Sideromycin Pathway Elucidation: Insights into Salmycin Biosynthesis, Transport Paradigms, and Drug Release

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Sideromycin Pathway Elucidation: Insights into Salmycin Biosynthesis, Transport Paradigms, and Drug Release
by
Gerry Sann M. Rivera

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
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Gerry Sann M. Rivera

Washington University in St. Louis

December 2019
Dedicated to me.
ABSTRACT OF THE DISSERTATION

Sideromycin Pathway Elucidation: Insights into Salmycin Biosynthesis, Transport Paradigms, and Drug Release

By

Gerry Sann M. Rivera

Doctor of Philosophy in Chemistry

Washington University in St. Louis, 2019

Professor Timothy A. Wencewicz, Chair

Antibiotic resistance is an increasing threat in today’s society. In order to overcome resistant bacteria, it is necessary to discover new drugs with novel mechanisms of action. This work focuses on the sideromycin pathway, encompassing the biosynthetic production, mechanism of entry and hydrolysis-mediated drug release. Sideromycins are an interesting approach to combat the rise of antibiotic resistance since they provide a different avenue that overcomes problems that arise when entering the cell. The dissertation is separated into distinct sections dealing with the various areas of interest in the sideromycin pathway, particularly for the sideromycin, salmycin, produced by Streptomyces violaceus. The first two sections encompass the mechanism of entry through the siderophore transport protein, FhuD2, in Staphylococcus aureus. We report a novel siderophore purification method that utilizes the displacement mechanism of FhuD2. In another section, we discuss the biosynthesis of trihydroxamate siderophores, revealing evidence for secondary gene clusters responsible for the diversity of siderophores that originate from naturally produced ferrioxamine-E. We also present in another section insights into the hydrolysis-mediated drug release mechanism, providing evidence of a relationship between iron-reduction/release, and hydrolysis of the drug.
Chapter 1: Introduction
1.1 Preface
This chapter was written by Gerry Sann Rivera (GSR) with feedback provided by Prof. Tim Wencewicz (TAW).

1.2 Antimicrobial Resistance in Bacterial Pathogens
The first antibiotic that was used in the clinic was discovered in 1928 by Alexander Fleming, revolutionizing human medicine and increasing the average life expectancy of the human population. Since then, there has been more antimicrobial resistance occurring in an abnormally fast pace with respect to the previous decades and centuries prior to the use of antibiotics in the clinic. As shown in Figure 1.1, the mechanisms of resistance can be categorized into the following: modification of the target site of the drug, prohibition and reduced uptake, activation of efflux motifs, modification of metabolic pathways, and degradation of the drug. Antimicrobial resistance has been an ongoing phenomenon in nature dating back to their very origins of antibiotics in nature. Bacteria have been able to develop a delicate balance between antibiotic resistance and susceptibility within the environment. The overuse of antibiotics by humans has tilted this balance in favor of selection towards multi-drug resistant strains. In addition to the misuse of antibiotics, there is a lack of investment towards R&D for new and innovative ways to combat this resistance. Many pharmaceutical companies have focused their attention on other avenues that provide more profit. The combination of these two main factors is leading to an estimated annual death toll of 10 million from antimicrobial resistant infections, outpacing cancer by the year 2050.

It is necessary to input the time and resources to discover new and innovative strategies to combat this resistance before the estimated trends become reality. There are currently many strategies that are being explored, some of which include natural product drug discovery and
combination drug therapy. The strategy that will be explored in this thesis is gaining insight into the various pathways bacteria undergo to obtain the nutrients necessary to survive and proliferate during an infection. A majority of clinically used antibiotics are β-lactams, macrolides, fluoroquinolones, tetracyclines, and aminoglycosides. A clearer understanding of the various pathways utilized by bacteria can lead to new antibiotics that are chemically novel and act on novel targets.

1.3 Bacterial Iron Acquisition

Bacteria require a plethora of nutrients in order to grow, so naturally they develop ways to obtain these nutrients that allows for growth. One class of nutrients that are required are metal ions; over one-third of proteins are believed to require to interact with a metal ion while performing its biological function. The metal ion of focus here that is necessary for both bacteria and humans is iron. Iron is very abundant in the Earth’s crust, but under aerobic pH 7.4 conditions, it exists mostly in its ferric hydroxide state. The available Fe$^{3+}$ concentration thus is about $10^{-18}$ M. In the human body, the concentration of Fe$^{3+}$ drops by a few orders of magnitude to about $10^{-24}$ M due to the presence of certain proteins such as transferrin, lactoferrin, heme, and ferritin. The intracellular concentration of iron necessary for bacterial growth is about $10^{-6}$.

Ferritin was discovered in 1937 and was found to be one of the major storages for iron because of the large cavity in the structure that allows for the sequestration of up to 4500 Fe$^{3+}$ atoms in its core. Lactoferrin has been studied since the 1930s and is shown to act as an immune response that is capable of disrupting antibacterial activity. It is released into the blood and tissue when an infection is present, and one of its important defensive properties is its ability to sequester iron, thereby lowering the overall Fe$^{3+}$ concentration. In addition, transferrin can also
be introduced to the environment during infection and sites of inflammation. It is able to bind up
to two Fe\(^{3+}\) ions (Figure 1.3). The primary role of transferrin is to bind Fe\(^{3+}\) and transport it from
the absorption centers in the duodenum and macrophages to a variety of tissues.\(^8\) Calprotectin
and heme containing enzymes (hemophores) are also known to have antimicrobial properties due
to their ability to sequester transition metals, including iron.

The low iron concentrations presents a difficult environment for bacteria to proliferate.
As such, they have evolved mechanisms to scavenge iron during these iron deficient conditions
through a variety of mechanisms. Many of these mechanisms involve interactions with the iron-
containing proteins listed previously, and removal of the iron from these systems. These include
the expression of surface receptor proteins that bind to transferrin/lactoferrin/heme/hemophores.
The last mechanism discussed here that is commonly used by bacteria is the production of high
iron-binding affinity secondary molecules called siderophores.\(^9,10\)

The first mechanism of iron acquisition that will be discussed is that associated with
heme and hemophores. Targeting heme and hemophores is a viable strategy due to the fact that
about 75% of the iron in the extracellular environment is sequestered by heme.\(^11\) Hemoglobin
(Hb) is an oxygen transporter hemophore, considered to be one of the most abundant
hemophores. The acquisition of Hb differs between that of gram-positive and gram-negative
pathogens because of the extra outer membrane that gram-negative organisms have.

An iron-regulated surface determinant system (Isd) is present in \(S.\ aureus\), a gram-
positive pathogen, which can utilize heme and hemophores such as Hb to obtain iron.\(^12\) Figure
1.2a illustrates that Isd proteins reside on the cell wall surface and bind to heme and Hb using the
near transporter (NEAT) domains. The NEAT surface proteins associated with \(S.\ aureus\) are
IsdB, IsdH, and IsdA. Each protein has differing binding affinities towards heme and Hb,
providing a system that can utilize the most common complexes involved.\textsuperscript{13-15} IsdC obtains the heme from IsdBHA, and shuttles it to IsdDEF, the proteins embedded in the membrane that transport the heme to the cytoplasm.\textsuperscript{16} IsdGI, the heme oxygenase proteins inside the cytoplasm, can then degrade the heme to obtain the available iron.\textsuperscript{17} Other gram-positive pathogens that differ from the \textit{S. aureus} system are found in \textit{B. anthracis} and \textit{C. diphtheria} that involve either additional proteins or a different heme uptake system.\textsuperscript{18}

Gram-negative bacteria need to account for the extra outer membrane layer. Thus, there are outer-membrane receptors, and inner membrane transporters by which together, they are able to shuttle heme to the cytoplasm.\textsuperscript{18} One example of a gram-negative system is \textit{P. aeruginosa} and its two systems for heme uptake are shown in \textbf{Figure 1.2b}; the \textit{Psuedomonas} heme uptake (Phu) system and the heme assimilation system (Has). The Phu system utilizes an outer membrane TonB-dependent protein PhuR that can bind and transport heme to the periplasm. Once in the periplasm, the heme can bind to PhuT which shuttles the heme to inner membrane ABC transport proteins PhuUV. Similar to IsdGI, PhuS and HemO can degrade heme to obtain the iron.\textsuperscript{18} The latter system, Has, secretes a protein HasA that can bind to heme and transport it back to the outer membrane protein, HasR.\textsuperscript{19} The two systems work in tandem to obtain heme and extract iron. Other examples of gram-negative bacteria that also undergo these heme acquisition systems are \textit{H. influenza} and \textit{N. meningitidis}.

