), production of shunt/fragment biosynthetic products (as for fimsbactin A–F), precursor-directed biosynthesis (as for fimsbactin incorporation of Thr or Ser), or biosynthetic tailoring reactions (as for acyl-enterobactins). Additionally, pathogens often produce siderophore transport proteins for siderophores produced by neighboring bacteria, so-called xenosiderophores. Pathogenic *A. baumannii* express the protein FhuD to enable the utilization of hydroxamate-based xenosiderophores including desferrioxamine B. Potential advantages of utilizing multiple siderophores include synergistic effects of siderophore combinations on iron acquisition, evasion of immune proteins such as siderocalin, suppression of competing microbial growth, stimulation of cooperative and synergistic microbial growth, expansion of metal uptake beyond iron, quorum sensing and cell signaling, nutritional passivation of necessary but potentially toxic metals, quenching of reactive oxygen species, and induction of metal-dependent cellular responses in the host.

We sought to investigate the interplay of two high-affinity *A. baumannii* siderophore-based iron acquisition systems, the PreAcb/Acb and Fim pathways. Prior to this work, there were no reports on the metal chelating properties of Fim, and growth promotion of *A. baumannii* by Fim had not been demonstrated. Here, we show that Acb or Fim alone stimulates *A. baumannii* growth under iron-deficient conditions. Both siderophores promote growth more efficiently in the holo, iron-bound forms. Surprisingly, we found that apo-Fim is antagonistic toward holo-Acb leading us to hypothesize that Acb and Fim have competing utilization pathways. We recently
reported the X-ray crystal structure of the periplasmic siderophore-binding protein BauB bound to a stable [Fe(Acb)₂]⁻ ferric complex, which provided a template for understanding siderophore molecular recognition in A. baumannii. In this work, we show that both isomers of acinetobactin, PreAcb and Acb, and Fim bind to BauB with nanomolar affinity. Intrinsic fluorescence quenching and direct binding via an immobilized BauB-LC-MS competition experiment validated a meaningful binding interaction between BauB and the apo- and holo-forms of PreAcb, Acb, and Fim. The substrate binding promiscuity of BauB is explained using density functional theory (DFT) calculated structures of the [Fe(PreAcb)₂]⁻ and [Fe(Fim)]⁻ complexes compared to the BauB-[Fe(Acb)₂]⁻ crystal structure. Structure comparison reveals how the three A. baumannii siderophores fulfill the octahedral iron(III) coordination sphere using ligands derived from the common 2,3-DHB-Thr/Ser phenolate oxazoline, catechol, and imidazole/hydroxamate motifs. All three ferric complexes share identical stereochemistry about the ferric metal center with clear overlapping of electron density in the ligand-to-metal coordination sphere. Pathway competition between Acb and Fim might explain in part why it is rare to find clinical isolates of pathogenic A. baumannii harboring biosynthetic operons for both siderophores.

7.4 Results and Discussion

Purification of Acb and Fim from A. baumannii ATCC 17978.

The fimsbactin siderophores were originally isolated from the environmental strain A. baylyi ADP1. Up to this point, Fim had not been isolated from any pathogenic strains of A. baumannii although comparative genomics predicts the presence of the Fim operon in at least 4 unique strains of A. baumannii in the NCBI database (Figure 7.5). One of these strains is human
pathogenic *A. baumannii* ATCC 17978, a clinical isolate that is predicted to be a producer of Acb, Fim, and baumannoferrin. We isolated crude Acb and fimsbactin A−F from *A. baumannii* ATCC 17978 grown in iron-deficient M9 minimal medium at 37 °C. LC-MS analysis of the crude fermentation broths from two separate trials showed the presence of Acb and all of the fimsbactin isomers, fimsbactin A−F, with fimsbactin A providing the largest signal in the optical absorbance and extracted ion chromatogram (EIC) traces (Figure 7.1b; Figures 7.6, 7.7). This validates coproduction of Acb and Fim by a human pathogenic strain of *A. baumannii*. Acb and fimsbactin A were purified via RP-C18 prep-HPLC allowing for isolation of both siderophores from a single fermentation. Acb was isolated in >95% purity by LC-MS at a mass recovery after purification of 31 mg/L averaged over two trials (Figure 7.8). No PreAcb was observed, which is consistent with the instability of PreAcb during extended fermentations at physiological pH and previous failed isolation attempts. However, PreAcb is readily available in our group through total synthesis. Fimsbactin A, hereon referred to as Fim, was isolated as a mixture of fimsbactin A (>90%) and fimsbactins B, C, and F (≤10%) according to LC-MS and NMR analysis. We validated that fimsbactin A is the primary component by HRMS and comparison of the 1D (1H, 13C) NMR spectra against the literature reported values (Table 7.1; Figures 7.9–7.13). The mass recovery of Fim after purification was 5 mg/L averaged over two independent trials. The greater mass production of Acb over Fim is consistent with the observations for siderophore production in *A. baylyi*. We were unable to detect any positively charged ions corresponding to apo- or holo-baumannoferrin in the *A. baumannii* ATCC 17978 supernatants despite the predicted biosynthetic capacity for this siderophore (Figure 7.5). It is possible that the baumannoferrins remain membrane associated or have a low ionization potential in positive mode electrospray ionization (ESI), due to the anionic
carboxylate/hydroxamate metal-chelating groups, thus proving to be undetectable by the low-resolution single quadrupole mass spectrometer employed in this study.\textsuperscript{50} We utilized synthetic PreAcb, natural Acb, and natural Fim (>90% fimsbactin A) for all studies in this work.

**Iron-Binding Properties of Fim.**

Fim is a mixed ligand bicatecholate-monohydroxamate siderophore that is structurally related to other NRPS siderophores including heterobactin\textsuperscript{26} and vibriobactin\textsuperscript{25} (Figure 7.14, 7.15). These related siderophores are hexadentate metal chelators with three bidentate ligands (either catecholates, phenolate oxazolines, or hydroxamates) branching from three arms of a tetrahedral atom, either nitrogen or carbon, creating a tripodal ferric coordination template. Vibriobactin is spermidine-based with a tetrahedral nitrogen at the apex of the tripod, while Fim and heterobactin are amino acid-based using the \(\alpha\)-carbon of L-Ser or L-Lys, respectively, as the tetrahedral branch point for the three metal-chelating ligands.

Fim is composed of hydroxamate, phenolate oxazoline, and catecholate metal-chelating groups derived from N-acetyl-N-hydroxy-cadaverine, serine-2,3-DHB oxazoline, and 2,3-DHB, respectively.\textsuperscript{11} Heterobactin contains hydroxamate, phenolate oxazoline, and catecholate ligands derived from cyclized N-hydroxy-ornithine, 2,5-dihydroxy-6-amino-benzoate, and 2,3-DHB, respectively.\textsuperscript{26} Heterobactin forms a stable 1:1 ferric complex with a ligand-to-metal charge transfer band in the optical absorbance spectra appearing at 520 nm.\textsuperscript{51} For comparison, the well-known 1:1 enterobactin tris-catecholate ferric complex has a characteristic ligand-to-metal charge transfer band at 498 nm.\textsuperscript{52} We sought to investigate the iron binding properties of Fim in comparison to PreAcb and Acb to better understand metal chelation dynamics of the mixed siderophore system in *A. baumannii.*

400
Ferric complexes of PreAcb, Acb, and Fim were prepared by treatment with Fe(acac)$_3$ using a method reported previously by our group.$^{20,22,33,53}$ Titration of Fim with Fe(acac)$_3$ or FeCl$_3$ in MeOH monitored by fluorescence quenching ($\lambda_{\text{excitation}} = 330$ nm; $\lambda_{\text{emission}} = 380$ nm) was consistent with the formation of a stable [Fe(Fim)] ferric complex (Figure 7.16). At 0.5 equiv of Fe(III), the titration curve indicates formation of 2:1 [Fe(Fim)$_2$] complex that converts to a 1:1 [Fe(Fim)] complex once stoichiometric Fe(III) is added. These observations are consistent with previous studies of mixed ligand siderophores, including heterobactin, where ligand-to-metal stoichiometry of ferric complexes varied with metal/ligand concentrations and pH.$^{51}$ LC-MS analysis of [Fe(Fim)] produced an [M + H]$^+$ ion with an observed $m/z = 627$ consistent 1:1 siderophore/iron(III) stoichiometry. Both PreAcb and Acb form 2:1 siderophore/iron complexes, [Fe(PreAcb)$_2$] and [Fe(Acb)$_2$], respectively, that lose iron upon ionization by ESI in positive ion mode to give [M + H]$^+$ ions with $m/z$ 347 corresponding to the expected mass of apo-Acb.$^{20,21}$ This difference in ionization modes reflects the increased relative stability of a 1:1 siderophore/iron complex, [Fe(Fim)], compared to 2:1 siderophore/iron complexes, [Fe(PreAcb)$_2$] and [Fe(Acb)$_2$], toward ESI. The optical absorbance spectrum of [Fe(Fim)] shows a broad ligand-to-metal charge transfer absorbance band at 445 nm (Figure 7.17). [Fe(PreAcb)$_2$] and [Fe(Acb)$_2$] show ligand-to-metal charge transfer absorbance bands at 515 and 570 nm, respectively, in the optical absorbance spectra.$^{20}$ The red spectral shift for [Fe(Fim)] ($\lambda_{\text{max}} = 445$ nm) compared to [Fe(PreAcb)$_2$] ($\lambda_{\text{max}} = 515$ nm) and [Fe(Acb)$_2$] ($\lambda_{\text{max}} = 570$ nm) implies that there are greater interactions between ferric iron and stronger-field ligands in the [Fe(Fim)] complex.$^{52}$

We measured the apparent Fe(III) affinity ($\log K_{\text{Fe}}$) of [Fe(Fim)] to be $27.1 \pm 0.2$ using an EDTA competition assay (Table 7.1, Figure 7.18). The apparent stability of the [Fe(Fim)]
The ferric complex is similar to [Fe(PreAcb)$_2$] (apparent log $K_{Fe} = 27.4 \pm 0.2$) and greater than [Fe(Acb)$_2$] (apparent log $K_{Fe} = 26.2 \pm 0.1$).$^{20}$ We confirmed that apparent $K_{Fe}$ for FimFe was greater than Acb$_2$Fe through a competitive iron exchange assay. Treatment of [Fe(Acb)$_2$] with an equimolar amount of apo-Fim resulted in formation of the [Fe(Fim)] complex as confirmed by optical absorbance spectroscopy (Figure 7.19). The metal exchange was slow, as expected, and complete conversion was achieved after 20 h suggesting that the 1:1 [Fe(Fim)] ferric complex is thermodynamically more stable than the 2:1 [Fe(Acb)$_2$] complex. The relevance of iron exchange between siderophores is unknown, but it could play a role in the overall iron acquisition process through a metal shuttle.$^{54}$ Similar differences in ferric complex stability of siderophores have been reported for mycobactin (more stable; phenolate oxazoline, bis-hydroxamate) and exochelin (less stable; imidazole-containing bis-hydroxamate) from Mycobacterium tuberculosis.$^{55}$ We sought to investigate the potential for siderophore cooperativity between combinations of Fim, PreAcb/Acb, and the corresponding ferric complexes by performing A. baumannii growth promotion studies.

**Fimsbactin A Stimulates A. baumannii Growth under Iron-Deficient Conditions.**

Formation of stable ferric complexes is the first requirement for siderophore-mediated iron acquisition.$^{56}$ The second requirement is import of the holo-siderophore across the bacterial cell envelope. To investigate the ability of A. baumannii to utilize [Fe(Fim)] as an iron source we performed kinetic growth promotion assays in M9 minimal medium supplemented with 2,2’-dipyridyl (DIP). The same strain of A. baumannii ATCC 17978 used to isolated Fim and Acb was used in the growth promotion studies. We fine-tuned DIP concentrations, as previously described,$^{20,22,53}$ to the point where only weak growth is observed unless an iron supplement is
added to the medium (Figure 7.20). As a positive control, we showed that both Acb and [Fe(Acb)₂] recover the growth of A. baumannii ATCC 17978 in a dose-dependent manner (Figure 7.20a,c; Figures 7.21 and 7.22). As expected from our previous work,²⁰ [Fe(Acb)₂] stimulated growth at lower concentrations and to a higher final cell density after 48 h at 37 °C compared to Acb. Similarly, both [Fe(Fim)] and Fim promoted A. baumannii growth in a time- and dose-dependent manner with [Fe(Fim)] providing faster and more enhanced growth promotion (Figure 7.20b,d; Figures 7.21 and 7.22). Clearly, preloading Acb and Fim with iron(III) is beneficial for growth promotion under these growth conditions. Since [Fe(Acb)₂] and [Fe(Fim)] were both prepared as pure ferric complexes with removal of all residual iron, the potent growth stimulating effects must be attributed to utilization of the siderophore ferric complexes as an iron source. These results show for the first time that [Fe(Fim)] can serve as a true iron source for A. baumannii ATCC 17978 and support the role of the fimsbactins as natural siderophore substrates.⁵⁷

**apo-Fim Antagonizes the Growth Promoting Effects of [Fe(Acb)₂].**

After establishing the individual roles of Fim and Acb as siderophores, we sought to probe the more challenging question of why A. baumannii produces multiple siderophores in the first place. More specifically, we thought it was curious that all pathogenic A. baumannii maintain the ability to produce and utilize PreAcb/Acb while <10% produce Fim. We hypothesized that siderophore competition, as opposed to cooperativity, might have led to evolutionary selection of PreAcb/Acb over Fim. To test for siderophore competition between Acb and Fim, we performed checkerboard kinetic growth promotion assays using combinations of Acb, Fim, [Fe(Acb)₂], and [Fe(Fim)] against A. baumannii ATCC 17978 under the same iron restrictive growth conditions described previously (Figures 7.23, 7.24). We were surprised to
discover that combinations of apo-siderophores Acb and Fim were growth inhibitory (Figure 7.23a) compared to each apo-siderophore alone (Figure 7.20). Increasing the concentration of Acb from 3.9 μM to 62.5 μM in the presence of 3.9 μM Fim slightly recovered growth. Increasing the concentration of Fim to 62.5 μM in the presence of 62.5 μM Acb returned the antagonistic effect on Acb-promoted growth. The addition of holo-siderophores recovered A. baumannii growth but increasing the concentration of apo-siderophores could still antagonize growth slightly. Figure 7.23b shows that 15.6 μM [Fe(Acb)₂] can recover A. baumannii growth in the presence of 3.9 μM or 62.5 μM Fim. However, [Fe(Acb)₂] at 0.5 μM was not capable of recovering growth in the presence of Fim at 3.9 or 62.5 μM. The addition of apo-Acb at 62.5 μM was also slightly antagonistic toward [Fe(Fim)] at 0.5 and 15.6 μM in the growth recovery assay (Figure 7.23c), although to much lesser extent than the effect of apo-Fim on [Fe(Acb)₂] (Figure 7.23b). We also investigated the effects of Fim/[Fe(Fim)] combinations on A. baumannii growth and discovered slightly reduced growth promotion with increasing concentrations of apo-Fim (Figure 7.25).