As previously stated, in order to combat the production of iron-binding proteins transferrin and lactoferrin, bacteria have developed systems that utilize transferrin and lactoferrin to obtain their iron. Transferrin is approximately 80kDa and has two binding sites that house Fe\textsuperscript{3+}. Lactoferrin is part of the transferrin family and has a mass of about 80kDa.\textsuperscript{20, 21} The main difference here is that lactoferrin is secreted towards inflammatory or mucosal areas. Bacteria
have developed receptors for these proteins on the outer membranes. The most well-known receptors found in pathogenic bacteria are from *Neisseriaceae* and *Pasteurellaceae*.\textsuperscript{22} Transferrin and lactoferrin binding protein receptors are found in both gram-negative and gram-positive bacteria with the latter being less studied.\textsuperscript{22} *Neisseria meningitidis*, a gram negative bacteria, was found to produce two transferrin binding proteins (Tbps) later named TbpA, the outer membrane receptor protein, and TbpB, the surface-associated lipoprotein. They can each bind transferrin independently but differ in that TbpB preferentially binds the holo-Tf while TbpA has equal binding affinity towards the apo- and holo-Tf. These receptors act in a similar way to the heme uptake systems but remove the iron from transferrin before entry through TbpA. Lactoferrin receptors have also been found in other pathogenic bacteria such as *Neisseriaceae* and *Moraxellaceae* by using a TonB-dependent outer membrane protein LbpA, and lipoprotein LbpB.

The iron acquisition strategy which will be explored in much more detail in this thesis is the utilization of siderophores. As previously stated, siderophores are secondary metabolites with high iron-binding affinities, up to $10^{50}$ in some cases, that are produced and secreted out into the extracellular environment by bacteria. The production of these siderophores are controlled through a ferric uptake regulator (Fur), which can turn on siderophore production when the intracellular iron concentrations are too low.\textsuperscript{23, 24} Siderophores are produced in a variety of both gram-positive and gram-negative bacteria in similar fashion. The uptake pathway of these molecules differs between the two types of bacteria (Figure 1.4). In gram-positive bacteria, holo-siderophore is transported into the cell via a siderophore-binding protein and an ATP-binding cassette (ABC) transporter on the cytoplasmic membrane (Figure 1.4b). In gram-negative bacteria, holo siderophore is transported into the outer membrane through outer membrane
receptors and the ability of TonB to transduce energy by moving between the two membranes
with the help of the proton motive force provided by ExbB and ExbD (Figure 1.4a). ExbB and
ExbD are found to be responsible for bringing back TonB from the outer membrane to the
cytoplasmic membrane. Once the holo-siderophore is inside the cytoplasm, the iron is then
removed in one of two ways. The first being an enzyme-catalyzed reduction of Fe$^{3+}$ to Fe$^{2+}$ by
reductase enzymes. The second mechanism by which iron is removed from the siderophores is
through enzymatic hydrolysis, a process that breaks down the siderophore and consequently
reducing the binding interaction between the ligands and the metal center. The first mechanism
allows for the recycling of intact siderophores whereas the second mechanism does not.

There are a multitude of siderophores that are diverse in structure but can be categorized
by their specific ligands attached to the metal center. The three main categories of siderophores
are the catecholate, carboxylate, and hydroxamate. Although the structures vary greatly,
siderophores can be described in similar fashion when discussing the overall biosynthetic
pathways. The biosynthesis usually involves a non-ribosomal peptide synthetase (NRPS) which
incorporates amino-acid like derivatives. The siderophore building blocks as a result are usually
amino acids and can also include chorimsate and citrate.

1.4 Biosynthesis, Chemistry, and Utilization of Siderophores in Bacteria

Molecular Structure of Siderophores

Taking a closer look at the siderophore structure, it is evident that the necessary
characteristics for iron binding include ferric iron selectivity and iron-siderophore complex
stability. A few examples of siderophores found in nature are shown in Figure 1.5. Regarding
the selectivity for ferric iron, it is advantageous that siderophores have less binding affinity for
other metals such as copper(II), aluminum(III), zinc(II), manganese(II), iron(II), and nickel(II). As previously mentioned, once inside the cell, iron is released from the siderophore typically via reduction of ferric iron to ferrous iron. Consequently, siderophores have a lower binding affinity towards all 2⁺ charged metals over 3⁺ charged metals. The difference in selectivity between aluminum(III) and iron(III) can be explained through the difference in the size of the metal center.²⁴

Siderophore ligands are able to have this selectivity for these hard acids by utilizing hard base atoms, primarily oxygen. Figure 1.5 shows the various types of siderophore ligands that appear in literature. Catechols possess high iron binding affinities because of the two high charge density ortho-phenolate atoms.²² Similarly, hydroxamate and alpha-hydroxycarboxylate ligand moieties also have high iron-binding affinities. Both these binding moieties have high pKa’s. Other less common binding moieties include both nitrogen and oxygen atoms that bind to the metal center.²⁶

With regard to the entire siderophore structure, the enhanced iron-binding affinity is also due to the multidentate ligand structure forming an octahedral complex around the metal center. The most common siderophore structure currently found is the hexadentate formation.²⁷ These can involve either one type of binding ligand or a variety of binding ligands. Although hexadentate siderophores are common, there are a variety of tetrade ntate and bidentate ligand systems that exist. Based on entropic considerations, a hexadentate siderophore would be preferential and allow for greater stability of the iron-siderophore complex.²⁸,²⁹ The appearance of tetrade ntate and bidentate ligands can be argued in that hexadentate ligands may exhibit more molecular strain as it wraps around the metal center.²⁷
Siderophore Biosynthesis

Siderophores are synthesized inside of the cell before being secreted out into the extracellular environment as their iron-free forms. Siderophore biosynthesis is characterized into two categories: Non-ribosomal peptide synthetase (NRPS) dependent siderophores and NRPS-independent siderophores (NIS). The former category of siderophores and their respective gene clusters have been studied in greater detail than NIS. The enzymes in the NRPS are modular and contain multiple domains that typically utilize amino acids as substrates and modify/link them. In addition to l-amino acids, NRPS enzymes have also been found to use non-proteinogenic amino acids, modified amino acids, fatty acids, and aryl acids.\textsuperscript{30} The main domains in NRPS enzymes are the adenylation, thiolation, and condensation domains.\textsuperscript{31} Figure 1.6 illustrates one of the better studied siderophores, enterobactin, and its corresponding biosynthesis and gene cluster. Other examples of siderophores that are synthesized by NRPS gene clusters are mycobactin, vibriobactin, and yersiniabactin.\textsuperscript{32-34}

Typical NRPSs work similarly to an assembly line where each substrate is passed along the various domains in a sequential order, where the order of domains dictates the order of chemistry and final product. There are also others called iterative NRPSs that can use a domain more than once by backtracking along the assembly line.\textsuperscript{35} The adenylation (A) domain functions by adenylating the substrate, which activates it for the rest of the modules. The adenylated substrate is transferred to a phosphopantetheine (Ppant) on a thiolation (T) domain, forming an aminoacyl-thioester. The last domain, condensation (C) domain, is responsible for catalyzing the peptide-bond formation between the different aminoacyl substrates made from the previous two domains. After the final product is formed through these domains, it is removed typically through the C-terminal domain that contains a thioesterase (TE) domain. This domain removes
the tethered product either through hydrolysis or through an intramolecular nucleophilic attack. Hydrolysis leads to a linear product whereas intramolecular nucleophilic attack leads to a cyclic product.

The example siderophore produced by NRPS enzymes that will be explained in further detail here is enterobactin. The overall structure of enterobactin is cyclic and highly symmetrical and produced solely from chorismate. In Figure 1.6a, EntABC take chorismate and form 2,3-Dihydroxybenzoate (2,3-DHB), which EntE, an A domain, transfers it to the T domain of EntB. The substrate is then taken through EntF iteratively until the terminal TE domain catalyzes intramolecular nucleophilic attack on a tethered trimer which untethers and cyclizes the product to form enterobactin. EntD is considered to be the phosphopantetheinyl transferase of the cluster which works to activate the T domains of EntB and EntF.

In contrast to the NRPS siderophore biosynthetic gene clusters, the NIS biosynthetic gene clusters are not as well studied. The enzymes in this class have one enzymatic function each and have been thought to take in substrates like citric acid or other derivatives that contain either an amine or an alcohol group. Siderophores produced by NRPS-independent gene clusters include desferrioxamines, aerobactin, putrebactin, and bisucaberin. Similarly to NRPS enzymes, the NIS enzymes also adenylate their substrates. A stark difference between the two is that the NIS enzymes contain a fold and active site form that NRPS enzymes do not contain. As such, the adenylating enzymes have been categorized into three classes based on the fold architecture (Figure 1.7). Class I adenylating enzymes, referred to as the NRPS enzymes, contain a large N-terminus and a small C-terminus that are connected by a small hinge. Class II adenylating enzymes consist of aminoacyl-tRNA synthetases, and finally Class III adenylating enzymes consist of the NIS enzymes. These enzymes are described and characterized as a “cupped hand”
with three separate domains denoted as the palm, thumb, and finger. NIS enzymes bind ATP in a fashion where the formed pyrophosphate remains in the binding pocket of the enzyme until the citryl intermediate is released.

Much is still to be elucidated amongst the various NIS siderophores. The example pathway for NIS siderophores, which will be discussed in greater detail in Chapter 3, is the desferrioxamine E pathway. In Figure 1.6b, DesABC are the first three enzymes in the pathway that catalyze the modification of lysine to \(N\)-hydroxy-\(N\)-succinyl cadaverine (HSC). DesD, the final enzyme in the pathway, catalyzes the oligomerization of three molecules of HSC to form desferrioxamine E using ATP and magnesium. These four enzymes are believed to be capable of catalyzing the formation of other desferrioxamines such as desferrioxamine B using two molecules of HSC and one molecule of \(N\)-hydroxy-\(N\)-acetyl-cadaverine (AHC). Chapter 3 will delve further into the Des enzymes and their true substrate tolerance and product formation.

**Siderophore Transport**

Siderophores, once produced inside the cell, are exported outside of the cell through efflux proteins. The literature knowledge is currently lacking examples of siderophore exporters; there are only a few examples known. The examples include efflux pumps within the major facilitator superfamily (MFS), resistance, nodulation, and cell division (RND) superfamily, and ATP-binding cassette (ABC) superfamily. The first example is the MFS which are known to transport a variety of molecules including primary and secondary metabolites, and organic and inorganic anions through the following transport paradigms; uniport, symport utilizing a proton or cation, and antiport utilizing a solute. \(E. coli\) utilizes a 12 alpha-helical transmembrane segment containing MFS called EntS with the secretion of enterobactin, a well-characterized
siderophore.\textsuperscript{36} Other examples of 12 TMS MFS siderophore exporters are LbtB in \textit{Legionella pneumophila}, YhcA in \textit{Erwinia chrysanthemi}, and PvsC in \textit{Vibrio parahaemolyticus}.\textsuperscript{49, 50} Amongst the known MFS efflux pumps, a 13aa motif (GX\textsubscript{2}ADRGR[R/K][R/K]X[L/I]) was found to be more highly conserved than the rest of the sequences. It is predicted that the conserved region is responsible for the promotion of conformational changes following binding of the substrate, which would allow for the substrates to enter through the membrane.\textsuperscript{50, 51}

\textbf{ABC} superfamilies are involved in both efflux and uptake pathways and perform similarly in both situations. They are comprised of four subunits, including two integral membrane domains and two cytoplasmic domains. In the case of efflux ABC transporters present in prokaryotes, the domains are usually fused into two polypeptide chains, with each containing an integral membrane domain and a cytoplasmic domain.\textsuperscript{52-54} The cytoplasmic domain protein consists of the ATP-hydrolyzing component which drives the transfer between the membrane and into the cytoplasm.

The final class of transport proteins that are found to export siderophores are the RND proteins which are used primarily for export of various molecules including heavy metals, lipids, drugs, and other small molecules.\textsuperscript{26, 55} RND proteins work solely through one mechanism unlike the MFS proteins, in that the transport is catalyzed through a proton antiport mechanism.\textsuperscript{55} The RND proteins consist of an N terminus that starts the transmembrane region, following an extracytoplasmic region, six more transmembrane regions, another extracytoplasmic region, and ends with five transmembrane regions at the C terminus.
Siderophore Uptake

As previously discussed in less detail, siderophore uptake differs greatly between gram-positive and gram-negative bacteria because of the extra membrane present in gram-negative bacteria. In gram-negative bacteria, the first recognition site for siderophores near the cell membrane is the OM receptor (Figure 1.4A). There are a variety of examples of OM receptors that have been studied and characterized in *E. coli* and *P. aeruginosa* for siderophores involving enterobactin, ferrichrome, and pyochelin using FepA, FhuA, and FptA respectively.\(^{56-58}\) Structural comparisons of the known OM receptors show that the conserved sites include a 22-B-barrel domain used to cap the entry and tethering to the membrane with a disulfide linkage.\(^{59}\) The interactions involving TonB, ExbB, and ExbD result in a conformational change with the OM receptor that allows for substrates to enter into the periplasm. Once in the periplasm, the siderophore is sequestered by periplasmic substrate binding proteins (PBPs) and shuttled through an ABC transport system. In this particular case, these substrate binding proteins will be called siderophore binding proteins (SBPs). The conformational changes and binding of NTP push the substrate across the membrane and into the cytoplasm where the iron is extracted from the siderophore.

In gram-positive bacteria, there is only one membrane that the siderophores have to traverse through in order to reach the cytoplasm (Figure 1.4B). Thus, the siderophore binding proteins are considered the first entry checkpoint in the overall pathway back inside the cell. Unlike the siderophore binding proteins in the periplasm in gram-negative bacteria, these siderophore binding proteins are tethered to the cell membrane.\(^{26}\) Similarly to that of the ABC transport proteins used for the efflux of siderophores, the uptake pathways of most gram-positive siderophores also involve ABC transport proteins. The entry pathway for siderophores are more
characterized and studied than the efflux pathways, and consequently, more SBPs and uptake pathways have been elucidated and are found to be similar to that of the substrate uptake pathway of vitamin B\textsubscript{12}.\textsuperscript{60} Examples of well-studied siderophore uptake systems are namely the HtsABC and SirABC transport systems for staphyloferrin A and B respectively, both in \textit{S. aureus}.\textsuperscript{51-64} This thesis will delve into the mechanistic intricacies of the xenosiderophore uptake system utilized in \textit{S. aureus} via the ferric hydroxamate uptake pathway FhuD2BGC.

\textbf{Iron Release Mechanism}

Once inside the cell, it is thought that iron release from the holo-siderophore occurs via two mechanisms. The first mechanism is through a reduction of Fe\textsuperscript{3+} to Fe\textsuperscript{2+}, catalyzed by reductase enzymes, which dissociates the siderophore-iron complex due to the lower binding constant for the ferrous iron. The reductase enzymes are found to only be useful when dealing with siderophore-iron complexes with a $K_D$ of $10^{30}$ or lower, with a couple exceptions such as bacillibactin that has a $K_D$ of $10^{47}$.\textsuperscript{26,65} The reasoning behind this is that most reductase enzymes aren’t specific towards siderophores; they are very general reducing enzymes that are used to reduce a number of other compounds, namely Fe-dicitrate or Fe-hydroxamates, most of which do not have high formation constants.\textsuperscript{66} Siderophores also tend to have very negative redox potentials when compared to those of other biological reducing agents such as glutathione.\textsuperscript{67} Since the reducing enzymes are thought to be used in other reductive processes for other molecules in the cell, not much is known as to the specific reducing enzymes used for siderophores. The main advantage for having this reduction occur is the efficiency in energy usage because the siderophore structure and efficacy isn’t hindered by this reduction, and thereby it can be recycled back to the extracellular environment to obtain more iron.
The second mechanism of iron release is modification of the siderophore scaffold through a hydrolysis mechanism that is used in cases where the siderophore-iron complex is too stable for reductase enzymes to work. The popular example here is enterobactin and its corresponding esterase, Fes, which is responsible for hydrolyzing enterobactin and destabilizing the siderophore-iron complex, forming a varying amount of monomer, dimer, and trimer products of 2,3 dihydroxybenzoylserine. The gene cluster IroA has two hydrolases, IroD and IroE, that have been characterized and are responsible for synthesizing using glucosylated enterobactin derivatives. IroD and Fes are similar in that they hydrolyze the substrates down to monomers, while iroE preferentially hydrolyzed the cyclic substrates into their linear forms. The advantage for having the hydrolysis of the siderophores over the reduction process to occur is that it can accommodate a wider variety of siderophores with higher binding constants to iron. Unlike the reduction mechanism, the hydrolysis mechanism doesn’t benefit through the reuse of the siderophores, and consequently needs a higher production of siderophores at a higher metabolic cost.