The discovery that Fim and Acb appear to be antagonistic adds to the complexity of understanding the delicate balance of metals and siderophores in pathogenic A. baumannii. This phenomenon is consistent with our hypothesis that Acb and Fim competition drove natural selection of the Acb pathway found in all A. baumannii clinical isolates. In natural environments siderophore competition plays a critical role in microbial population dynamics.32,42 Siderophore producers often produce siderophore cocktails and express siderophore transport and utilization proteins for xenosiderophores in order to gain a competitive advantage.32 Some siderophores are inhibitory toward competing bacteria by withholding metals from the extracellular or intracellular environment.36 Some siderophores are attached to antibiotics, so-called
sideromycins, to deliver a toxic payload into susceptible competitor cells.\textsuperscript{58} Some siderophores are thought to directly compete for uptake at the receptor level through endogenous pathways.\textsuperscript{37,53} Thus, we turned our attention to probing for pathway competition between Acb and Fim in \textit{A. baumannii} to gain further insight into the cause of the antagonistic siderophore relationship.

\textbf{Fim, PreAcb, and Acb Binding to BauB.}

Bioinformatic analysis of the fimsbactin biosynthetic operon revealed some potential gaps in the uptake pathway, including no dedicated periplasmic siderophore-binding protein (SBP) and no inner membrane permease (\textbf{Figure 7.4}). Outer membrane receptors (OMRs) are often highly selective for binding cognate substrates, while SBPs can be more promiscuous in terms of binding small molecules.\textsuperscript{33,56,59} Thus, we hypothesized that the SBP responsible for binding PreAcb/Acb might also bind Fim, which shares structural homology to PreAcb/Acb through the phenolate oxazoline and catecholate moieties. We turned our focus toward BauB, the highly conserved periplasmic SBP responsible for binding PreAcb/Acb and shuttling the corresponding holo-siderophores to the membrane permease, BauCDE, to facilitate import to the cytoplasm. We hypothesized that Fim might compete with PreAcb/Acb for BauB binding and inhibit the import of ferric siderophore complexes to the cytoplasm. To test this hypothesis, we pursued \textit{in vitro} reconstitution of BauB and evaluated the ability of apo- and holo-forms of PreAcb, Acb, and Fim to compete for binding.

We confirmed binding of recombinant BauB to authentic PreAcb and Acb using an intrinsic tryptophan fluorescence quenching assay.\textsuperscript{21} We observed dose-dependent fluorescence quenching for both the apo- and holo-forms of both acinetobactin isomers. The binding of both
apo- and holo-siderophores by outer membrane siderophore receptors and periplasmic siderophore-binding proteins is common.\(^{33,60}\) The apparent \(K_d\) values for apo-PreAcb and [Fe(PreAcb)\(_2\)] were 380 ± 110 nM and 750 ± 160 nM, respectively (Table 7.1, Figure 7.26).\(^{21}\) The apparent \(K_d\) values for apo-Acb and [Fe(Acb)\(_2\)] were 300 ± 100 nM and 160 ± 80 nM, respectively (Table 7.1).\(^{21}\) The apparent trend in binding affinities of apo-\(K_d > holo-K_d\) for Acb appear to be reversed for the measurements obtained for PreAcb where apo-\(K_d < holo-K_d\).

Treatment of BauB with apo- and holo-forms of Fim resulted in dose dependent fluorescence quenching with apparent \(K_d\) values falling within the margin of error for those observed for Acb (Table 7.1). The apparent \(K_d\) values for apo-Fim and [Fe(Fim)] were 360 ± 140 nM and 240 ± 90 nM, respectively. All apparent \(K_d\) values were calculated using a one-site binding model to fit the binding stoichiometry observed in the BauB·[Fe(Acb)\(_2\)] crystal structure.\(^{21}\) These results are consistent with our hypothesis that PreAcb, Acb, and Fim bind to BauB. We next turned to investigating the reversibility and competitiveness of BauB binding using a siderophore displacement assay with resin-immobilized BauB.

**Fimsbactin A and Acinetobactin Directly Compete for Binding to BauB.**

To validate that Fim binding to BauB is authentic, we performed a competitive binding experiment with [Fe(Acb)\(_2\)] and [Fe(Fim)] using resin-immobilized BauB. Our group has reported this type of binding assay previously in the study of ferrioxamine siderophore competition for binding FhuD2, the xenosiderophore receptor displayed on the cell surface of pathogenic *Staphylococcus aureus*.\(^{61}\) N-His\(_6\)-tagged BauB was adhered to a column of Ni-NTA resin. Saturation of the resin and the quantity of immobilized BauB was determined by SDS-PAGE analysis of column flow through from the protein-loading step. Treatment of the BauB resin with a solution of [Fe(Acb)\(_2\)] resulted in a decrease of ion counts in the EIC trace for \(m/z\)
347, corresponding to the \([M + H]^+\) ion for Acb (this is the dominant ion in the ESI-MS spectrum of \([\text{Fe(Acb)}_2]\))^{20}. Treatment of the BauB-\([\text{Fe(Acb)}_2]\) resin with a solution of \([\text{Fe(Fim)}]\) recovered ion counts corresponding to \([\text{Fe(Acb)}_2]\), indicating that the excess \([\text{Fe(Fim)}]\) displaced \([\text{Fe(Acb)}_2]\) to produce the resin bound BauB-\([\text{Fe(Fim)}]\) complex with elution of \([\text{Fe(Acb)}_2]\) (Figure 7.27a). Similarly, treatment of the BauB resin with \([\text{Fe(Fim)}]\) resulted in a decrease of ion counts in the EIC trace for \(m/z\) 627, corresponding to the \([M + H]^+\) ion for \([\text{Fe(Fim)}]\) (this is the dominant ion in the ESI-MS spectrum of FimFe) after washing the column with buffer. Treatment of the BauB-\([\text{Fe(Fim)}]\) resin with a solution of \([\text{Fe(Acb)}_2]\) recovered ion counts for \([\text{Fe(Fim)}]\) in the elution, indicating that the excess \([\text{Fe(Acb)}_2]\) displaced \([\text{Fe(Fim)}]\) from the resin resulting in resin bound BauB-\([\text{Fe(Acb)}_2]\) with elution of \([\text{Fe(Fim)}]\) (Figure 7.27b).

We found that the BauB resin could be cycled between treatment with \([\text{Fe(Acb)}_2]\) and \([\text{Fe(Fim)}]\) multiple times with reversible siderophore displacement. Unfortunately, these competition studies are limited to the use of holo-siderophores since apo-siderophores will bind directly to the Ni-NTA resin.\(^{61}\) These competition experiments validate that BauB binds tightly and reversible to both \([\text{Fe(Acb)}_2]\) and \([\text{Fe(Fim)}]\). Since BauB binding is reversible, the delicate balance between concentrations of apo-siderophores PreAcb, Acb, Fim, and corresponding holo ferric complexes will ultimately determine which siderophore species dominates the BauB binding equilibrium. Our results here show that Fim antagonizes \(A.\ baumannii\) growth promotion by \([\text{Fe(Acb)}_2]\), suggesting that occupancy of BauB in the periplasm by high concentrations of Fim might exclude utilization of \([\text{Fe(Acb)}_2]\) as an iron source. This suggests that the local concentrations of Acb and Fim in the cytoplasm, periplasm, and extracellular space are regulated to minimize pathway competition and optimize iron scavenging under infection conditions.

*Structural Similarity of \([\text{Fe(Fim)}]\), \([\text{Fe(PreAcb)}_2]\), and \([\text{Fe(Acb)}_2]\) Ferric Complexes.*
Accommodation in the BauB substrate binding site requires similarity in the molecular volume, stereochemistry, and geometrical orientation of ligands around the ferric iron center for [Fe(PreAcb)₂], [Fe(Acb)₂], and [Fe(Fim)]. We investigated these properties using density functional theory (DFT) calculations to create energy minimized structural models of [Fe(PreAcb)₂]⁻ and [Fe(Fim)]⁻ using the experimentally observed geometry of the [Fe(Acb)₂]⁻ monoanion in the BauB·[Fe(Acb)₂]⁻ crystal structure (Figure 7.28).²¹ We assumed that [Fe(PreAcb)₂]⁻, [Fe(Acb)₂]⁻, and [Fe(Fim)]⁻ exist as the mono anions in the ferric complexes. BauB appears to recognize one enantiomer of the *cis*-[Fe(Acb)₂]⁻ isomeric form. The DFT-computed energy minimized structures of [Fe(Fim)]⁻ (Figure 7.28a) and *cis*- [Fe(PreAcb)₂]⁻ (Figure 7.28b) were strikingly similar to the experimentally observed *cis*- [Fe(Acb)₂]⁻ complexed to BauB (Figure 7.28c). An overlay of the three structures revealed how the common phenolate oxazoline fragment aligns nicely and the L-Ser stereochemistry enables [Fe(Fim)]⁻ to map the hydroxamate and catecholate ligands to the same ferric binding sites as the imidazole and catecholate ligands of *cis*- [Fe(Acb)₂]⁻, respectively. As shown in Figure 7.28e, BauB binds one-half of the *cis*- [Fe(Acb)₂]⁻ complex through hydrophobic interactions with one of the Acb ligands, while the second Acb ligand remains largely solvent exposed. The computed structures of *cis*- [Fe(PreAcb)₂]⁻ and [Fe(Fim)]⁻ would appear to fit this same binding mode to BauB and might explain how all three siderophores can compete for binding to periplasmic BauB. Since [Fe(Fim)] is the only stable 1:1 complex, this could indicate that BauB cannot facilitate transport of 1:1 siderophore/iron complexes across the inner membrane through the associated membrane permease BauCDE hinting at a potential shuttling mechanism for 2:1 PreAcb/Acb−iron complexes (discussed below in more detail).⁵⁴,⁶²,⁶³

*A New Model for Siderophore Competition in A. baumannii.*
Our findings are largely consistent with the current understanding of PreAcb/Acb biosynthesis and utilization in pathogenic *A. baumannii* (Figure 7.29). PreAcb is the kinetic biosynthetic product, while Acb is formed via nonenzymatic isomerization of PreAcb as the thermodynamic biosynthetic end product. Both PreAcb and Acb can reach the extracellular space, presumably via efflux mediated in part by BarAB. The TonB-dependent OMR BauA has been shown to be essential for PreAcb/Acb utilization, presumably providing import of holo-siderophores to the periplasm. The periplasmic SBP BauB, which might be membrane anchored, although this has not been experimentally confirmed, is then thought to shuttle holo-siderophores to the ABC-type membrane permease BauCDE where import to the cytoplasm is driven by ATP hydrolysis. BauF is a putative flavin-dependent oxidoreductase responsible for reduction of the ferric holo-siderophore complexes to release ferrous iron for incorporation into protein scaffolds such as the iron–sulfur cluster generating protein SufU. The apo-siderophores, PreAcb or Acb, can then reenter the transport cycle.

Our findings provide new insight into the role of PreAcb, Acb, and Fim in overall siderophore-mediated iron acquisition by *A. baumannii*. The Fim biosynthetic operon contains genes encoding for an efflux pump, FbsOQ, a TonB-dependent OMR, FbsN, and a putative reductase, FbsP. The reductase FbsP is predicted to be secreted to the periplasm due to the presence of an N-terminal signal sequence, similar to the siderophore-interacting periplasmic reductase FpvC in *Pseudomonas aeruginosa*. We propose that Fim is exported in part by FbsOQ after biosynthesis in the cytoplasm. Extracellular [Fe(Fim)] is presumably imported to the periplasm by FbsN where periplasmic reduction by FbsP releases Fim for efflux and ferrous iron for import to the periplasm via the endogenous ferrous transport system FeoABC. This transport model is analogous to the well-studied utilization pathway for the siderophore
pyoverdine in pathogenic *P. aeruginosa*. Furthermore, *P. aeruginosa* balances the use of multiple siderophores for adaptive iron acquisition during infection where some siderophores stay in the periplasm (pyoverdine) and some enter the cytoplasm (pyochelin). Analogously, *A. baumannii* might utilize Fim as the periplasmic siderophore and PreAcb/Acb as the cytoplasmic siderophore. This model suggests that Fim never makes it to the cytoplasm, which is consistent with the observation that fimsbactin analogs conjugated to antibiotics with periplasmic targets (β-lactams, daptomycin, vancomycin) show potent, iron-dependent growth inhibitory activity against *A. baumannii* while the same siderophores conjugated to antibiotics with cytoplasmic targets (fluoroquinolones) show no antibacterial activity. One unique structural feature of fimsbactin A is the presence of an L-Ser-2,3-DHB ester, which resembles a fragment of the L-Ser-2,3-DHB trilactone scaffold of enterobactin. Similar to enterobactin, it is possible that hydrolysis of the L-Ser-2,3-DHB ester in the periplasm may play a role in iron release. The product of fimsbactin A hydrolysis is fimsbactin F, which is the second most concentrated member of the fimsbactin mixture present in *A. baumannii* ATCC 17978 supernatants (Figures 7.3, 7.6, 7.7).