1.5 Sideromycins: Siderophore-Antibiotic Conjugates

Natural growth conditions usually involve a plethora of organisms living and scavenging off a limited supply of nutrients. Bacteria compete for these nutrients through a variety of mechanisms, one of which is through the acquisition of xenosiderophores through siderophore binding proteins. Xenosiderophores are siderophores produced by other bacteria, which the host bacteria are able to obtain by expressing import protein systems. This provides a competitive advantage because the host organism obtains the Fe$^{3+}$ without the need to produce the siderophores itself, while also starving out the competing bacteria by utilizing the siderophores.
In order to circumvent the siderophore utilization of competing bacteria, other organisms have developed and produced a different class of siderophores which have an antibiotic covalently attached to the siderophore. This sideromycin acts in a “Trojan horse” type strategy where the sideromycin is delivered to the thieving bacteria through its own siderophore transport system, bypassing any existing innate cell membrane defenses (Figure 1.8). Once inside the cell, the whole siderophore-antibiotic conjugate can act on the intracellular target, or the antibiotic is cleaved from the siderophore and can also act on the intracellular target. Due to their ability to enter the cell through a nonconventional pathway, sideromycins are intriguing natural products that have been studied to further the advancement of combating antibiotic resistance mechanisms.

There are currently four main classes of naturally produced sideromycins: albomycins, salmycins, ferrimycins, and microcins (Figure 1.9). Albomycins are the most studied of the sideromycins, with a fully elucidated structure and biosynthetic gene cluster. Albomycin is a hydroxamate siderophore similar to that of fungal ferrichromes, with a thioribosyl pyrimidine antibiotic that inhibits tRNA synthetase. The three albomycins have minor modifications to the antibiotic portion, but all conserve the same methylated pyrimidine at the N3 position. Albomycin S1 contains an N4 carbamoylated cytosine that is not present in albomycin e. Albomycin S2 contains an N3-methyluracil as opposed to an N3-methylcytosine. All three albomycin compounds are considered to be broad-spectrum antibiotics against both gram-positive and gram-negative bacteria. As for all sideromycins, albomycin enters the cell via a siderophore transport pathway, and once the sideromycin is inside the cell, the antibiotic is cleaved off from the siderophore before the antibiotic reaches its biological target.
Salmycins are currently known to be produced by *Streptomyces violaceus*, and are not very well studied when compared to albomycin. There are four salmycins (A-D) that have been identified, with identical siderophore backbone structure and slight variations to the antibiotic portion. Similarly to albomycins, salmycins also contain a hydroxamate siderophore backbone, specifically that of danoxamine. The antibiotic conjugated to salmycin is an aminodisaccharide predicted to act like an aminoglycoside and inhibit protein synthesis. It is active against gram-positive bacteria only. Unlike albomycin, the biosynthetic gene cluster and the biological target are not known.

Ferrimycins are less studied but are known to be produced by *Streptomyces griseoflavus*. There have been three isolated versions of ferrimycin (A1, A2, and B), but there is weak structural analysis for all of them. It is certain that the siderophore backbone of ferrimycin is derived from the clinically used siderophore, desferrioxamine B. The biosynthetic gene cluster and target are not completely elucidated, but there is evidence that the ferrimycins also inhibit protein synthesis exclusively in gram-positive bacteria.

The last class of sideromycins is called microcins, which are antimicrobial peptimers synthesized by the ribosome in gram-negative bacteria. Differing from the previous three classes of sideromycins, there have been quite a few natural products classified under microcins and as such, have led to the classification of these microcins based on size and post-translational modifications. Microcins under 5 kDa but contain post-translational modification along the backbone are classified under class I microcins. Microcins ranging between 5-10 kDa are classified under Class II microcins. Class II is further separated into Class IIa and Class IIb with the former containing no post-translational modifications other than the inclusion of disulfide bonds. Class IIb are linear microcins that may contain post-translation modification along the C-
terminus and are typically the catecholate microcins. As opposed to the other three classes of sideromycins that exhibit broad-spectrum antibiotic activity, microcins are found to be considered a narrow-spectrum antibiotic.

With these four classes of siderophores discovered, it gave inspiration to synthetically produce siderophore-drug conjugates that can work in a variety of ways. One promising example that has come from this fruitful inspiration is cefiderocol, a catechol siderophore conjugated to a cephalosporin antibiotic. It is found to be effective against gram-negative bacteria such as *enterobacteriaceae*, *P aeruginosa*, and *Acinetobacter baumannii*. Most interestingly, the potency is maintained against multi-drug resistant bacteria. Cefiderocol has been shown to overcome traditional resistance mechanisms for β-lactam antibiotics such as porin OprD deficiency, production of β-lactamase and/or efflux pumps due to its uptake pathway unique to other β-lactam antibiotics, and its improved stability towards β-lactamas due to its conjugation with a siderophore. By modeling after and studying naturally produced sideromycins, it is possible to develop new drugs with similar and effective properties. Designing new sideromycins involve careful selection of the siderophore, the antibiotic, and the linker region, all three which are currently being extensively studied.
1.6 Tables and Figures

**Figure 1.1** General schematic for bacterial antibiotic resistance mechanisms. Both degradation and modification/inactivation of antibiotics reduces or nullifies antibiotic potency. Target alteration and efflux pumps do not chemically modify the antibiotic, and instead alters the binding target or reduces the concentration of antibiotic in the cell which also leads to reduced activity. Figure adapted from Munita et al.³
Figure 1.2 (A) Import pathway of hemes in gram-positive bacteria S. aureus. (B) Import pathway of hemes in gram-negative bacteria, P aeruginosa. PhuS/HemO and IsdG/I act in similar fashions to obtain Fe$^{2+}$ once inside the cell. Figure adapted from Sheldon et al.\textsuperscript{11}
Figure 1.3 Crystal structure of transferrin bound to iron and a carbonate ion. PDB#: 1D3K
Figure 1.4 (A) Siderophore import in gram-negative bacteria. (B) Siderophore import in gram-positive bacteria. OMR=Outer Membrane Receptor. PBP=Periplasmic Binding Protein. ABC=ATP-binding cassette. SBP=Siderophore-Binding Protein. Figure adapted from Letain et al.25
Figure 1.5 Common binding motifs of siderophores used to bind iron. The sections highlighted in red on the right column represent the ligands on the siderophores that interact with the metal center.
Figure 1.6 (A) Gene cluster and biosynthetic pathway of enterobactin utilizing NRPS enzymes. A = Adenylation domain. C = Condensation domain. T = Thiolation domain. TE = Thioesterase domain. (B) Gene cluster and biosynthetic pathway of desferrioxamine E utilizing NRPS-independent enzymes.
Figure 1.7 Crystal structures of the three classes of adenylating enzymes. (A) Class I enzyme, referred as NRPS enzyme, EntE in the enterobactin cluster. (PDB#: 6IYK) (B) Class II enzyme, LeuRS. (PDB#: 1OBC) (C) Class III enzyme, DfoC in the desferrioxmine E cluster. (PDB#: 5O7O)

Figure 1.8 A schematic for the generic sideromycin pathway from the producing bacteria to the competing bacteria. Molecule highlighted in blue is the antibiotic.
Figure 1.9 Panel of naturally produced sideromycins. Sections highlighted in black are the siderophore moieties. Sections highlighted in red are considered to be the antibiotic portion of the sideromycin that is proposed to cleave off upon entry into the competing bacterial cell.
1.7 References


Chapter 2: Immobilized FhuD2 Siderophore-Binding Protein Enables Purification of Salmycin Sideromycins from Streptomyces violaceus DSM 8286
2.1 Preface

This chapter was adapted with permission from [Rivera, G.S., Beamish, C.R., & Wencewicz, T.A. ACS Infect. Dis. 4, 5, 845-859 (2018)]. Copyright © 2018 American Chemical Society. All authors contributed to writing of the manuscript. GSR and CRB performed all experiments. TAW served as principal investigator and oversaw experimental design.

2.2 Abstract

Siderophores are a structurally diverse class of natural products common to most bacteria and fungi as iron(III)-chelating ligands. Siderophores, including trihydroxamate ferrioxamines, are used clinically to treat iron overload diseases and show promising activity against many other iron-related human diseases. Here we present a new method for the isolation of ferrioxamine siderophores from complex mixtures using affinity chromatography based on resin-immobilized FhuD2, a siderophore-binding protein (SBP) from Staphylococcus aureus. The SBP-resin enabled purification of charge positive, charge negative, and neutral ferrioxamine siderophores. Treatment of culture supernatants from Streptomyces violaceus DSM 8286 with SBP-resin provided an analytically pure sample of the salmycins, a mixture of structurally complex glycosylated sideromycins (siderophore-antibiotic conjugates) with potent antibacterial activity towards human pathogenic Staphylococcus aureus (MIC = 7 nM). Siderophore affinity chromatography could enable the rapid discovery of new siderophore and sideromycin natural products from complex mixtures to aid drug discovery and metabolite identification efforts in a broad range of therapeutic areas.
2.3 Introduction

Natural products continue to be a major source of lead molecules for drug discovery efforts in many therapeutic areas. Siderophores are iron(III)-chelating natural products biosynthesized and excreted by bacteria and fungi to aid in iron acquisition. Siderophores typically have high-affinity and selectivity for iron(III) imparted by oxygen-rich chelating groups such as catecholates, hydroxamates, and α-hydroxycarboxylates. Siderophores have been investigated in clinical applications for metal chelation therapies aimed at treating iron overload diseases. Desferal®, also known as desferrioxamine B, is a naturally occurring linear trihydroxamate siderophore from Streptomyces pilosus that is FDA-approved for the treatment of human iron overload diseases, including β-thalassemia (Figure 2.1). Ferrioxamine siderophores have also been explored as treatments for neurological disorders such as Parkinson’s disease, chronic pulmonary disease (COPD), wound healing, malaria, and cancer.

Natural functions of microbial siderophores include metal transport, toxic metal sequestration, quorum sensing, protection from oxidative stress, virulence, and chemical defense. The biosynthesis of bacterial siderophores is under transcriptional control of ferric uptake regulator (FUR) proteins. Under iron-restrictive conditions, siderophore biosynthesis takes place in the cytoplasm followed by efflux of the mature siderophore scaffold to the extracellular space. Siderophores bind iron(III) with extraordinary affinity (stability constants can be as high as $10^{42}$) and can solubilize and/or strip the metal directly from environmental and biological sources (e.g. transferrin). In Gram-negative bacteria, high-affinity outer membrane-imbedded receptor proteins (OMRs) bind the soluble siderophore-iron(III) complex and facilitate import with assistance from the TonB-ExbB-ExbD protein complex and a proton-motive force. Soluble periplasmic siderophore-binding proteins (SBPs) ferry the siderophore-iron(III) complex
to ATP-dependent ABC-type transporters that couple ATP-hydrolysis to cytoplasmic transport. Enzymatic reduction of iron(III) to iron(II) secures the intracellular iron and provides recycled apo-siderophore. Alternatively, reductive iron release can take place in the periplasm of Gram-negative bacteria. In some cases, enzymatic degradation of the siderophore scaffold is necessary to retrieve iron from the stable siderophore chelate. Analogous siderophore utilization pathways are operative in Gram-positive bacteria with the exception of OMRs and the TonB-ExbB-ExbD protein complex. Instead, SBPs are displayed on the cell surface as membrane-anchored lipoproteins that directly sequester extracellular siderophore-iron(III) complexes and interface with ATP-hydrolyzing ABC-transporters to drive import. Some OMRs and SBPs are expressed to enable the utilization of siderophores produced by neighboring organisms, so called xenosiderophores, which provide a competitive growth advantage. Xenosiderophore utilization can be countered by the production of sideromycins, siderophore-antibiotic conjugates (SACs), which deliver a potentially toxic antibiotic upon internalization. Natural sideromycins and synthetic SACs represent an attractive approach for pathogen-targeted antibiotic delivery and have served as tools and inspiration for the study of siderophore pathways in bacteria and fungi.

More than 500 naturally occurring siderophore structures have been reported in the literature. The ferrioxamine family of siderophores is common to many soil-dwelling Streptomyces and marine bacteria. Ferrioxamine siderophores are composed of repeating units of N-hydroxy-putrescine and/or cadaverine joined by succinyl groups that provide the carbonyl for the metal chelating hydroxamate ligand. Three sequential hydroxamate ligands in the ferrioxamine backbone provides an ideal template for chelating iron(III) with octahedral geometry, 1:1 siderophore:iron(III) stoichiometry, and stability constants (K_{Fe}) on the order of
Ferrioxamine biosynthesis is driven by the conserved operon desABCD, which has enabled chemoenzymatic synthesis and virtual mining for new ferrioxamine siderophores. However, many siderophore biosynthetic gene clusters (BGCs) remain silent under standard culture conditions and many siderophore-producing microbes are difficult to grow in the laboratory using standard cultivation techniques. The discovery of new natural products, including siderophores, is further limited by laborious scale-up and purification from microbial cultures. Historically, siderophore production and purification has been guided by the extraordinary affinity for iron(III). The chrome azurol s (CAS) assay is a colorimetric test for siderophore production that can be applied to culture supernatants or directly incorporated into bacterial growth media. Technological advances in high-resolution mass spectrometry, comparative metabolomics, microbial cultivation, synthetic biology, comparative transcriptomics, metagenomics, microbial genome-mining, and high-throughput screening have enabled the discovery of new natural products, including siderophores. Recently, a new structural class of siderophores, the crochelins, was discovered in Azotobacter chroococcum culture supernatants using the “chelome” metabolomics platform that searches high-resolution LC-MS/MS data for the mass isotopes of iron. Similarly, the siderophore nicoyamycin A was isolated from a library of 32,879 natural product extracts using a screen for growth inhibition of uropathogenic E. coli (UPEC) under low iron conditions. In both cases, preparative purification of the metal-chelating siderophores from microbial fermentations required challenging chromatographic steps.

Natural product isolation and purification is challenging no matter how the metabolite is discovered. Efforts to improve the purification of natural products from complex mixtures include advancements in fermentation, strain prioritization, heterologous expression,
bioengineering, chromatography, and fractionation. The unique structural complexity of natural products can be leveraged for various types of affinity chromatographies. Chemoselective enrichment chromatography has been used to covalently capture target natural product classes from complex extracts. Methods for the chemoselective enrichment of natural products containing hydroxyl, polyol, carboxylate, amine, thiol, ketone/aldehyde, and conjugated diene functional groups have been reported. However, chemoselective functionalization of complex natural product scaffolds is a prerequisite for covalent capture on an immobilization resin and this remains a challenge in synthetic organic chemistry.

Furthermore, natural product functionalization must be reversible in order to release the immobilized metabolite and obtain the native structure for accurate characterization. Metal chelation has been exploited as a reversible, chemoselective method to purify metal-chelating natural products, including siderophores. Nickel(II)-based immobilized affinity chromatography (IMAC) has been used to purify hydroxamate-containing desferrioxamine siderophores from microbial cultures, in a similar manner as conventional purification of polyhistidine-tagged proteins. Boronate affinity chromatography has also been used to purify catechol-based siderophores. Both boronate and Ni-IMAC methods are selective for metal-free siderophores, which can be problematic for siderophores that readily form high-affinity and thermodynamically stable metal chelates.