If fimsbactins accumulate in the periplasm, this would create a scenario for competition between Fim and PreAcb/Acb to bind BauB that is consistent with our current findings. A buildup of excess Fim in the periplasm could shift the BauB equilibrium toward a complex of BauB and [Fe(Fim)] and inhibit the import of [Fe(PreAcb)₂] and [Fe(Acb)₂] leading to the growth inhibitory effect that we observed for the siderophore combinations. It is also possible that Fim, PreAcb, and Acb compete for iron and form mixed siderophore ferric complexes that act as transport inhibitors. A crystal structure of the *A. baumannii* OMR BauA bound to a mixed [Fe(PreAcb)(Acb)] ferric complex was recently reported.
BauA·[Fe(PreAcb)(Acb)] structure shows complexation of the iron by a PreAcb ligand through phenolate oxazoline, hydroxamate, and imidazole filling four of the size coordination sites in the octahedral ferric center. The final two coordination sites are apparently filled by the 2,3-DHB catecholate of the Acb ligand. The structure suggests that BauA only interacts with the PreAcb ligand while the Acb dangles outside of the binding pocket. Our group recently reported a competitive inhibitor of siderophore uptake based on an oxidized analog of PreAcb that induces an iron-dependent growth inhibitory effect on *A. baumannii* that is similar to the effect of apo-Fim.\(^{53}\) The oxidized PreAcb features an aromatic oxazole in place of the PreAcb oxazoline that stabilizes the PreAcb structure and prevents isomerization to Acb. The BauA·[Fe(PreAcb)(Acb)] structure suggests that apo- and holo-variants of the oxazole PreAcb or fimsbactin A might also bind to BauA in a similar manner, although this requires experimental validation. One possibility is that PreAcb stays bound to BauA and BauB as a cofactor to facilitate iron trafficking with ligand exchange.\(^{54,62,63}\)

Our work suggests that competition for periplasmic BauB is possible, but it is not entirely clear whether competition for BauA plays a role in the growth inhibitory effect of apo-Fim. We tested the effect of excess apo-Fim on the growth of *A. baumannii* ATCC 19606T, a strain that does not produce Fim but does produce PreAcb/Acb and is predicted to produce baumannoferrin (*Figure 7.30*).\(^{12,64}\) Treatment of wild-type *A. baumannii* ATCC 19606T with apo-Fim under iron-deficient minimal medium conditions weakly promoted growth at low doses; an effect that diminishes at higher doses of apo-Fim (*Figures 7.31 and 7.32*). Addition of [Fe(Fim)] promoted the growth of *A. baumannii* ATCC 19606T wild-type and mutant variants s1 (insertional mutant in *basD*, deficient in PreAcb/Acb biosynthesis), t6 (insertional mutation in *bauA*, deficient in PreAcb/Acb import to periplasm), and t7 (insertional mutation in *bauD*,...
deficient in PreAcb/Acb import to cytoplasm) at low doses. This suggests that [Fe(Fim)] can serve as an iron source in the absence of the dedicated Fim utilization proteins FbsOQNP and Fim bioactivity is independent of acinetobactin biosynthesis and the presence of functional BauA/BauD. It is possible that [Fe(Fim)] can be imported through one of the other ∼20 TonB-dependent outer membrane receptors present in pathogenic strains of A. baumannii.15 We hypothesize that periplasmic accumulation of Fim might contribute to inhibition of A. baumannii growth promotion by holo-siderophores, including Acb. A recent study in Mycobacterium tuberculosis showed that a siderophore efflux mutant accumulated toxic concentrations of mycobactin siderophores supporting the idea that maintaining the appropriate balance of intracellular siderophores is critical to balance metal homeostasis.75 Regardless of the underlying cause of apo-Fim inhibition of A. baumannii growth, pathway competition between PreAcb, Acb, and Fim seems to be important even for nonproducers of the fimsbactins, including the majority of A. baumannii clinical isolates.

7.5 Outlook and Conclusions

Pathogenic bacteria often produce multiple siderophores to enhance iron acquisition, expand capacity for metal scavenging, evade the human immune system, and gain a competitive edge in mixed microbial environments. All genome sequenced clinical isolates of the MDR Gram-negative pathogen A. baumannii produce the siderophores PreAcb, Acb, and baumannoferrin. PreAcb and Acb both enhance iron acquisition and contribute to pathogen virulence, while the role of baumannoferrin has not yet been investigated. A small percentage (<10%) of A. baumannii clinical isolates produce a third family of siderophores known as the fimsbactins. We have shown that A. baumannii ATCC 17978 produces Acb in greater quantities
than Fim and both siderophores can be purified from culture supernatants. Here, we show that fimsbactin A, the primary component of the fimsbactin siderophore mixture, antagonizes *A. baumannii* growth promotion by Acb and [Fe(Acb)$_2$]. The two siderophores compete directly for binding to the periplasmic siderophore-binding protein BauB implying that pathway competition might contribute to the antagonistic effect of Fim. This competition might explain why the majority of *A. baumannii* clinical isolates have lost the ability to produce the fimsbactins, but have maintained genes for Acb biosynthesis and utilization in a highly conserved manner. Due to the structural similarities of Fim, PreAcb, and Acb, sharing common NRPS-derived fragments from phenolate oxazolines and catecholates from 2,3-DHB, we propose that the fimsbactin scaffold serves as an ideal template for designing new siderophore–antibiotic conjugates that can outcompete native siderophores for cell entry under infection conditions, although this antibiotic delivery strategy should be reserved for antibiotics with periplasmic targets since our current understanding of the fimsbactin pathway suggests import is limited to the periplasm.$^{70-72}$ The inherent growth inhibitory effect of apo-Fim toward *A. baumannii* also presents the opportunity to design competitive inhibitors of siderophore uptake that might serve as antivirulence agents to starve the pathogen of iron.$^{36,53}$ Our findings suggest that regulation of relative siderophore concentrations, perhaps through control of gene transcription,$^{76}$ in pathogens that produce multiple siderophores might be important for minimizing competition and enhancing cooperation between the associated iron uptake pathways. Further studies on the effects of natural siderophore combinations are needed to reveal the subtle details of cooperativity and competition at the transcriptional, biochemical, and cellular level of bacterial iron acquisition.
7.6 Materials and Methods

Strains, Materials, and Instrumentation.

Growth studies were conducted using \textit{A. baumannii} ATCC 17978, ATCC 19606T. The s1, t6, and t7 mutant strains of \textit{A. baumannii} ATCC 19606T were obtained from Prof. Luis Actis (Miami University). Precultures and 96-well plate \textit{A. baumannii} growth assays were performed in filter-sterilized M9 minimal media. M9 minimal media was prepared for all experiments as previously described. Samples for LC-MS were prepared in 0.45 μM PTFE mini-UniPrep vials from Agilent. All preparatory HPLC was performed using a Beckman Coulter SYSTEM GOLD 127P solvent module and 168 detector with a Phenomenex Luna 10u C18(2) 100A column, 250 mm × 21.20 mm, 10 μm with guard column. Prep HPLC was performed with a mobile phase of 5 mM ammonium acetate in (A) water and (B) acetonitrile, and data were processed using 32 Karat software, v7.0. LC-MS was performed on an Agilent 6130 quadrupole LC-MS with G1313 autosampler, G1315 diode array detector, and 1200 series solvent module. A Phenomenex Gemini C18 column, 50 mm × 2 mm, 5 μm with guard column was used for all LC-MS separations. LC-MS mobile phases were 0.1% (v/v) formic acid in (A) water and (B) acetonitrile, and data were processed using G2710 ChemStation software. NMR was performed on a Varian Unity Inova-600 MHz instrument with a cold probe. Bacterial growth studies were performed using polystyrene 96-well plates with polystyrene lids. OD\textsubscript{600} measurements were taken on a Molecular Devices SpectraMax Plus 384 plate reader.

Isolation and Purification of Acb and Fim.

PreAcb was synthesized as described previously by our group. Acb and Fim were isolated and purified from \textit{A. baumannii} ATCC 17978 cultures using a modified literature
procedure. Briefly, 1 L cultures of *A. baumannii* ATCC 17978 were grown overnight in M9 minimal media. Cells were pelleted, and the supernatant was adjusted to pH ≈ 6 using citric acid. XAD-7HP resin was added to the supernatant, and the mixture was shaken gently. The mixture was filtered, and the resin was washed with methanol. The methanol washings were combined and concentrated via rotary evaporation under reduced pressure. Acb (retention time 12 min, 31 mg/L) and Fim (retention time 15 min, 5 mg/L) were purified from the crude residue by preparatory HPLC (gradient of 0% B to 95% B (v/v) over 17 min, then 95% B to 100% B (v/v) over 8 min). Fim represents a mixture of fimsbactin A (>90%) and fimsbactins B, C, and F (≤10%) as judged by NMR and LC-MS analysis (Table 7.1; Figures 7.6, 7.7, 7.9–7.13). Acb was >95% pure as judged by NMR and LC-MS (Figure 7.8). The holo-siderophores were prepared by mixing PreAcb, Acb, and Fim with excess Fe(acac)₃ in methanol followed by concentration and trituration with Et₂O to provide the pure [Fe(PreAcb)₂], [Fe(Acb)₂], and [Fe(Fim)] ferric complexes.

*Determination of FimFe Complex Stoichiometry.*

A 300 μL solution of 570 μM Fim in methanol was prepared. A fluorescence emission spectrum was recorded (λ<sub>excitation</sub> = 330 nm; λ<sub>emission</sub> = 380 nm). To determine stoichiometry of the complex between Fim and Fe(III), 1 μL aliquots of a methanolic solution of 10 mM Fe(acac)₃ or FeCl₃ were added 0.044 equiv at a time via Hamilton syringe, and emission spectra were recorded after each addition. Peak fluorescence (Abs<sub>380nm</sub>) was plotted against Fe(III) equivalents to reveal a 1:1 final stoichiometry for the [Fe(Fim)] complex (Figure 7.16).

*Determination of Apparent K<sub>Fe</sub> for [Fe(Fim)].*
A stock solution of 100 μM [Fe(Fim)] was prepared in 10 mM HEPES buffer (10 mM HEPES, 600 mM NaCl, 100 mM KCl, pH 7.4), and an optical absorbance spectrum was obtained from λ = 300−800 nm (Figure 7.17). While continuously monitoring optical absorbance at 500 nm, EDTA was added at a final concentration of 120 μM (1.2 equiv relative to [Fe(Fim)]). The apparent iron-binding affinity (\(K_{Fe}\)) was determined based on the change in optical absorbance at 500 nm after 800 min for two independent trials using the equations provided in the Equations 7.1 (Figure 7.18).

**Iron Exchange between [Fe(Acb)\(_2\)] and Fim.**

[Fe(Acb)\(_2\)], 300 μL of a 100 μM solution in phosphate buffer (50 mM potassium phosphate pH 8.0, 150 mM NaCl, 1 mM DTT, 5% glycerol), was added to a quartz cuvette. The solution was mixed and allowed to stand for 20 min before an optical absorbance spectrum was collected (400−700 nm). One microliter aliquots of apo-Fim were added consecutively to increase the concentration of apo-Fim from 30 to 210 μM in 30 μM increments. After each addition of apo-Fim, the solution was allowed to stand for 20 min at RT before an optical absorbance spectrum was collected (400−700 nm). Once the final concentration of 210 μM apo-Fim was reached, the solution was allowed to stand for 20 h at RT before an optical absorbance spectrum was collected (400−700 nm) (Figure 7.19).

**A. baumannii Growth Studies.**

Stock solutions of Acb, Fim, [Fe(Acb)\(_2\)], and [Fe(Fim)] were prepared in M9 media at 250 μM (up to 2.5% (v/v) final DMSO). Each well of a 96-well plate was filled with 50 μL of M9 media, and 50 μL of the 250 μM test compound stock solutions was added to the first row of a 96 well plate to provide 100 μL total volume per well. Compounds were serially diluted down...
the plate to 3.9 μM by removing 50 μL from the first row of wells and diluting down the plate, discarding the last 50 μL, to afford 50 μL in each well. An inoculum was made by adding 100 μL of 0.5 McFarland standard (A. baumannii ATCC 17978, ATCC 19606T, wt, s1, t6, and t7 mutants) to 4.0 mL of M9 minimal media supplemented with 350 μM 2,2′-dipyridyl (DIP). Inoculum (50 μL) was added to each well resulting in a final volume of 100 μL per well with a final concentration of 175 μM DIP per well and a serial dilution of test compounds ranging from 62.5−1.95 μM. Bacterial growth was monitored at 37 °C by measuring OD$_{600}$ using a microplate reader (Molecular Devices SpectraMax Plus 384 plate reader). Control experiments were performed in M9 media with 175 μM DIP and no test compounds. DIP concentrations were optimized prior to each experiment by serial dilution against A. baumannii under the growth conditions described in this section. All experiments were performed in triplicate as independent trials.

Combinations of Acb, Fim, and the corresponding ferric complexes were evaluated for bacterial growth promotion in a similar manner at 37 °C. A 96-well plate was first filled with 40 μL of M9 minimal media per well and 5 μL of each test compound solution. Fifty microliters of the inoculum in M9 media was added to each well giving a final concentration of 175 μM DIP per well and a total volume of 100 μL per well. Compounds were tested in combinations ranging from 0.5−62.5 μM as summarized in Table 7.2. All experiments were performed in duplicate as independent trials.

**Determination of Apparent $K_d$ Values for BauB.**

BauB was expressed as the N-terminal hexahistidine fusion, N-His$_6$-BauB, in E. coli BL21 (DE3) as described previously (Figure 7.33).²¹ N-His$_6$-BauB was recovered on ice from a
−80 °C freezer stock. A 400 nM BauB stock solution was prepared in assay buffer (25 mM Tris-
HCl, 8 g/L NaCl, 0.2 g/L KCl, pH 7.4). For each measurement, 300 μL of the BauB stock
solution was transferred to a fluorescence cuvette (HellmaAnalytics High Precision Cell cuvette
made of quartz SUPRASIL; light path 10 mm × 2 mm) in the presence of substrate (PreAcb,
[Fe(PreAcb)₂], Acb, [Fe(Acb)₂], Fim, Fe(Fim)) at concentrations ranging from 100 to 1200 nM.
Emission spectra were recorded at λ_{emission} = 300−400 nm using a PerkinElmer LS 55
luminescence spectrometer (slit width 10 nm; scan speed 400 nm/min) at λ_{excitation} = 280 nm.
Fluorescence intensity at 320 nm was plotted versus substrate concentration (nM), and apparent
Kₐ was calculated using nonlinear regression and a one binding site model in GraphPad Prism
v7.0b (Figure 7.26).²¹ All experiments were performed in duplicate as independent trials.