Protein-ligand affinity chromatography is a highly specific purification method that can exploit the strong binding affinity of a natural product for its biological target. Affinity chromatography has been applied in both targeted and untargeted approaches to isolate numerous natural product classes. Affinity selection-mass spectrometry (ASMS) has emerged as a promising method for small molecule drug discovery. ASMS utilizes an immobilized target
that is exposed to a library of compounds followed by elution with known ligands. Alternatively, immobilized or tagged natural products, including siderophores, can be utilized for protein pull-down assays to reveal biological targets from cell lysates.\textsuperscript{84, 85} For example, a biotinylated version of the siderophore petrobactin was immobilized to avidin resin to enable the direct identification of the petrobactin import protein in pathogenic Bacillus anthracis.\textsuperscript{86} Siderophores have also been immobilized on surfaces for applications in metal sequestration,\textsuperscript{87} surface adhesion,\textsuperscript{88} and pathogen detection.\textsuperscript{89-92} For these types of applications, covalent modification of the siderophore is often required and carries the risk of perturbing the affinity for target proteins. However, the reversibility, stoichiometric binding, and low dissociation constants (typically nanomolar\textsuperscript{31}) makes the siderophore-SBP interaction attractive for affinity applications. Immobilization of SBPs using standard protein-resin immobilization techniques presents the opportunity for sequestration of natural, unmodified siderophore metabolites. To the best of our knowledge, immobilized SBPs have never been used to sequester siderophores from complex mixtures (at least not intentionally since heterologous expression and affinity purification of some SBPs can result in co-purification with a siderophore ligand).\textsuperscript{93, 94} However, in one report soluble CntA, an SBP from S. aureus, was incubated with S. aureus culture supernatant to sequester a Ni-bound metabolite later identified as the metallophore staphylopine, which is an important S. aureus virulence factor.\textsuperscript{95, 96}

Here, we sought to streamline the isolation of siderophores from complex mixtures by leveraging the high specificity and affinity of SBPs for siderophore ligands (Figure 2.2). We developed a versatile affinity chromatography platform for sequestering ferrioxamine siderophores by adhering an N-His\textsubscript{6}-tagged SBP (FhuD2 from S. aureus) to Ni(II)-charged nitriloacetic acid agarose (Ni-NTA) resin. We used the SBP-resin to purify charge positive,
charge negative, and charge neutral ferrioxamine siderophores. The SBP-resin is stable and can be cycled repetitively by loading siderophores of interest and displacing with sacrificial siderophores that are easily separated based on net charge using ion exchange chromatography. We used the FhuD2 SBP-resin to isolate the salmycins, a mixture of glycosylated sideromycins, from fermentation extracts of *Streptomyces violaceus* DSM 8286. The use of affinity chromatography greatly simplifies the purification of the salmycins and rapidly provides analytically pure material suitable for quantitative biological assays. Our SBP-resin could be interfaced with existing natural product discovery platforms to selectively survey for ferrioxamine siderophores in diverse environments.

2.4 Results and Discussion

*Designing a siderophore-binding resin.*

Bacteria rely on two types of siderophore receptors as gatekeepers for cell entry, OMRs from the transmembrane beta-barrel superfamily and soluble SBPs from the type III class of the substrate-binding protein superfamily.97 OMRs present challenges for *in vitro* study due to lack of solubility using standard heterologous protein expression.16 SBPs are well known for having high aqueous solubility and stability. SBPs from Gram-negative bacteria are expressed with an N-terminal signal sequence that directs secretion to the periplasm with signal peptidase-mediated cleavage of the signal peptide. Periplasmic SBPs are highly soluble and efficiently expressed using heterologous methods.17, 31 SBPs from Gram-positive bacteria are co-expressed with an N-terminal signal sequence that is cleaved during secretion. SBPs are displayed as covalently anchored lipoproteins on the surface of Gram-positive bacteria.29 Typically, SBPs are selective for binding a general structural class of siderophores (e.g. ferrioxamines), but broadly bind
structural analogs within the class. The high solubility and broad substrate-binding ability of SBPs make these proteins excellent candidates for heterologous expression and in vitro study. We identified FhuD2 as a promising SBP for siderophore-affinity chromatography. FhuD2 is selective for binding trihydroxamate siderophores, including the ferrioxamines, in both the iron-free and iron-bound forms. Pathogenic strains of *S. aureus*, including methicillin-resistant *S. aureus* (MRSA), display the SBP FhuD2 on the cell surface as a virulence factor during infection. The hydrophobic, charge-neutral siderophore-binding site in FhuD2 accommodates a wide range of ferrioxamine siderophores including charge positive, charge negative, neutral, and sterically bulky analogs. Truncated FhuD2 lacking the lipopeptide signal sequence is soluble and maintains the ability to bind ferrioxamine siderophores and sideromycins carrying large antibiotic cargos with nanomolar affinity. We envisioned a simple strategy for using immobilized FhuD2 to sequester ferrioxamine siderophores in analogy to established methods for performing affinity chromatography (Figure 2.2a). We hypothesized that adhering the soluble domain of FhuD2 to Ni-NTA resin via a hexahistidine linker would provide an SBP-resin selective for binding ferrioxamine siderophores. The SBP-resin could be treated with a solution of the siderophore of interest ($S_1$) to load $S_1$ in the FhuD2 binding pocket. After washing with buffer, $S_1$ could be eluted by treating the SBP-resin with a solution of a sacrificial siderophore ($S_2$) that can displace $S_1$ from the FhuD2 binding site. Excess $S_2$ could be removed from $S_1$ using simple ion exchange chromatography if the net charge of the two siderophores is different. In theory, any SBP could be immobilized for biased and unbiased siderophore sequestration (Figure 2.2b). We selected FhuD2 to enable unbiased isolation of hydroxamate-based siderophores because of its known siderophore promiscuity. Other SBPs are highly selective and might enable biased, targeted isolation of siderophores. Genes encoding for SBPs are often
clustered in operons with siderophore biosynthesis genes. Heterologous expression and immobilization of SBPs from siderophore BGCs might enable biased isolation of the cognate siderophore from cultures of the producing microbe.

**Functional validation of SBP-resin.**

We used heterologous expression in *E. coli* BL21(DE3) to overproduce a truncated version of FhuD2, FhuD2Δ24, replacing the first 24 amino acids with a *N*-terminal hexahistidine motif to facilitate purification using Ni-NTA resin (Table 2.1). Protein purity was assessed to be >95% by ESI-MS and SDS-PAGE analysis (Figure 2.3, 2.4). ESI-MS analysis of purified *N*-His$_6$-FhuD2Δ24 showed an intact hexahistidine motif and loss of the *N*-terminal methionine residue. *N*-His$_6$-FhuD2Δ24 is highly soluble in aqueous buffer and is stable towards repeated freeze-thawing. After dialysis of the purified protein into phosphate buffer, we loaded fresh Ni-NTA resin to capacity with *N*-His$_6$-FhuD2Δ24 forming the SBP-resin (~0.2 μmol FhuD2Δ24/mL; resin loaded to capacity was judged by SDS-PAGE analysis of column flowthrough). We chose the model siderophores ferrioxamine (FO), succinylferrioxamine (SFO), and acetylferrioxamine (AcFO) as iron(III) complexes to validate capacity of the FhuD2 SBP-resin to reversibly bind trihydroxamate siderophores (Figure 2.1). FhuD2Δ24 is proposed to catalyze the exchange of iron(III) between ferrioxamine siderophores. Desferrioxamine (DFO) has been shown to form coordination complexes with Ni-NTA resin. To avoid complications from iron exchange and non-specific chelation to the Ni-NTA resin, we used the iron(III)-bound forms of the siderophores. FO (net +1 charge), SFO (net -1 charge), and AcFO (neutral) were chosen based on differences in polarity and net charge in order to facilitate ion exchange separation of target siderophore (S$_1$) and sacrificial siderophore (S$_2$) from the SBP-
resin column elution. Passing FO, SFO, and AcFO through Ni-NTA resin without immobilized FhuD2Δ24 resulted in no detectable, non-specific adherence to the resin. We used an intrinsic fluorescence-quenching assay to confirm that our recombinant N-His₆-FhuD2Δ24 protein binds FO, SFO, and AcFO with the anticipated nanomolar affinity (Figure 2.5).³¹ FhuD2Δ24 tryptophan fluorescence is strongly quenched upon ferrioxamine siderophore binding, presumably due in part to W197, which directly contacts the ferrioxamine backbone through a stabilizing hydrogen bond.⁹⁹ Apparent $K_d$ values of N-His₆-FhuD2Δ24 for FO, SFO, and AcFO were 53 ± 6 nM, 46 ± 7 nM, and 31 ± 4 nM, respectively, indicating that all three siderophores form 1:1 stoichiometric complexes with soluble N-His₆-FhuD2Δ24.