**Siderophore Competition Studies with Immobilized BauB.**

*N*-His₆-BauB was immobilized on Ni-NTA resin following a literature protocol for
performing siderophore-affinity chromatography.⁶¹ Saturation of the Ni-NTA resin (2.3 cm × 1
cm resin volume) with BauB was confirmed by SDS-PAGE analysis of the column flow through.
The Ni-NTA-BauB column was washed thoroughly with phosphate buffer (50 mM potassium
phosphate, pH 8.0, 150 mM NaCl, 1 mM BME, 5% glycerol). A solution of [Fe(Acb)₂] (5 mL,
0.1 mg mL⁻¹) was loaded onto the column, and column was rocked gently for 30 min at 4 °C.
Column flow through was collected, and the resin was washed with excess phosphate buffer (5 ×
5 mL). [Fe(Fim)] (5 mL, 0.1 mg mL⁻¹) was then loaded, and the column was gently rocked for
30 min at 4 °C. The flow through was collected, and the resin was washed with excess phosphate
buffer (5 × 5 mL). [Fe(Acb)₂] (5 mL, 0.1 mg mL⁻¹) was then added back onto the column, and
after rocking for 30 min, the flow through was collected. Aliquots of the column loading
solutions and elutions were analyzed by LC-MS for the presence of [Fe(Acb)₂] and [Fe(Fim)].
For [Fe(Acb)₂], the extracted ion chromatogram (EIC) for m/z = 347, corresponding to the [M + H]⁺ for apo-Acb, was plotted. For [Fe(Fim)], the EIC for m/z = 627 corresponding to the [M + H]⁺ for holo-[Fe(Fim)] was plotted.

_DFT Calculations._

Stable holo-siderophore complexes with ferric iron were calculated using density functional theory (DFT) following a protocol described previously by our group.¹¹ We used the crystal structure of the monoanionic [Fe(Acb)₂]⁻ (S = 5/2) ferric complex bound to BauB (PDB 6FML) as starting geometry of the monoanionic [Fe(PreAcb)₂]⁻ and [Fe(Fim)]⁻ ferric complexes.¹¹

7.7 Acknowledgements

We thank B. Evans at the Proteomics & Mass Spectrometry Facility at the Donald Danforth Plant Science Center, St. Louis, MO for assistance with the acquisition of the QTRAP LC-MS/MS spectra (supported by the National Science Foundation under Grant No. DBI-0521250). We thank J.-S. Taylor (WUSTL, Dept. of Chemistry) for assistance with fluorescence quenching studies. We thank L. Actis (Miami University) for the s1, t6, and t7 mutant strains of _A. baumannii_ ATCC 19606T. We thank A. Gulick and D. Bailey (State University of New York at Buffalo) for the pET28a-TEV plasmid used for expression of BauB. We thank J. Kao and M. Singh (WUSTL Chemistry) for help with the acquisition of 2D NMR data. The research reported here was supported by NSF CAREER Award 1654611 to TAW.
7.8 Figures and Tables

(1) \[ K_L = \frac{[FeL]}{[Fe^{3+}][L]} \] for the following equilibrium: \[ Fe^{3+} + L \rightleftharpoons FeL \]

(2) \[ K_{FeEDTA} = \frac{[FeEDTA]}{[Fe^{3+}][EDTA]} \] for the following equilibrium: \[ Fe^{3+} + [EDTA] \rightleftharpoons [FeEDTA] \]

(3) \[ K_{Exchange} = \frac{K_L}{K_{FeEDTA}} \] for the following equilibrium: \[ [FeEDTA] + [L] \rightleftharpoons [FeL] + [EDTA] \]

(4) \[ K_{Exchange} = \frac{[FeL][EDTA]}{[FeEDTA][L]} \]

(5) \[ \Delta = \frac{Abs_{FeL} - Abs_{FeL+EDTA}}{e_L} \]

(6) \[ K_L = K_{FeEDTA} \times \frac{[FeL][EDTA]}{[FeEDTA][L]} \]

(7) \[ [FeL] = \frac{Abs_{FeL}}{e_L} \]

(8) \[ [EDTA] = [EDTA]_T - \Delta \quad \text{where} \quad [EDTA]_T = \text{total EDTA added} \]

(9) \[ [FeEDTA] = \Delta \]

(10) \[ [L] = \Delta \]

(11) \[ K_{Fe} = \text{apparent } K_L \]

**Equations 7.1.** As described in the experimental methods section of the main text, an EDTA competition experiment was used to measure the apparent \( K_{Fe} \) for FimFe at pH 7.4. The following equations were used to calculate apparent \( K_{Fe} \) based on the change in optical absorbance observed at 500 nm for FimFe in the presence of 1.2 equivalents of EDTA. A \( K_{Fe} \) value of \( 10^{25.1} \) was used for EDTA at pH 7.4 in final calculations. \(^7\)
Table 7.1: NMR characterization data for purified fimsbactin A from this work compared with previously reported data from the original isolation and characterization of fimsbactin A.\(^{78}\)

<table>
<thead>
<tr>
<th>( ^{13}C - \text{Reported} )</th>
<th>( ^{13}C - \text{Observed} )</th>
<th>( ^{1}H - \text{Reported} )</th>
<th>( ^{1}H - \text{Observed} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 148.2</td>
<td>148.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 145.7</td>
<td>145.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 119.3 119.5</td>
<td>6.968 (dd) 6.97 (dt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 118.6 118.7</td>
<td>6.74 (t) 6.74 (t)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 117.7 117.9</td>
<td>7.08 (dd) 7.07 (dd)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 110.1 110.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 166.3 163.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 69.1 69.3</td>
<td>4.52 (dd) 4.52 (dd)</td>
<td>4.61 (dd) 4.60 (m)</td>
<td></td>
</tr>
<tr>
<td>10 66.9 67.2</td>
<td>5.06 (dd) 5.06 (dd)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 169.9 168.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 8.72 (dd) 8.74 (d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 51.5 51.7</td>
<td>4.72 (m) 4.72 (m)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 64.3 64.5</td>
<td>4.39 (m) 4.39 (dd)</td>
<td>4.61 (m) 4.60 (m)</td>
<td></td>
</tr>
<tr>
<td>15 168.7 167.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 112.8 112.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 149.5 149.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 146.0 146.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 120.7 120.9</td>
<td>6.97 (dd) 6.97 (dt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 118.6 118.8</td>
<td>6.59 (t) 6.59 (t)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 119.6 119.8</td>
<td>7.15 (dd) 7.15 (dd)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22 167.7 166.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23 8.23 (dt) 8.26 (t)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 38.3 38.5</td>
<td>3.10 (m) 3.09 (m)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 25.9 26.4</td>
<td>1.38 (m) 1.38 (m)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26 23.5 23.7</td>
<td>1.49 (m) 1.49 (m)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 46.3 46.5</td>
<td>3.45 (t) 3.45 (t)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28 170.1 170.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29 20.1 20.3</td>
<td>1.96 (s) 1.96 (s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 31 32</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 7.2: Concentrations of test compounds used in combination for *A. baumannii* growth studies. For each combination of compounds 1 and 2, all possible concentrations were tested in duplicate as independent trials using a checkerboard arrangement in a 96-well plate.

<table>
<thead>
<tr>
<th>Compound 1</th>
<th>Compound 2</th>
<th>[Compound 1] (µM)</th>
<th>[Compound 2] (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acb</td>
<td>Fim</td>
<td>3.9, 15.6, 62.5</td>
<td>3.9, 15.6, 62.5</td>
</tr>
<tr>
<td>[Fe(Acb)]</td>
<td>Fim</td>
<td>0.5, 3.9, 15.6</td>
<td>3.9, 15.6, 62.5</td>
</tr>
<tr>
<td>Acb</td>
<td>[Fe(Fim)]</td>
<td>3.9, 15.6, 62.5</td>
<td>0.5, 3.9, 15.6</td>
</tr>
<tr>
<td>Fim</td>
<td>[Fe(Fim)]</td>
<td>3.9, 15.6, 62.5</td>
<td>0.5, 3.9, 15.6</td>
</tr>
</tbody>
</table>
Figure 7.1: Siderophores from *A. baumannii*. (a) Structures of preacinetobactin (PreAcb), acinetobactin (Acb), fimsbactin (Fim), and baumannoferrin. PreAcb/Acb and Fim share a common motif derived from condensation of 2,3-DHB with L-Thr (PreAcb/Acb) or L-Ser (Fim). (b) DAD at 263 nm (black), EIC at m/z 347 (blue), and EIC at m/z 575 (red) chromatograms from LC-MS analysis of crude *A. baumannii* ATCC 17978 supernatant after acidification, treatment with XAD-7HP resin, and methanol elution. Baumannoferrin was not detected.
Figure 7.2: Predicted biosynthesis of acinetobactin and fimsbactin in *A. baumannii* ATCC 17978. Non-ribosomal peptide synthetase (NRPS) assembly lines for acinetobactin (top) and fimsbactin A (bottom) share a common precursor, 2,3-DHB, and a common phenolate oxazoline motif. Pre-acinetobactin (PreAcb) is the kinetic product released from the NRPS assembly line. PreAcb undergoes spontaneous isomerization to the thermodynamic product acinetobactin (Acb).
Figure 7.3: Structures and m/z values for [M+H]^+ molecular ions of fimbactin A–F. Structural differences are highlighted in red.
Figure 7.4: Biosynthetic gene clusters for acinetobactin, fimsbactin, and baumannoferrin from A. baumannii ATCC 17978 along with annotated genes.
Fimsbactin Biosynthetic Gene Clusters

AbPK1 (GenBank: GCA_002753915.1)

Query sequence

BGC0000352: Fimsbactin biosynthetic gene cluster (100% of genes show similarity)

AB042 (GenBank: GCA_001941765.1)

Query sequence

BGC0000352: Fimsbactin biosynthetic gene cluster (100% of genes show similarity)

D36 (GenBank: GCA_001399655.1)

Query sequence

BGC0000352: Fimsbactin biosynthetic gene cluster (100% of genes show similarity)

Strain ATCC 17978 (GenBank: GCA_001593425)

Query sequence

BGC0000352: Fimsbactin biosynthetic gene cluster (100% of genes show similarity)

ATCC 17978-mmf (GenBank: GCA_001077675.1)

Query sequence

BGC0000352: Fimsbactin biosynthetic gene cluster (94% of genes show similarity)
Acinetobactin Biosynthetic Gene Clusters

AbPK1 (GenBank: GCA_002753915.1)

Query sequence

BGC0000294: Acinetobactin biosynthetic gene cluster (78% of genes show similarity)

AB042 (GenBank: GCA_001941765.1)

Query sequence

BGC0000294: Acinetobactin biosynthetic gene cluster (100% of genes show similarity)

D36 (GenBank: GCA_001399655.1)

Query sequence

BGC0000294: Acinetobactin biosynthetic gene cluster (100% of genes show similarity)

Strain ATCC 17978 (GenBank: GCA_001593425)

Query sequence

BGC0000294: Acinetobactin biosynthetic gene cluster (100% of genes show similarity)

ATCC 17978-mmf (GenBank: GCA_001077675.1)

Query sequence

BGC0000294: Acinetobactin biosynthetic gene cluster (100% of genes show similarity)
Acinetoferrin/Baumannoferrin Biosynthetic Gene Clusters

AbPK1 (GenBank: GCA_002753915.1)

Query sequence

BGC0000295: Acinetoferrin biosynthetic gene cluster (40% of genes show similarity)

AB042 (GenBank: GCA_001941765.1)

Query sequence

BGC0000295: Acinetoferrin biosynthetic gene cluster (30% of genes show similarity)

D36 (GenBank: GCA_001399655.1)

Query sequence

BGC0000295: Acinetoferrin biosynthetic gene cluster (30% of genes show similarity)

Strain ATCC 17978 (GenBank: GCA_001593425)

Query sequence

BGC0000295: Acinetoferrin biosynthetic gene cluster (30% of genes show similarity)

ATCC 17978-mmf (GenBank: GCA_001077675.1)

Query sequence

BGC0000295: Acinetoferrin biosynthetic gene cluster (30% of genes show similarity)

Figure 7.5: AntiSMASH\textsuperscript{81} analysis of putative fimsbactin \emph{A. baumannii} producers identified from BLASTp analysis of \emph{A. baumannii} genomes reveals conservation of acinetobactin and baumannoferrin biosynthetic gene clusters (BGCs). The acinetoferrin BGC is the reference in the antiSMASH database. Acinetoferrin and baumannoferrin BGCs share homology, so comparison for all strains was made to the acinetoferrin BGC.\textsuperscript{82}
Figure 7.6: Diode array optical absorbance detection (DAD) and extracted ion chromatograms (EICs) for fimbactin A–F [M+H]$^+$ ions from LCMS analysis of *A. baumannii* ATCC 17978 culture supernatant extractions (Trial #1) using ESI ionization in positive ion mode. The x-axis represents retention time (min) for all chromatograms.
Figure 7. Diode array optical absorbance detection (DAD) and extracted ion chromatograms (EICs) for fimbactin A–F [M+H]^+ ions from LCMS analysis of *A. baumannii* ATCC 17978 culture supernatant extractions (Trial #2) using ESI ionization in positive ion mode. The x-axis represents retention time (min) for all chromatograms.
Figure 7.8: Diode array optical absorbance detection (DAD) and extracted ion chromatograms (EICs) for Acb [M+H]$^+$ ions from LCMS analysis of HPLC-purified Acb from *A. baumannii* ATCC 17978 using ESI ionization in positive ion mode. The x-axis represents retention time (min) for all chromatograms.
Figure 7.9: Diode array optical absorbance detection (DAD) and extracted ion chromatograms (EICs) for fimsbactin A–F [M+H]⁺ ions from LCMS analysis of HPLC-purified fimsbactin A from A. baumannii ATCC 17978 (Trial #1) using ESI ionization in positive ion mode. The x-axis represents retention time (min) for all chromatograms.
Figure 7. 10: Diode array optical absorbance detection (DAD) and extracted ion chromatograms (EICs) for fimsbactin A–F [M+H]^+ ions from LCMS analysis of HPLC-purified fimsbactin A from A. baumannii ATCC 17978 (Trial #2) using ESI ionization in positive ion mode. The x-axis represents retention time (min) for all chromatograms.
Figure 7. 11: High-resolution ESI MS (positive ion mode) of fimsbactin A purified by prep-HPLC from A. baumannii ATCC 17978 culture supernatant (Trial #1). Expected [M+H]^+ for C_{26}H_{31}N_{4}O_{11} 575.1984, found 575.2056.
**Figure 7.12:** $^1$H-NMR (600 MHz, DMSO-$d_6$) spectrum of purified fimbactin A purified by prep-HPLC from *A. baumannii* ATCC 17978. The x-axis is chemical shift given in parts per million (ppm). The y-axis is arbitrary peak intensity.
Figure 7.13: $^{13}$C-NMR (151 MHz, DMSO-$d_6$) spectrum of purified fimbactin A purified by prep-HPLC from *A. baumannii* ATCC 17978. The x-axis is chemical shift given in parts per million (ppm). The y-axis is arbitrary peak intensity.
Figure 7.14: Structures of Fim (A. baumannii), heterobactin (Rhodococcus erythropolis), and vibriobactin (Vibrio cholerae) siderophores. Metal chelating groups are shown in blue. The tetrahedral atom is highlighted by a salmon sphere.
Figure 7.15: Structures and microbial producers of amino acid-based siderophores fimsbactin A$^{76}$, heterobactin A$^{83}$, and JBIR-16$^{84}$, and spermidine-based siderophores vibriobactin$^{85}$, vulnibactin$^{86}$, fluvibactin$^{87}$, agrobactin$^{88}$, parabactin$^{89}$, and protochelin$^{90}$. Iron chelating groups are shown in blue. The tetrahedral branch point for metal chelating ligands is highlighted by a yellow circle.
**Figure 7.16:** Titration of Fim with (A) Fe(acac)$_3$ and (B) FeCl$_3$ in methanol. Graphs depict fluorescence ($\lambda_{\text{excitation}} = 330$ nm; $\lambda_{\text{emission}} = 380$ nm) vs equivalents of ferric iron source showing a titration end point correlating with a 1:1 final stoichiometry for the [Fe(Fim)] ferric complex. Titration with FeCl$_3$ was performed as two independent trials. Error bars represent standard deviation from the mean.

**Figure 7.17:** Optical absorbance spectrum of the holo-[Fe(Fim)] complex at 100 $\mu$M in phosphate buffer (50 mM potassium phosphate pH 8.0, 150 mM NaCl, 1 mM DTT, 5% glycerol). The molar extinction coefficient ($\varepsilon$) of holo-[Fe(Fim)] was determined to be 4255 M$^{-1}$ cm$^{-1}$ at 445 nm. The inset shows the visible color of the [Fe(Fim)] solution at 100 $\mu$M.
Figure 7. 18: Decay plots for EDTA competition assays with [Fe(Fim)]. Graphs depict absorbance at 500 nm (y-axis) vs time (x-axis) for (A) EDTA (1.2 equiv) plus [Fe(Fim)], (B) [Fe(Fim)], and (C) buffer only. Each experiment was performed in duplicate as independent trials. The apparent $K_{Fe}$ for [Fe(Fim)] was calculated using the final absorbance values after 150 minutes according to equations (1)–(11) shown on Equations 7.1. The final absorbance values at 500 nm were 0.3873 (trial #1 w/ EDTA), 0.3870 (trial #2 w/ EDTA), 0.4292 (trial #1 w/o EDTA), and 0.4357 (trial #2 w/o EDTA).
Figure 7.19: Titration of 100 μM [Fe(Acb)₂] with Fim reveals slow exchange of iron leading to complete formation of [Fe(Fim)]. Optical absorbance spectra were collected for each concentration after 20 min in phosphate buffer (50 mM potassium phosphate pH 8.0, 150 mM NaCl, 1 mM DTT, 5% glycerol). The final optical absorbance spectrum with 210 μM apo-Fim added was measured after 20 hours.
Figure 7.20: Influence of apo- and holo-siderophores on A. baumannii growth. Line graphs depict the growth of A. baumannii ATCC 17978 determined by measuring the optical density at 600 nm (OD$_{600}$) at 37 °C as a function of time in the presence of (a) Acb or [Fe(Acb)$_2$] and (b) Fim or [Fe(Fim)]. Bar graphs depict the comparison of OD$_{600}$ values after 30 h in the presence of variable concentrations of (c) Acb or [Fe(Acb)$_2$] and (d) Fim or [Fe(Fim)]. Error bars represent standard deviations from the mean for three independent trials, ****p < 0.0001.
Figure 7.21: Dose dependent growth promotion of *A. baumannii* ATCC 17978 by (A) apo-acinetobactin (Acb), (B) holo-acinetobactin (Acb_Fe), (C) apo-fimsbactin (Fim), and (D) holo-fimsbactin (Fim_Fe). Line graphs depict the growth of *A. baumannii* ATCC 17978 in M9 minimal medium supplemented with 175 μM 2,2’-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time in the presence of variable siderophore concentrations. All experiments were performed in triplicate. Error bars are shown in Figure 7.22. Data from these plots were used to create the line and bar graphs shown in Figure 7.20 in the main text.
Figure 7.22: Dose dependent growth promotion of *A. baumannii* ATCC 17978 by (A) apo-acinetobactin (Acb), (B) holo-acinetobactin (Acb_Fe), (C) apo-fimsbactin (Fim), and (D) holo-fimsbactin (Fim_Fe). Line graphs depict the growth of *A. baumannii* ATCC 17978 in M9 minimal medium supplemented with 175 μM 2,2’-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time in the presence of variable siderophore concentrations. Error bars represent standard deviations from the mean for three independent trials. Line graphs are shown without error bars for clarity in Figure 7.21. Data from these plots were used to create the line and bar graphs shown in Figure 7.20 in the main text.
Figure 7.23: Influence of apo- and holo-siderophore combinations on A. baumannii growth. Bar graphs depict the comparison of A. baumannii ATCC 17978 growth measured by optical density at 600 nm (OD600) values after 30 h at 37 °C in the presence of variable concentrations of (a) Fim and Acb, (b) Fim and [Fe(Acb)2], and (c) [Fe(Fim)] and Acb. Error bars represent standard deviations from the mean for two independent trials; ****p < 0.0001; ns = not significant.
### A. Acinetobactin + Fimbactin A

<table>
<thead>
<tr>
<th></th>
<th>[Acb]</th>
<th>[Fms A]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3.9 uM</strong></td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
</tr>
<tr>
<td><strong>15.6 uM</strong></td>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
</tr>
<tr>
<td><strong>62.5 uM</strong></td>
<td><img src="image5.png" alt="Graph" /></td>
<td><img src="image6.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>3.9 uM</th>
<th>15.6 uM</th>
<th>62.5 uM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time (hr)</strong></td>
<td><img src="image7.png" alt="Graph" /></td>
<td><img src="image8.png" alt="Graph" /></td>
<td><img src="image9.png" alt="Graph" /></td>
</tr>
</tbody>
</table>
B. Acinetobactin + Fimsbactin A - Fe

<table>
<thead>
<tr>
<th>[Acb]</th>
<th>3.9 uM</th>
<th>15.6 uM</th>
<th>62.5 uM</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Fims A - Fe]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.9 uM</td>
<td><img src="image1" alt="Graph" /></td>
<td><img src="image2" alt="Graph" /></td>
<td><img src="image3" alt="Graph" /></td>
</tr>
<tr>
<td>15.6 uM</td>
<td><img src="image4" alt="Graph" /></td>
<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
</tr>
<tr>
<td>62.5 uM</td>
<td><img src="image7" alt="Graph" /></td>
<td><img src="image8" alt="Graph" /></td>
<td><img src="image9" alt="Graph" /></td>
</tr>
</tbody>
</table>

Time (hr)

OD
Figure 7.24: Influence of apo- and holo-siderophore combinations on the growth of A. baumannii ATCC 17978. Line graphs depict the growth of A. baumannii ATCC 17978 in M9 minimal medium supplemented with 175 $\mu$M 2,2′-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time in the presence of variable concentrations of siderophore mixtures. For all graphs, siderophore concentration gradients are provide on the x-axis and y-axis of the checkerboard. The black line graph represents bacterial growth without addition of siderophores. The red line graph represents bacterial growth in the presence of variable concentrations of (A) apo-Fim and apo-Acb, (B) holo-FimFe and apo-Acb, (C) apo-Fim and holo-Acb$_2$Fe, or (D) apo-Fim and holo-FimFe. Error bars represent standard deviations from the mean for two independent trials. Data from these plots were used to create bar graphs shown in Figure 7.23 in the main text.
Figure 7. 25: Influence of apo-Fim holo-FimFe combinations on *A. baumannii* growth. Bar graphs depict the comparison of *A. baumannii* ATCC 17978 growth measured by optical density at 600 nm (OD$_{600}$) values after 30 hours at 37 °C in the presence of variable concentrations of (a) Fim and Acb, (b) Fim and Acb$_3$Fe, and (c) FimFe and Acb. Error bars represent standard deviations from the mean for two independent trials. ns = not significant
Figure 7.26: Siderophore-dependent fluorescence quenching of N-His$_6$-BauB. Graphs depict intrinsic tryptophan fluorescence quenching (y-axis: $\lambda_{\text{excitation}} = 280$ nm; $\lambda_{\text{emission}} = 320$ nm) of 400 nM N-His$_6$-BauB in the presence of variable siderophore concentrations (x-axis). Apparent $K_d$ values were calculated using a single-binding mode curve-fitting model in GraphPad Prism version 7.0b. Error bars represent standard deviations for two independent trials.
Figure 7. 27: [Fe(Acb)$_2$] and [Fe(Fim)] compete for BauB binding. N-His$_6$-BauB was immobilized on Ni-NTA resin and loaded with (a) [Fe(Acb)$_2$] or (b) [Fe(Fim)], washed with phosphate buffer, and eluted with a competing holo-siderophore. Column elutions were analyzed by LC-MS for [Fe(Acb)$_2$] ($m/z = 347$) and [Fe(Fim)] ($m/z = 627$) after each step. Extracted ion chromatograms (EIC) are shown for the initially bound holo-siderophore. EICs are representative for two independent trials.
Figure 7.28: Structural comparison of [Fe(PreAcb)₂]⁻, [Fe(Acb)₂]⁻, and [Fe(Fim)]⁻ complexes. DFT calculated structures of the monoanionic (a) 1:1 [Fe(Fim)]⁻ and (b) 2:1 cis-[Fe(PreAcb)₂]⁻ complexes (see Materials and Methods for DFT parameters). (c) Experimentally observed structure of the monoanionic cis-[Fe(Acb)₂]⁻ complex bound to the siderophore-binding protein BauB (PDB 6MFL). (d) Overlay of all three structures highlighting similarity of geometry and placement of ligands (ox, oxazoline; cat, catecholate; hx, hydroxamate; im, imidazole) around the ferric iron center. (e) Surface view of the siderophore-binding pocket of BauB occupied by cis-[Fe(Acb)₂]⁻ (PDB 6MFL).
Figure 7.29: Schematic overview of the PreAcb/Acb and Fim iron acquisition pathways in *A. baumannii*. The Fim pathway has not been experimentally characterized and is hypothesized based on homology to related pathways in Gram-negative bacteria. Periplasmic BauB is highlighted to show interactions with both PreAcb/Acb and Fim connecting the two pathways through competition for the siderophore-binding protein.
Figure 7.30: Antismash analysis of two deposited genomes of *A. baumannii* ATCC 19606 turned up acinetobactin and acinetoferrin/baumannoferrin as the only siderophore BGCs present in the genome. No fimsbactin BGCs were detected.
Figure 7.31: Dose dependent influence of apo-Fim and holo-FimFe on A. baumannii ATCC 19606T growth. Line graphs depict the growth of wild-type A. baumannii ATCC 19606T in M9 minimal medium supplemented with 175 μM 2,2′-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD$_{600}$) at 37 °C as a function of time in the presence of variable siderophore concentrations. Error bars represent standard deviations from the mean for three independent trials.
Figure 7.32: Dose dependent growth promotion of *A. baumannii* ATCC 19606T strains by *holo*-FimFe. Line graphs depict the growth of wild-type (wt), s1-mutant (insertional mutant in *basD*, deficient in PreAcb/Acb biosynthesis), t6-mutant (insertional mutation in *bauA*, deficient in PreAcb/Acb import to periplasm), and t7-mutant (insertional mutation in *bauD*, deficient in PreAcb/Acb import to cytoplasm) strains of *A. baumannii* ATCC 19606 in M9 minimal medium supplemented with 175 μM 2,2′-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD$_{600}$) at 37 °C as a function of time in the presence of variable siderophore concentration. Error bars represent standard deviations from the mean for three independent trials.
N-His$_6$-BauB in pET28bTEV (cleavable N-term His-tag):

Nucleotide:

\[
\begin{align*}
\text{AAAAGAGATATA} & \text{CAGAGCAGCATACATACATCATACACAGCGGCGGAAACC} \\
\text{GCGGTCAGCGTGCA} & \text{AGCCAAAGGTGCGATACCCAGCGGAGCCAA} \\
\text{AATGCGACATTAGGTTAAGCGACGCCTGGCGACACCGTGATGACACACCTGCC} \\
\text{GCCGACGGTGTTAGGACGTCGGAACCGTGGACGCCAACAAGCTGTCGAC} \\
\text{ATCAGGTTGTGCTCACAACAACCGTGTTAGCGACTAAGCGTGTTACGTT} \\
\text{CAAAGCTTGCAGTTAAGACCGGGCGAGCGTGTTGTTGACACACGGTGAC} \\
\text{ACCAGTTAGCAACGCCGATATCCTGATAATTGATCCTG} \\
\text{ACCGCGGCTGTAATGACACCCTGCGAACATCAAACCGGCGCAGCGTGAA} \\
\text{AAGCCCGCTGCGATGACTGATCAGTTAAGACGACCTGAGAAGCTGGAAGCCCG} \\
\text{ACCCACAAAG} \\
\text{AAAAGCTT}
\end{align*}
\]

Amino Acid:

\[
\begin{align*}
\text{GSSHHHHHHSSSSGNNLYQGGHMCQQKVAADTVQAASQKLAEPTVHKLVTGTYIDHPQRVA} \\
\text{VLDMNEADQDLNPMGKDYFLEKKDAQIDGLGAVPMMEYALKPDLYLMTPLKQNYQELSKPATIIHYDINFNNSHHGLVLKHDMLGKIFKEDLARQKVSE} \\
\text{LDEQVQKQVAVTANRPERALVLLHHNGAFLSNFGQPQRYGFIFNAFGVAPSGVVDSTLHGGQPISSEIKKADPILYIDVRTAVMEHRPNINAAASVENPLLRQTKAWKNGRVIIFVDADAW} \\
\text{YTTASPTSLKIVMEDVKKGYQ}
\end{align*}
\]

Figure 7. 33: Nucleotide and amino acid sequence of N-His$_6$-BauB in pET28bTEV (TEV-cleavable N-term hexahistidine-tag). Start and stop codons are underlined. The TEV cleavage site is indicated by an arrow ↓. The 969-bp bauB gene from Acinetobacter baumannii (Genbank Accession Number AAT52185) was used as the sequence template for subcloning.
7.9 References


involved in biosynthesis and transport of acinetobactin, a siderophore produced by Acinetobacter baumannii ATCC 19606T. *Microbiology* 150, 2587–2597.