In order for the SBP-resin to function as an affinity chromatography platform, the resin must bind to a target siderophore $S_1$ and subsequently release $S_1$ in the presence of a sacrificial siderophore $S_2$ that is added in excess. Presumably, excess $S_2$ can competitively displace $S_1$ from the SBP-resin resulting in $S_2$-saturated resin and an elution containing a mixture of $S_1$ and $S_2$ (Figure 2.2a). We validated this approach using all six possible combinations of FO, SFO, and AcFO as $S_1$ and $S_2$ and confirmed elution of $S_1$ from the SBP-resin using LC-MS (Figure 2.6a). Siderophore $S_1$ was loaded at 0.1 mg/mL and, after sufficient column washing, was eluted with an equal volume of siderophore $S_2$ at 0.1 mg/L. When FO was used as $S_1$ the SBP-resin was saturated with FO and excess FO was detected in the column flowthrough (Fig. 2.6b; blue trace). No FO was detected in the column wash (Fig. 2.6b; red trace). Addition of SFO as $S_2$ eluted FO from the SBP-resin (Fig. 2.6b; green trace). The same batch of SBP-resin was immediately used for another cycle of siderophore purification with bound SFO now serving as $S_1$. Excess SFO was detected in the column flowthrough suggesting full saturation of the SBP-resin (Fig. 2.6c; blue trace). No SFO was detected in the column wash (Fig. 2.6c; red trace).
Addition of FO as S₂ eluted SFO from the SBP-resin (Fig. 2.6c; green trace). Subsequent cycles with remaining S₁:S₂ siderophore pairs were performed in analogous fashion (FO:AcFO, Fig. 2.6d; AcFO:SFO, Fig. 2.6e; SFO:AcFO, Fig. 2.6f; AcFO: FO, Fig. 2.6g).

These proof-of-principle experiments show that SBP-resin can be cycled with different S₁ and S₂ siderophore combinations for sustained siderophore purification without the need to use fresh resin. The FhuD2Δ24 SBP-resin is robust and does not appear to lose efficiency during repeated use. All combinations of FO, SFO, and AcFO as S₁ and S₂ siderophores provided the desired S₁ siderophore upon elution from the SBP-resin with S₂. The broad scope of the SBP-resin is consistent with the observation that FhuD2 enables broad utilization of trihydroxamate siderophores for iron acquisition in S. aureus.³¹ Similar FhuD2 K₅₅ values for FO, SFO, and AcFO suggest that the siderophores competitively bind FhuD2 regardless of net siderophore charge (Figure 2.5). To demonstrate this competitive binding, we treated an equimolar mixture of FO, SFO, and AcFO with FhuD2 SBP-resin followed by elution with a fourth ferrioxamine siderophore, danoxamine (Dan) (Table 2.2). This resulted in competitive binding and elution of all three siderophores with only modest changes in ion counts from load to elution (Figure 2.7; Table 2.3). The siderophore-binding pocket of FhuD2 is mostly hydrophobic and charge neutral, which supports the capacity for accommodating ferrioxamine siderophores with different polarity and electrostatic properties.⁹⁹

**Separation of S₁ and S₂ by ion exchange chromatography.**

One remaining challenge for using SBP-resin to purify siderophores is that column elutions contain a mixture of the desired siderophore S₁ and the sacrificial siderophore S₂. In our case, both S₁ and S₂ are from the ferrioxamine family and give similar retention times on RP-
C18 HPLC. We were able to separate mixtures of siderophores by preparative HPLC, but the large excess of the $S_2$ siderophore limited injection volume size and decreased peak resolution. We selected $\text{FO}$, $\text{SFO}$, and $\text{AcFO}$ for these proof-of-principle studies because of differences in net charge (charge positive, charge negative, and neutral, respectively) that we predicted would enable final separation of $S_1$ and $S_2$ siderophores via ion exchange chromatography, which can be performed on FPLC or HPLC platforms.

The eluent from the SBP-resin requires desalting prior to ion exchange chromatography. This can be accomplished using C18 chromatography or by lyophilizing the sample and redissolving siderophores from the bulk solid using methanol. We used a diethylamine cation exchange resin (DEAE) and a Cellex P anion exchange resin to separate various combinations of cationic ($\text{FO}$), anionic ($\text{SFO}$), and neutral ($\text{AcFO}$) siderophores (Fig. 2.8a). Presumably other commercially available cation and anion exchange solid phases would also be suitable for this type of separation. An aqueous solution containing a mixture of $\text{FO}$ and $\text{SFO}$ was loaded onto a DEAE resin (Fig. 2.8b, c; blue traces). The column was washed with water to provide cationic $\text{FO}$ that does not adhere to the cationic DEAE resin (Fig. 2.8b, c; red traces). $\text{SFO}$ is left bound to the DEAE resin and can be eluted by addition of 4% ammonium hydroxide solution (Fig. 2.8b, c; green traces). Mixtures of $\text{AcFO}$ and $\text{FO}$ (Fig. 2.8d, c; blue traces) were treated with Cellex P resin to bind cationic $\text{FO}$ and provide charge neutral $\text{AcFO}$ in the column wash (Fig. 2.8d, c; red traces). $\text{FO}$ was eluted by the addition of 4% ammonium hydroxide solution (Fig. 2.8d, c; green traces). Lastly, mixtures of $\text{AcFO}$ and $\text{SFO}$ were separated using DEAE cation exchange resin (Fig. 2.8f, g; blue trace). Anionic $\text{SFO}$ adhered to the DEAE resin while $\text{AcFO}$ eluted in the column washes (Fig. 2.8f, g; red traces). $\text{SFO}$ was eluted by addition of 4% ammonium hydroxide in a manner similar to that described for the $\text{SFO/FO}$ mixture (Fig. 2.8f,
The use of ion exchange chromatography can expedite the final separation of $S_1$ and $S_2$ siderophores obtained from SBP-resin elutions. FO, SFO, and AcFO represent three distinct charge states for siderophores (mono-cationic, mono-anionic, and neutral, respectively) that can be used as the $S_2$ elution siderophore in applications of the FhuD2Δ24 SBP-resin in the purification of ferrioxamine siderophores from complex environmental samples and microbial fermentations.

**Purification of salmyns A–D from S. violaceus DSM 8286.**

To demonstrate the utility of the FhuD2Δ24 SBP-resin we sought to purify a natural siderophore from a microbial culture (Figure 2.9). We chose a challenging and structurally complex class of sideromycins, the salmyns, which contain a ferrioxamine siderophore component that is recognized by FhuD2. The salmyns are a mixture of glycosylated sideromycins (Sal A–D) produced by *Streptomyces violaceus* DSM 8286. The siderophore portion of Sal A–D is a trihydroxamate siderophore from the ferrioxamine family known as danoxamine (Dan). Danoxamine is composed of two N-hydroxy-cadaverine subunits and a terminal N-hydroxy-5-aminopentan-1-ol, or alternatively a terminal N-hydroxy-4-aminobutan-1-ol, joined via succinoyl linker groups. In Sal A–D the aminodisaccharide antibiotic is covalently linked to danoxamine through a succinoyl ester bond that is likely to be hydrolyzed upon internalization. The potency of the salmyns (minimum inhibitory concentration of ~10 nM in liquid media against planktonic *S. aureus*) is thought to arise from high intracellular concentrations driven by active transport through FhuBCDG. Mutations in the *fluD2* gene confer resistance to the salmyns in *S. aureus* consistent with FhuD2 selecting for cell
These findings make our FhuD2Δ24 SBP-resin a promising method for purification of the salmycins from *S. violaceus* cultures.