47. Qi, B., and Han, M. (2018) Microbial siderophore enterobactin promotes mitochondrial iron uptake and development of the host via interaction with ATP synthase. *Cell* 175, 571–582.


Chapter 8: Conclusions and Perspectives
8.1 Preface

This chapter was written by Luting Fang (LF) and edited by Timothy Adam Wencewicz (TAW). Portions of figures in Sections 8.6, 8.7, and 8.8 appeared in the dissertation written by Dr. Tabbetha Bohac in 2019. Funding for research in this chapter was provided by the National Institute of Allergy and Infectious Diseases (NIAID-NIH R01-123394) for the tetracycline projects, the National Science Foundation through a CAREER Award (grant 1654611) for the siderophores projects, and the Children’s Discovery Institute at St. Louis Children’s Hospital (grant MI-PD-II-2018-748), the Research Corporation for Science Advancement through a Cottrell Scholar Award (CS-24056), the Alfred P. Sloan Foundation through a Sloan Fellowship Award (FG201810935), and the Dreyfus Foundation through a Camille Dreyfus Teacher-Scholar Award (TC-19-079) for the β-lactam projects.

8.2 Introduction

My dissertation research presented in the previous chapters collectively introduced the study, development, and application of β-lactams, tetracyclines, and siderophores as molecular-based approaches to overcoming antibiotic resistance. I realize the challenges that remain in this field, but also see the bright future and opportunities for innovation. In this final chapter, I will discuss the potential future directions for my research projects and preliminary studies that establish the start of new projects in the Wencewicz lab.

8.3 Summary of Dissertation

In Chapter 2, we studied the mechanism of glutamine synthetase (GS) inhibition by the novel beta-lactam antibiotic, tabtoxinine-β-lactam (TβL). We proposed that TβL can be
developed into an anti-TB drug using dipeptide prodrug formulations that penetrate the waxy \textit{Mtb} cell envelope as shown in Chapter 3. With our improved understanding of T\betaL mechanism of action, we can see the bright future for the analogs of this molecule, such as the naturally occurring C4-halogenated analog Cl-T\betaL. In Chapter 4, we isolated and purified Cl-tabtoxin, the Ala-Cl-T\betaL dipeptide form, from natural producer \textit{Streptomyces}. We cleaved the dipeptide prodrug to release free drug Cl-T\betaL using recombinant dipeptidase PepA from \textit{E. coli}. We identified the biosynthetic gene cluster (BGC) of Cl-T\betaL and C-tabtoxin using whole genome sequencing and comparative genomics. We validated this BGC using functional \textit{in vitro} reconstitution of biosynthetic enzyme activity. Specifically, we characterized three enzymes from the Cl-tabtoxin BGC (CabB, CabD, and CblA) to establish early steps in the biosynthetic pathway that break from lysine biosynthesis through CblA-catalyzed methylation of the C5-carbon of acetyl/succinyl-THDPA. This information allowed us to propose a unified complete biosynthetic pathway for T\betaL and Cl-T\betaL and divergent pathways for construction of the prodrug forms tabtoxin and Cl-tabtoxin, respectively, in different producing microbes (Pseudomonads and \textit{Streptomyces}, respectively). The genome mining method used in this project can help us to discover more T\betaL analogs that are produced using homologous pathways in nature. The \beta-lactams found in this way will tell a new story different from classic \beta-lactams such as penicillin and provide us with more potential inhibitors against a broad family of enzymes belong to ATP-dependent carboxylate-amine ligase superfamily. With the target expended from GS to other ATP-dependent carboxylate-amine ligases, we can not only treat diseases like TB, but also a wide variety of diseases where these enzymes play an important role in pathogenesis.
Tetracyclines have been one of the most widely used antibiotics around the world. However, we recently observed a new resistance mechanism against tetracyclines—enzymatic degradation by TDases. In order to prevent the upcoming global resistance against tetracyclines using this new mechanism, our lab put efforts in the development of the inhibitors against the TDases discussed in Chapter 5. In Chapter 6 we sought to understand the evolution of TDases in order to predict and prevent the emergence of super TDases. Chapter 6 also introduced tetracycline analogs that are able to be developed into diagnostic coloring agents for TDases.

In Chapter 7, we investigated a different path for drug development from antibiotics. We studied siderophores, which are small molecule ferric iron chelators that facilitate the transport of iron across the bacterial cell envelope. We introduced two siderophores in Chapter 7, fimsbactin A (FimA) and acinetobactin (Acb) from human pathogenic A. baumannii. We studied the functional relationship between these two endogenous siderophores to solve an apparent redundancy in iron acquisition pathways. We propose a unified model for the siderophore uptake pathways of FimA and Acb in A. baumannii. With the antibiotic resistance problem being more and more severe, siderophore are becoming attractive as potential antibacterial agents that can disrupt metal homeostasis in target pathogens through metal withholding or inhibition of siderophore utilization at by binding to multiple proteins in the transport pathway. Instead of killing bacteria outright by inhibiting one specific enzyme like traditional antibiotics, siderophore-based approaches can starve bacteria of nutrients to let native human microbes win the growth competition. FimA and Acb are only a tip of the iceberg for the siderophore world. More siderophores are awaiting discover and study. The relationships among siderophores is also an interesting subject to study since it is very common that bacteria can produce a bunch of different siderophores for iron uptake.
8.4 Investigation of folate pathway inhibitors

Introduction

Studying the mechanistic basis for TβL inhibition of GS in Chapter 2 inspired a new avenue for the development of β-lactam molecules as inhibitors against ATP-dependent carboxylate-amine ligase superfamily. Dihydrofolate Synthetase (DHFS) is a member of this protein superfamily. Same as GS catalyzed reaction (Figure 8.1a), DHFS catalyzes the coupling of L-glutamate with 7,8-dihydropteroate to form the dihydrofolate with the involvement of ATP (Figure 8.1b). DHFS is an important enzyme involved in folate biosynthetic pathway, which is only present in plants and microorganisms, but not mammals. Mammals must obtain nutritional folate from their diet through specific receptors for folate uptake. This feature makes the enzymes involved in folate biosynthetic pathway attractive targets for drug development since they are absent in human cells. The interruption of folate biosynthesis can restrict folate supply, which is required for the biosynthesis of pyrimidines and purines, amino acids such as methionine and glycine, as well as vitamins like pantothenic acid. In fact, the sulfonamides (Figure 8.2a), which act by inhibiting dihydropteroate synthase (DHPS), is a widely used clinical class of antibiotics. Sulfonamides are typically used in combination with inhibitors of dihydrofolate reductase (DHFR) such as Trimethoprim (Figure 8.2b) due to the synergistic nature of simultaneously blocking folate biosynthesis and reduction. Surprisingly, DHPS is the only clinically exploiting target in the folate biosynthetic pathway despite validation that inhibiting other folate biosynthetic enzymes like DHFS can also restrict folate supply (Figure 8.3).

Results
The opportunity to develop DHFS inhibitor as antibiotics inspired our lab to focus on the study of DHFS drug discovery since it is a potential new target for the strains that are resistant to well-developed drugs against certain enzymes in folate pathway. By studying the mechanism of TβL inhibition of GS (Figure 8.4a), we came up with a possible DHFS inhibitor (Figure 8.2c) that exploits the 3-hydroxy-beta-lactam (3-HβL) derived from the natural product TβL to leverage the mechanism-based inhibition mechanism shown in Figure 8.4b. The only difference between our proposed DHFS 3-HβL inhibitor (Figure 8.2c) from the natural substrate, pteroic acid, for DHFS (Figure 8.2e) is the incorporation of the 3-HβL ring in place of the carboxylate. We hypothesize that the 3-HβL inhibitor will bind competitively to the DHFS active site and undergo phosphoryl group transfer from ATP to form a tight-binding transition state mimic that prevents further coupling with glutamate and irreversibly blocks folate biosynthesis.

To support our proposed DHFS inhibitor has inhibition effect on DHFS enzyme, the dhfs gene from E. coli was cloned into pET28 protein expression vector and transformed into E. coli BL21 for over expression as the N-His₆-tagged construct. The protein was purified by Ni-NTA chromatography (Figure 8.5). Due the challenges of synthesizing folate analogs we sought to utilize a prodrug approach for accessing our proposed 3-HβL DHFS inhibitor. Brett Virgin-Downey in the Wencewicz lab synthesized 3-(p-NH₂-phenyl)-3-HβL as a prodrug that when treated with HPPK/DHPS enzymes can be converted to the corresponding DHFS 3-(p-NH₂-phenyl)-3-HβL inhibitor containing the folate pterin heterocycle (Figure 8.2d). HPPK and DHPS were cloned, expressed and purified in the same way as DHFS (Figure 8.6 and Figure 8.7).
Brett-Virgin Downey performed *in vitro* biochemical characterization of recombinant HPPK, DHPS, and DHFS. **Figure 8.8a** shows great activity of our recombinant HPPK and DHPS based on the formation of dihydropteroate confirmed by LC-MS analysis of enzyme reaction mixtures. We were also able to detect our proposed 3-HβL DHFS inhibitor by reacting synthetic 3-((p-NH₂-phenyl)-3-HβL with HMDP in the presence of HPPK/DHPS (**Figure 8.8b**). The activity of DHFS was confirmed by the detection of dihydrofolate formation (**Figure 8.8c**). With all three active enzymes and our potential inhibitor in hand, we are ready to test whether our proposed inhibitor can indeed inhibit DHFS. These studies are currently being performed by Brett Virgin-Downey in the Wencewicz lab.

**Conclusions**

We were able to reconstitute the enzymatic activity of recombinant HPPK, DHPS, and DHFS enzymes from *E. coli*. We were able to synthesize 3-((p-NH₂-phenyl)-3-HβL and enzymatically convert this molecule to our proposed 3-HβL DHFS inhibitor by treatment with HPPK and DHPS. Future studies are aimed at validating 3-HβLs as antifolate DHFS inhibitors to demonstrate that the 3-HβL ring can be a universal pharmacophore for the design and development of drugs against ATP-dependent carboxylate-amine ligase superfamily.

**8.5 Exploration of other enzymes in Cl-tabtoxin BGC**

In Chapter 4, we characterized 3 enzymes from the Cl-tabtoxin BGC: CabB, CblA, and CabD. We also proposed a complete pathway for the biosynthesis of TβL and a portion of Cl-tabtoxin (**Figure 8.9**). In the future, we would like to investigate all of the enzymes involved in...
this BGC, and validate the proposed pathway including the enzyme function as well as the
enzyme reaction order.

CabC (or TabC) and CabA (or TabA) are the two enzymes following CabD in the
proposed pathway. CabC is predicted to take succinyl/acetyl group off from CH$_3$-succinyl/acetyl-
DAP, by functioning as a peptidase. This prediction is made by comparing with the lysine
biosynthetic pathway (Figure 8.10), where desuccinylation/deacetylation happens right after the
aminotransferation by DapC (homologue of TabD and CabD). After that, the decarboxylation is
carried out by LysA, the homologue of CabA and TabA (Table 4.2). Thus, we propose that for	
tabtoxin pathway, the CabA functions as decarboxylase to release the carboxyl group from CH$_3$-
DAP following CabC (Figure 8.10).

In order to test our hypothesis, we ordered synthetic genes in a pET28 vector from
GenScript encoding CabC and CabA proteins (Table 8.1). No further action was made after this
point. For future experimental plans, we will transform the plasmids into E. coli BL21 for over-
exression, followed by purification and filtered concentration. Unlike TabB and TabD, there is
not any previous study available about TabC and TabA from P. syringae. Based on our current
knowledge, a possible problem for CabC expression is stability given it is predicted to be a
peptidase that could potentially result in self-cleavage. Another possible issue for all new
proteins is the solubility. As reported in Calderone’s study about TabB and TabD, they had
problems in obtaining other enzymes in the gene cluster due to the insoluble expression.$^5$
Assuming soluble active proteins are obtained, we will incubate enzymes individually or in
combination with additional biosynthetic enzymes including CabC or CabA with CabB, CblA,
and CabD using LC-MS to search for product formation. If enzyme conversion is good enough,
we might also be able to get enough purified products from enzyme reactions for NMR analysis.
With LC-MS and NMR characterization of reaction products we will be able to propose functions, preferred substrates, and exact identities of biosynthetic intermediates for Cl-tabtoxin.

The parallel biosynthesis of lysine and TβL comes to an end at the step of CabA. The enzymes after CabA: TblD (CblD), TblC (CblC), TblS (CblS) and TabP are predicted to be involved in β-lactam ring formation. Once TβL is produced, it could be loaded on to the T domain of NRPS system for the ligation with alanine and the addition of the chlorine atom to form Cl-tabtoxin as proposed in Chapter 4. Further experiments are needed to support the β-lactam ring formation and the NRPS system hypothesis.

8.6 Fimsbactin A analogs

Introduction

In Chapter 7, we introduced Fimsbactin A (FimA), a siderophore produced by A. baumannii, which can promote the bacteria growth. We were also interested in the mechanism for the transportation and binding with siderophore binding protein BauB for FimA. For this purpose, three FimA analogs (Figure 8.11 and Figure 8.12) were synthesized by Dr. Tabbetha Bohac, a former graduate student in the Wencewicz lab, and Victoria Banas, current graduate student in the Wencewicz lab. These analogs have different properties at natural pH: analog 1 is negatively charged, analog 2 is neutrally charged, and analog 3 is positively charged. We sought to investigate the impact of charge on Fimsbactin’s function. The earlier studies indicated that Fimsbactin can only reach periplasm, instead of cytoplasm.6,7 We would like to know whether the change of charge can help Fimsbactin get into the cell. Can this change create growth inhibitor instead of growth promoter, or can this change increase the level of growth promotion?
With this study, we hopefully are able to obtain a hint for the development direction for Fimsbactin analogs.

**Result and Discussion**

The whole cell study of all three FimA analogs on *A. baumannii* growth shows that all of them can promote the growth of *A. baumannii* in a dose-dependence manner at the similar promotion level ([Figure 8.13](#) and [Figure 8.14](#)). Same as FimA, for all the analogs, the *holo*-variants has better promotion level than *apo*-variants. Consistently, the iron affinity and BauB binding affinity shows similar level among the three analogs, along with FimA.

Based on the similar behavior between FimA and analogs, we further investigated whether the analogs utilized the same pathway as FimA. For this purpose, we synthesized gallium (Ga) loaded FimA analogs. Ga-siderophores mimic the Fe-siderophores contacts, but cannot be reduced in periplasm or cytoplasm, thus siderophores will be trapped with Ga. Ga-siderophores can then inhibit the growth of *A. baumannii* because of the limited free siderophores, and limited iron transported by siderophores ([Figure 8.15](#), “Ga” group). We then added the *holo*-siderophores: FimFe and AcbFe with the addition of variable concentrations of Ga-FimsA analogs. In theory, if the analogs utilize the same siderophores uptake pathway as FimA and Acb, then the growth inhibition will get recovered by the addition of *holo*-siderophores since they will compete with the Ga-FimsA analogs, and can supply bacteria with iron. In fact, we did observe the growth recovery ([Figure 8.15](#), “Ga + FimFe” and “Ga + AcbFe” groups). Actually, the growth recovery is dose-dependent based on the concentrations of Ga-FimA analogs. Specifically, the growth recovery effect is worse under the high concentration of Ga-FimA analogs conditions than low concentration conditions ([Figure 8.16](#), [8.17](#), and [8.18](#)).
The better growth recovery with addition of FimFe than AcbFe indicates a direct competition of FimA analogs with FimA, and confirmed the analogs not only structurally mimics FimA, but also functionally mimics FimA. The growth recovery by AcbFe confirmed the hypothesis in Chapter 7 that Acb and FimA utilize the same siderophores uptake pathway, and compete with each other for the periplasmic siderophore binding protein BauB.

Conclusions

Although we didn’t find the relationship of growth recovery with the ion charge property for the three FimA analogs, we were able to support the hypothesis in Chapter 7 that Acb and FimA compete on the binding of BauB, and utilize the same siderophore pathway. We also confirmed that FimA analogs not only structurally, but also functionally mimics FimA. Based on this result, for future directions, we are able to obtain more FimA analogs with this simplified structure to study the mechanism of FimA uptake pathway.

8.7 FimF investigation

Introduction

In Chapter 7, we introduced the two siderophores produced by A. baumannii: acinetobactin (Acb) (Figure 8.19a) and Fimsbactin A (FimA) (Figure 8.19b). However, they are not the only siderophores produced by A. baumannii. A. baumannii also produces a class of Fimsbactin isomers B-F. In particular, we are interested in FimF (Figure 8.19c), which misses the seryl-O-2,3-DHB ester from FimA. We hypothesized FimF might be formed from FimA by an esterase cleavage. We also sought to investigate the relationship among Acb, FimA, and FimF to explore the siderophore redundancy impact on A. baumannii growth.
Results and discussion

The isolation and purification of FimA, FimF, and Acb from \( A. \text{baumannii} \) ATCC 17978 was performed by Dr. Tabbetha Bohac. In the \( A. \text{baumannii} \) whole cell assay, we observed a dose-dependent growth promotion with the addition of FimF under the iron-limited media condition (Figure 8.20a). The growth promotion is enhanced with the \textit{holo}-variant (FimF\textsubscript{Fe}) (Figure 8.20c), which also shows dose-dependent feature (Figure 8.20b).

In Chapter 7, we found the competition relationship for siderophore combinations, where high concentration of FimA with low or high concentration of Acb can cause the inhibition of \( A. \text{baumannii} \) growth. Herein, we are interested in the impact for FimF on the combinations with other siderophores. We performed checkerboard-combination growth studies with FimF in combination with Acb or FimA, along with the \textit{holo}-variant of three compounds. In specific, the combinations performed are: FimA & FimF, FimA\textsubscript{Fe} & FimF, FimA & FimF\textsubscript{Fe} (Figure 8.21 and Figure 8.22); Acb & FimF, Acb\textsubscript{Fe} & FimF, Acb & FimF\textsubscript{Fe} (Figure 8.23 and Figure 8.24). Interestingly, we observed that high concentration of FimA can cause the inhibition on \( A. \text{baumannii} \) growth with low concentration of FimF, which is the same result with the combination of FimA and Acb. However, different from FimA & Acb combination, the growth can be recovered by increasing the concentration of FimF, where high concentration of Acb can’t recover the growth. On the other hand, the combination of Acb with FimF doesn’t have any inhibition on \( A. \text{baumannii} \) growth. Instead, higher concentration of siderophores increases the growth promotion across the board. This result is different from Acb & FimA combination, which has the competitive relationship. Instead, Acb and FimF has a cooperative relationship. The new relationship of FimF with both FimA and Acb makes us to look at the relationship of
siderophores in a different way other than competitive. The relationship is more complicated than we thought, and it is more about balance.

Based on our current observation and understanding, we proposed a potential pathway regarding FimA, FimF, and Acb in the transportation of Fe and binding to the siderophores binding proteins (Figure 8.25). FimA is biosynthesized inside of the cell, and efﬂuxed out of the cell to bind iron in the environment. It is known that FimA is unable to enter the inner-membrane, and it can only have access to the periplasm. Based on this information, we propose that the holo-FimA is then transported into the periplasm, followed by the release of iron, which is transported to the cytoplasm for further use, as well as the conversion into FimF by esterase. Apo-FimF can then form a 2:1 complex with iron, may be taken from holo-Acb, bind to BauB, and transport inside of the cell to release iron. Now apo-FimF might be recycled into FimA and repeat the cycle for iron acquisition. The additional step of FimA conversion into FimF is beneﬁcial for FimA recycle, and also help explain the cooperative relationship between FimF and Acb since they both help to transport iron into the cells. This theory can also explain the non-competitive relationship between FimA and FimF since FimF not only doesn’t compete with FimA for the binding with BauB, which is the case of FimA and Acb, but also help the transportation of iron into cells, and help the recycle of FimA.

Based on the structure similarity between FimA and FimF, we proposed that FimF is formed from FimA by an esterase cleavage. By investigating the Fimsbactin gene cluster reported by Bode’s work in 2013, we think the enzyme we are looking for is FbsM (predicted function as thioesterase). The gene encoding FbsM was cloned in pET28, transformed into E.coli BL21 for over-expression, followed by Ni-NTA puriﬁcation (Figure 8.26). We also cloned out and obtained BesA with the same procedure (Figure 8.27). BesA is a well-known esterase for
ferri-bacillibactin\textsuperscript{9}, which we think also have the potential to convert FimA to FimF. We used nitrophenyl acetate (Figure 8.28) as a test model to confirm the activity of FbsM and BesA as esterase, which showed great cleavage of ester bond with obvious visually color change as well as the detection of product with LC-MS. No further experiments were conducted at this stage. Future studies will utilize recombinant FbsM or BasA with FimA as the substrate in an attempt to produce FimF.

**Conclusions and Future directions**

We isolated and purified a siderophore FimF, and showed it has the ability to promote the growth of *A. baumannii*. The combination study of FimF with FimA and Acb provides a new way to look at the relationships among different siderophores, and help us have a better understanding in the redundancy of siderophores production in *A. baumannii*. We invested in a potential enzyme that convert FimA to FimF: FbsM, but further study is needed to support this hypothesis.

For future direction, we would like to investigate the whole gene cluster to study the biosynthetic pathway of Fimsbactin, including all of the isomers A-F. We can also study more combinations of siderophores to have a better understanding of the balance of siderophore relationships, as well as the siderophore transportation/uptake mechanism. With more knowledge of the siderophores uptake mechanism, we can design inhibitors to interrupt the siderophores uptake pathway, and thus kill the bacteria by starving it of its iron source. Since siderophores are unique to bacteria, and aren’t present in plants or mammals, it can avoid the issue of off-target effects to human, animals, and plants.
8.8 Oxidized Pre-Acinetobactin as A. baumannii growth inhibitor

It was reported that Pre-Acinetobactin (PreAcb) (Figure 8.29a) is a growth promoter, however, the oxidized PreAcb (OxypreA) (Figure 8.29b), is the growth inhibitor. In order to understand the reason for this huge functional change with the little structural change, we preformed the OxypreA-Ga study. The presence of OxypreA-Ga causes the growth inhibition (Figure 8.30). However, the inhibition is recovered with the addition of holo-siderophores (Fim-Fe, Acb-Fe, and OxypreA-Fe) (Figure 8.31 and Figure 8.32). The growth recovery indicates that the earlier growth inhibition without holo-siderophores was not caused by the toxicity from Ga. Instead, it was because OxypreA-Ga interrupted the siderophore pathway by competing with other siderophores produced by A. baumannii (Figure 8.33). In fact, we found that OxypreA-Ga were able to bind BauB, a siderophore binding protein, at the same level as natural siderophores. Based on this result, we proposed that the inhibition on A. baumannii growth caused by OxypreA was because it can interrupt the natural siderophore uptake system by competitively binding to BauB. More details about this project can be found in the thesis of Dr. Tabbetha Bohac, Chapter 6.

8.9 Conclusion

Mechanistic study of TβL inhibition on GS provided us a new direction for developing β-lactams into ATP-dependent carboxylate-amine ligases’ inhibitors. We proposed an inhibitor against DHFS following the strategy of TβL. We obtained active HPPK, DHPS and DHFS enzymes, and are ready to perform the experiment to test whether it can be an inhibitor of DHFS.
This study will not only provide a new path for anti-folate drug development, but also confirmed our hypothesis that β-lactams can be developed into drugs against a wide variety of enzymes in ATP-dependent carboxylate-amine ligase superfamily. We would like to investigate our proposed whole TβL/Cl-TβL biosynthetic pathway, and we will start with CabA and CabC as the next two enzymes to characterize.

We synthesized three FimA analogs and showed it has the same function as natural FimA. These analogs are easily accessible via chemical synthesis and represent an attractive chassis for the development of siderophore-based therapeutics. We confirmed our hypothesis that FimA shared a portion of the same siderophore uptake pathway with Acb by competing for the periplasmic siderophore-binding protein BauB. We would like to investigate more naturally produced siderophores, and the relationships among siderophores in order to answer the question that why bacteria produce a various of siderophores and how these apparent redundancies are managed. By studying the biosynthetic intermediate/shunt product FimF, we gained a better understanding of this subject. It is not only about competition when multiple siderophores are at play, but more about balance. The topic about OxyPreA as a siderophore disrupting agent is very interesting, and we would like to find out the reason making it a growth inhibitor instead of promoter, which most of the siderophores are.
### 8.10 Figures and Tables

<table>
<thead>
<tr>
<th></th>
<th>Genscripts (start with: cat; end with: taaaagctt)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CabA (TAW3_06392)</td>
<td>5’ atgcggattccgatgcgttgtgcggggctgtgatagctgacttcggccacccgttctacggttattcgcggatcgcgtttgccctacgagccgatggcgattgcggaggcggtgcgtggtgttgcgtatttaccaacgagccgatggcgattgcggaggcggtgcgtggtgttgcgttctggccgccagcggcgagctacgcgctgggttgcctggacctggcggcggcgaccccggatctgggtgatggtttcgtcgtgcgctggcgggtctgcgtggcgcggtggaagcggagtggcagcgtgttaaggaaatcggtattccggcgcgtcgtcgtttcaacgagccgatggcgattgcggaggcggtgcgtggtgttgcgaaacacctgccgggtgaagcggatcgtcgtgcggcggagctgcgtgcgaacgcggtgcagga</td>
<td>MPIPDAFAARLEP LLGDVMVAYGTP FHVYDAAGIADTY RAMTDAFAGEPFR QYFAVKALPNPHV LALLLAEGSGLDC ASPVELELAASLG AGPDGTVFTSSNT TRAEFELAIKTGLS ITFDDRTALDRTD PLPETVAFRVSPH GLAAGSRLMGDA RATKFGVPVDQLA DAYRQAKARGAR RFGIHGMCANEML DVDRAAARATDV VELGARIAEVEGIE LDYVNVGGGLGIP YRPGDPVFDRRY AEAVALAARRAAFP RSAPRILMCEGRY VTPGHGLVTTVV NRCGKGEIVGVDR ASMSALMRPGFY GAYHHVTLPFAH GRAQAPYDVVGS LCNEMDKFAIDRP LPDREPGDLVLLVH DTGAHGLAMGFT YNRLRPAELLTL REGDVVEIRRAETYDDYVATVDPDR GPVLPAAARHA</td>
<td></td>
</tr>
<tr>
<td>CabC (TAW3_06394)</td>
<td>5’ atgcggattccgatgcgttgtgcggggctgtgatagctgacttcggccacccgttctacggttattcgcggatcgcgtttgccctacgagccgatggcgattgcggaggcggtgcgtggtgttgcgtatttaccaacgagccgatggcgattgcggaggcggtgcgtggtgttgcgttctggccgccagcggcgagctacgcgctgggttgcctggacctggcggcggcgaccccggatctgggtgatggtttcgtcgtgcgctggcgggtctgcgtggcgcggtggaagcggagtggcagcgtgttaaggaaatcggtattccggcgcgtcgtcgtttcaacgagccgatggcgattgcggaggcggtgcgtggtgttgcgaaacacctgccgggtgaagcggatcgtcgtgcggcggagctgcgtgcgaacgcggtgcagga</td>
<td>MPDRFDYPSNLAR AGSYRTHRSAFWP PAASYALGCLDLA AATPDLGDGFRRA LAGLRGAVEAEW GAYHHVTLPFAH GRAQAPYDVVGS LCNEMDKFAIDRP LPDREPGDLVLLVH DTGAHGLAMGFT YNRLRPAELLTL REGDVVEIRRAETYDDYVATVDPDR GPVLPAAARHA</td>
<td></td>
</tr>
</tbody>
</table>
Table 8.1: DNA and protein sequence of codon optimized genes and CabA and CabC proteins.
**Figure 8.1:** Reactions catalyzed by glutamine synthetase (GS) and dihydrofolate synthetase (DHFS). a) Mechanism of GS catalyzed reaction. b) Mechanism of DHFS catalyzed reaction (stereochemistry is not shown here).
Figure 8.2: Structures of important compounds related to the development of new antifolates. c) also named as “proposed 3-HβL DHFS inhibitor”, d) also named as “3-(p-NH₂-phenyl)-3-HβL”.

Figure 8.2: Structures of important compounds related to the development of new antifolates. c) also named as “proposed 3-HβL DHFS inhibitor”, d) also named as “3-(p-NH₂-phenyl)-3-HβL”.
Figure 8.3: A portion of the folate biosynthetic pathway in bacteria (only the steps we are interested in are shown in this figure).
Figure 8. 4: a) Mechanism of ATP-dependent TβL inhibition of GS. b) Proposed mechanism of ATP-dependent inhibition of DHFS by our proposed 3-HβL inhibitor. (b-7,8-dihydropteroate also named as “proposed 3-HβL DHFS inhibitor”, “TbL” and “TbL-Pi” also named as “TβL” and “TβL-Pi”)
Figure 8.5: SDS-PAGE analysis of recombinant N-His$_6$-tagged DHFS (45kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gels were stained with Coomassie blue.)
**Figure 8. 6:** SDS-PAGE analysis of recombinant N-His$_6$-tagged HPPK (18kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
**Figure 8.7:** SDS-PAGE analysis of recombinant N-His$_6$-tagged DHPS (31kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 8.8: HPPK, DHPS and DHFS enzyme assay results. a) HPPK/DHPS coupled reaction to form dihydropteroate from natural substrate \( p \)-ABA and HMDP. b) HPPK/DHPS coupled reaction to form proposed DHFS inhibitor \( \beta \)-dihydropteroate from synthesized substrate \( \beta \)-\( p \)-ABA and natural substrate HMDP. c) The formation of dihydrofolate from DHFS catalysed reaction with glutamate and dihydropteroate as substrate. Credit: Brett Virgin-Downey.
Figure 8.9: Proposed TβL biosynthetic pathway in *P. syringae*.
Figure 8.10: Comparison of Lysine and TβL biosynthetic pathways. (Red shows the difference between products and substrates from the enzymatic reaction, dashed line indicates the same steps between two pathways.)
**Figure 8.11:** Natural Fimsbactin A and synthetic Fimsbactin Analogs. Common metal binding moieties are highlighted – catechol (blue) and hydroxymate (red). Each compound contains a similar tetrahedral center denoted with a *. Credit: Dr. Tabbetha Bohac
Figure 8.12: Naturally produced siderophore Fimsbactin A and three synthetic analogs. Each analog contains a different sidechain substituent which are negatively (analog 1), neutral (analog 2) and positively (analog 3) charged, respectively, as neutral pH. Credit: Dr. Tabbetha Bohac

Figure 8.13: Influence of apo- and holo-fimsbactin analogs on A. baumannii growth. Bar graphs depict the growth of A. baumannii ATCC 17978 determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time in the presence of 15.625 µM concentration of either the apo- or holo- form of each Fimsbactin analog. Error bars represent standard deviations from the mean for three independent trials. ****p < 0.0001. Full growth curves at variable concentration seen in Figure 8.14. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
Figure 8.14: Influence of apo- and holo-fimsbactin analogs on A. baumannii growth. Line graphs depict the growth of A. baumannii ATCC 17978 determined by measuring the optical density at 600 nm (OD\textsubscript{600}) as a function of time in the presence of variable concentration of either the apo- or holo- form of each Fimsbactin analog. Error bars represent standard deviations from the mean for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
Figure 8.15: Growth inhibition by each Fimsbactin analog-Ga complex and the growth recovery of variable Fimsbactin analog-Ga (0.975µM or 31.25µM) and 3.9µM FimA-Fe or Acb-Fe. Bar graphs depict the growth of *A. baumannii* ATCC 17978 determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time. Error bars represent standard deviations from the mean for three independent trials. ****p < 0.0001, ns: non-significant. Full growth curves at variable concentration seen in Figure 8.16, 8.17 and 8.18. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
Figure 8. 16: a) Dose dependent growth inhibition by Fimsbactin analog 1-Ga complex. b) 3.9µM Fim-Fe and variable Fimsbactin analog 1-Ga complex. c) 3.9µM Acb-Fe and variable Fimsbactin analog 1-Ga complex. Line graphs depict the growth of A. baumannii ATCC 17978 determined by measuring the optical density at 600 nm (OD\text{600}) as a function of time. Error bars represent standard deviations from the mean for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabetha Bohac, 2019.
Figure 8. 17: a) Dose dependent growth inhibition by Fimsbactin analog 2-Ga complex. b) 3.9µM Fim-Fe and variable Fimsbactin analog 2-Ga complex. c) 3.9µM Acb-Fe and variable Fimsbactin analog 2-Ga complex. Line graphs depict the growth of *A. baumannii* ATCC 17978 determined by measuring the optical density at 600 nm (OD_{600}) as a function of time. Error bars represent standard deviations from the mean for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabetha Bohac, 2019.
Figure 8. 18: a) Dose dependent growth inhibition by Fimsbactin analog 3-Ga complex. b) 3.9µM Fim-Fe and variable Fimsbactin analog 3-Ga complex. c) 3.9µM Acb-Fe and variable Fimsbactin analog 3-Ga complex. Line graphs depict the growth of *A. baumannii* ATCC 17978 determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time. Error bars represent standard deviations from the mean for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
Figure 8. 19: Structures of siderophores from human pathogenic A. baumannii. a) Acinetobactin (Acb). b) Fimsbactin A (FimA). c) Fimsbactin F (FimF).
Figure 8. Dose dependent growth promotion of *A. baumannii* ATCC 17978 by a) apo-Fimsbactin F and b) holo-Fimsbactin F. Line graphs depict the growth of *A. baumannii* ATCC 17978 in M9 minimal media supplemented with 175 µM 2,2'-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD$_{600}$) as a function of time in the presence of variable siderophore concentrations. All experiments were performed in triplicate. Data from these plots were used to create the bar graphs in c) influence of apo- and holo-siderophore on *A. baumannii* growth depicting the comparison of OD$_{600}$ values after 30 hours in the presence of variable concentrations of FimF and FimF$_{Fe}$. Error bars represent standard deviations from the mean for three independent trials. ****p<0.0001, ***p<0.001. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
Figure 8.21: Influence of apo- and holo-siderophore combinations on A. baumannii growth. Bar graphs depict the comparison of A. baumannii ATCC 17978 growth measured by optical density at 600 nm (OD$_{600}$) values after 30 hours in the presence of variable concentrations of a) FimA and FimF, b) FimAFe and FimF, and c) FimA and FimFFe. Error bars represent standard deviations from the mean for two independent trials. ****p<0.0001; **p<0.01; *p<0.1; ns = not significant. Figure 8.22 shows full growth curve data. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
A. Fimsbactin F + Fimsbactin A

<table>
<thead>
<tr>
<th></th>
<th>3.9 uM</th>
<th>15.6 uM</th>
<th>62.5 uM</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Fims F]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.9 uM</td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
<td><img src="image3.png" alt="Graph" /></td>
</tr>
<tr>
<td>15.6 uM</td>
<td><img src="image4.png" alt="Graph" /></td>
<td><img src="image5.png" alt="Graph" /></td>
<td><img src="image6.png" alt="Graph" /></td>
</tr>
<tr>
<td>62.5 uM</td>
<td><img src="image7.png" alt="Graph" /></td>
<td><img src="image8.png" alt="Graph" /></td>
<td><img src="image9.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[Fims A]</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9 uM</td>
<td><img src="image10.png" alt="Graph" /></td>
<td><img src="image11.png" alt="Graph" /></td>
<td><img src="image12.png" alt="Graph" /></td>
</tr>
<tr>
<td>15.6 uM</td>
<td><img src="image13.png" alt="Graph" /></td>
<td><img src="image14.png" alt="Graph" /></td>
<td><img src="image15.png" alt="Graph" /></td>
</tr>
<tr>
<td>62.5 uM</td>
<td><img src="image16.png" alt="Graph" /></td>
<td><img src="image17.png" alt="Graph" /></td>
<td><img src="image18.png" alt="Graph" /></td>
</tr>
</tbody>
</table>
B. Fimbactin F + Fimbactin A-Fe
C. Fimsbactin F-Fe + Fimsbactin A

<table>
<thead>
<tr>
<th>Fims F - Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 uM</td>
</tr>
<tr>
<td>3.9 uM</td>
</tr>
<tr>
<td>15.6 uM</td>
</tr>
</tbody>
</table>

[Graphs showing growth of A. baumannii ATCC 17978 with various siderophore concentrations.]

**Figure 8.22:** Influence of apo- and holo-siderophore combinations on the growth of *A. baumannii* ATCC 17978. Line graphs depict the growth of *A. baumannii* ATCC 17978 in M9 minimal media supplemented with 175 µM 2,2’-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD₆₀₀) as a function of time in the presence of variable concentrations of siderophore mixtures. For all graphs, siderophore concentration gradients are provide on the x-axis and y-axis of the checkerboard. The black line graph represents bacterial growth without addition of siderophores. The red line graph represents bacterial growth in the presence of variable concentrations of (A) apo-FimA and apo-FimF, (B) holo-FimAFe and apo-FimF, or (C) apo-FimA and holo-FimF₂Fe. Error bars represent standard deviations from the mean for two independent trials. Data from these plots were used to create bar graphs shown in Figure 8.21. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
Figure 8.23: Influence of apo- and holo-siderophore combinations on *A. baumannii* growth. Bar graphs depict the comparison of *A. baumannii* ATCC 17978 growth measured by optical density at 600 nm (OD<sub>600</sub>) values after 30 hours in the presence of variable concentrations of a) Acb and FimF, b) AcbFe and FimF, and c) Acb and FimFFe. Error bars represent standard deviations from the mean for two independent trials. **p<0.01; *p<0.1; ns = not significant. Figure 8.24 shows full growth curve data. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
B. Fimsbactin F + Acb-Fe

<table>
<thead>
<tr>
<th>[Fims F]</th>
<th>3.9 uM</th>
<th>15.6 uM</th>
<th>62.5 uM</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Acb - Fe]</td>
<td>0.5 uM</td>
<td>3.9 uM</td>
<td>15.6 uM</td>
</tr>
<tr>
<td></td>
<td>0.5 OD</td>
<td>1.5 OD</td>
<td>1.5 OD</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Time (hr)</td>
<td>0</td>
<td>20</td>
<td>40</td>
</tr>
</tbody>
</table>

512
Figure 8.24: Influence of apo- and holo-siderophore combinations on the growth of *A. baumannii* ATCC 17978. Line graphs depict the growth of *A. baumannii* ATCC 17978 in M9 minimal media supplemented with 175 µM 2,2'-dipyridyl (DIP) determined by measuring the optical density at 600 nm (OD<sub>600</sub>) as a function of time in the presence of variable concentrations of siderophore mixtures. For all graphs, siderophore concentration gradients are provided on the x-axis and y-axis of the checkerboard. The black line graph represents bacterial growth without addition of siderophores. The red line graph represents bacterial growth in the presence of variable concentrations of (A) apo-Acb and apo-FimF, (B) holo-AcbFe and apo-FimF, or (C) apo-Acb and holo-FimFFe. Error bars represent standard deviations from the mean for two independent trials. Data from these plots were used to create bar graphs shown in Figure 8.23. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
Figure 8.25: Hypothesized potential relationship between FimA and FimF. We hypothesize Fimsbactin can compete with some acinetobactin transport proteins, as we already demonstrated a competition for siderophore binding protein, BauB. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
Figure 8. 26: SDS-PAGE analysis of recombinant N-His$_6$-tagged FbsM (31kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 8.27: SDS-PAGE analysis of recombinant N-His$_6$-tagged BasA (33kDa). (Bio-Rad precision plus protein dual Xtra prestained protein standards #1610377 is used as ladder. Gel was stained with Coomassie blue.)
Figure 8.28: BesA/FbsM test reaction using the module molecule nitrophenyl acetate.

Figure 8.29: Chemical structures of pre-acinetobactin (PreAcb) and oxidized pre-acinetobactin (OxypreA).
Figure 8. 30: OxPreAcb_Ga inhibits *A. baumannii* ATCC17978 growth. Growth curves of *A. baumannii* in M9 minimal medium supplemented with 125 μM 2,2'-dipyridyl (DIP) and gradient concentrations of OxPreAcb_Ga. Error bars represent s.d. for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.

Figure 8. 31: Growth studies of *A. baumannii* ATCC17978 in the presences of OxPreAcb-Ga (1-Ga) (7.8 μM) and Fims-Fe (31.25 μM, 0.975 μM); OxPreAcb-Ga (1-Ga) (7.8 μM) and (Acb)_2-Fe (31.25 μM, 0.975 μM); OxPreAcb-Ga (1-Ga) (7.8 μM) and OxPreAcb-Fe (31.25 μM, 0.975 μM); OxPreAcb (250 μM, 6.25 μM); OxPreAcb-Ga (250 μM, 7.8 μM). Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabbetha Bohac, 2019.
Figure 8. 32: 7.8 μM OxPreAcb-Ga with variable Fims-Fe, Acb-Fe and OxPreAcb-Fe growth studies against A. baumannii ATCC17978. Growth in M9 minimal medium supplemented with DIP. Error bars represent s.d. for three independent trials. Data generated by Luting Fang. Figure appeared in dissertation by Dr. Tabetha Bohac, 2019.
Figure 8.33: OxPreAcb inhibits growth of *A. baumannii* by disruption of the siderophore pathway, as shown by the ability of OxPreAcb to bind periplasmic siderophore binding protein, BauB. Credit: Dr. Tabetha Bohac
8.11 References