The salmycins were originally isolated in 1995 from the culture supernatant of *S. violaceus* DSM 8286. In the original report by Vértesy and coworkers and two subsequent patents from the same group a series of hydrophobic resins, ion exchange columns, and RP-C18 chromatography steps provided analytically pure salmycins suitable for full characterization. A large scale up (185 L of culture) was used to provide milligram quantities of *Sal A–D* and enabled the separation of isomers. In our hands, and others, reproducing this purification scheme has failed to provide analytically pure samples of the salmycins. During our purification attempts from 12 x 1 L batch cultures of *S. violaceus* DSM 8286 following patent protocol we identified an unknown contaminant with retention time and *m/z* values by LC-MS that co-elute with the salmycins (Figure 2.10). As a result, LC-MS analysis of *S. violaceus* DSM 8286 culture supernatants and solutions from various purification steps was challenging and we were never able to obtain a salmycin sample free of the unknown impurity, even after multiple rounds of preparative HPLC chromatography using RP-C18 and HILIC columns. The unknown impurity lacks antibacterial activity, so bioactivity guided fractionation was informative during purification of the salmycins. Agar diffusion antibacterial susceptibility assays using salmycin-sensitive *S. aureus* ATCC 11632 provided a sensitive assay for the presence of salmycins when coupled to a sister medium containing FO, which antagonizes the growth inhibitory activity of the salmycins by competing for FhuD2-mediated uptake. We aimed to use FhuD2Δ24 SBP-resin and bioactivity guided fractionation to purify the salmycins directly from *S. violaceus* DSM 8286 cultures to overcome co-elution of the impurities during traditional chromatographic steps.
We started with 10 L of *S. violaceus* DSM 8286 culture supernatant that was filtered through celite, concentrated, and washed with MeOH. Bioactivity assays suggested that the MeOH washings contained the salmycins. The MeOH washings were concentrated and dissolved in pure H$_2$O in preparation for treatment with SBP-resin. LC-MS analysis of the column loading solution showed a strong signal in the Extracted Ion Chromatogram (EIC) for the $m/z$ value (1053) corresponding to the [M+H]$^+$ ion of the Sal A iron(III) complex (Fig. 2.12; blue trace, retention time = 4.9 min). Mass signatures for other salmycin isomers were also observable by LC-MS (Figure 2.11). Unknown impurities with broad peak patterns were also present in the $m/z$ 1053 EIC of the load sample. When tested against *S. aureus* ATCC 11632 on solid media the load solution gave a strong zone of growth inhibition. The flowthrough from the SBP-resin showed decreased ion counts for $m/z$ 1053 peak corresponding to Sal A, but still contained the broad unknown impurity peaks (Figure 2.12; red trace) that was accompanied by a decrease in the size of the growth inhibition zone when tested against *S. aureus* ATCC 11632. Buffer washes of the SBP-resin gave no detectable ion counts for $m/z$ 1053 corresponding to Sal A in the EIC (Fig. 2.12; green trace) and showed no growth inhibition against *S. aureus* ATCC 11632. After elution of the SBP-resin with a dilute solution of Dan the ion counts for $m/z$ 1053 in the EIC returned (Fig. 2.12; magenta trace) along with a noticeable zone of growth inhibition when tested against *S. aureus* ATCC 11632. The zone of growth inhibition for the elution was hazy due to the excess Dan in the sample, which antagonizes salmycin transport in *S. aureus*. Further purification by RP-C18 preparative HPLC (Figure 2.13) provided a pure sample of the salmycins free of Dan with potent antibacterial activity (Fig. 2.12; olive trace). Purification of the salmycins by FhuD2Δ24 SBP-resin proceeded smoothly in a similar manner observed for combinations of FO, SFO, and AcFO used in the proof-of-principle studies. Dan was used as
the eluting siderophore $S_2$ instead of FO to increase separation of retention times on RP-C18 HPLC (Figure 2.13). The SBP-resin was tolerant of the complex media and could be recycled as done for the model siderophores. The amount of salmycin that can be purified from S. violaceus DSM 8286 is limited by the amount of FhuD2 employed due to 1:1 stoichiometric binding of salmycin by FhuD2.\textsuperscript{99} Thus, scaling up the purification will require scaling up FhuD2 production. Fortunately, FhuD2 expresses at high levels in our E. coli BL21 expression system and is highly soluble, stable, and tolerant of handling/immobilization.\textsuperscript{31} Industrial scale precedent with maltose-binding protein (MBP),\textsuperscript{114, 115} a substrate-binding protein that is structurally related to FhuD2, suggests that this type of scale-up is achievable. FhuD2Δ24 SBP-resin does not separate individual salmycin isomers, but enrichment using SBP-resin enhances HPLC peak resolution that might aid in final chromatographic separation of individual isomers (Figure 2.14).

We also explored the treatment of S. violaceus DSM 8286 culture supernatant with HP20 and DEAE resins prior to siderophore-affinity chromatography (Figure 2.15). We found that this treatment was not necessary when using FhuD2Δ24 SBP-resin, but was required for the use of centrifugal filtration since the culture supernatant frequently clogged the 30K molecular weight cut-off semi-permeable membranes. We used centrifugal filtration to scale up the salmycin purification and provide enough material for characterization. The molecular formulas of Sal A–D were confirmed by high-resolution LC-mass spectrometry (Fig. 2.11, 2.16). We also measured the optical absorbance properties, FhuD2 $K_d$ binding constant, iron(III) binding affinity ($K_{Fe}$), and MIC value towards S. aureus ATCC 11632 (Figure 2.17). The UV-Vis absorbance spectrum was consistent with other trihydroxamate siderophores bound to iron(III) with a characteristic peak at 427 nm arising from ligand-to-metal interactions (Figure 2.17a). Assuming an extinction
coefficient of 3000 M$^{-1}$cm$^{-1}$ (common for most 1:1 ferrioxamine:iron(III) complexes), we quantified stock solutions of the salmycins to measure FhuD2Δ24 $K_d$, $K_{Fe}$, and MIC values. The salmycins quenched the intrinsic fluorescence of N-His$_6$-FhuD2Δ24 similar to FO, SFO, and AcFO (Figure 2.17b). Fluorescence quenching of N-His$_6$-FhuD2Δ24 was used to estimate the apparent $K_d$ value of 43 ± 6 nM for the salmycins in close proximity with the apparent $K_d$ values for FO, SFO, and AcFO (53 ± 6 nM, 46 ± 7 nM, and 31 ± 4 nM, respectively). The aminoglycoside moiety does not appear to reduce affinity for FhuD2, which is consistent with binding studies performed with structurally related SACs. We used an EDTA competition assay to measure the apparent $K_{Fe}$ for the salmycins (Figure 2.17c). Treatment of iron(III)-bound salmycins with a slight excess of EDTA led to rapid exchange of iron and equilibration as judged by continuous monitoring of optical absorbance at 427 nm. The log $K_{Fe}$ was calculated to be 25.5 ± 0.1, which is similar to the log $K_{Fe}$ reported for a synthetic danoxamine-ciprofloxacin (25.6 ± 0.1) and the parent siderophore Dan (27.8 ± 0.3). The log $K_{Fe}$ value of FO is 30.3 ± 0.4 with the gain in iron(III) affinity arising from the simple change of a terminal hydroxyl group to a terminal primary amine that is protonated at physiological pH. FO is known to promote the growth of S. aureus ATCC 11632 more efficiently than Dan under iron-limiting conditions. FO is also more antagonistic towards a danoxamine-ciprofloxacin SAC in antibacterial susceptibilities assays for S. aureus. We showed the same effect for the salmycins using an agar diffusion antibacterial susceptibility assay with S. aureus ATCC 11632 (Figure 2.17d). The salmycins alone gave a well-defined zone of growth inhibition against S. aureus ATCC 11632. In the presence of a 20-fold excess of Dan we observed a small, hazy zone of growth inhibition. In the presence of a 20-fold excess of FO there was no visible zone of growth inhibition and the antibacterial activity of the salmycins was abolished. The salmycins contain a
secondary amine as part of the aminoglycoside antibiotic that is predicted to make the salmycin iron(III) complex mono-cationic under physiological conditions. We previously showed that mono-cationic ferrioxamine siderophores, like FO, outperform mono-anionic ferrioxamine siderophores, like Dan, in S. aureus growth promotion assays. Our current findings are consistent with this observation and suggest that mono-cationic salmycins will be competitive with other ferrioxamine siderophores in microenvironments to gain cell entry through ferrioxamine uptake systems. With analytically pure salmycins in hand we also set out to confirm the literature reported MIC value of ~10 nM against strains of S. aureus. We used the broth microdilution method in Mueller-Hinton No. 2 broth made iron-deficient by the addition of 2,2-dipyridyl to measure an MIC value of 7.8 nM against S. aureus ATCC 11632, confirming the original literature value.

2.5 Conclusions

We have developed a new affinity chromatography strategy for purifying siderophores by immobilizing a His-tagged SBP on Ni-NTA resin. We utilized FhuD2 from pathogenic S. aureus as the SBP because of its ability to broadly bind trihydroxamate siderophores from the ferrioxamine family. Our FhuD2Δ24 SBP-resin enabled sequestration of mono-cationic (FO), mono-anionic (SFO), and neutral (AcFO) ferrioxamines. Siderophore binding was reversible and all siderophores could be displaced from the SBP-resin using an excess of competing siderophore with similar binding affinity for FhuD2Δ24. To validate the utility of SBP-resin in natural product isolation, we purified the salmycins from S. violaceus DSM 8286 culture supernatants to greatly simplify the isolation of these structurally complex sideromycins. There is growing interest in the discovery of new siderophores and sideromycins to treat a variety of
human diseases, including infectious diseases.\textsuperscript{12, 34, 116} SBP-resin coupled with microbial cultivation, genome mining, metabolomic, transcriptomic, and high-throughput screening might expedite the discovery of new siderophores. SBP-resin might also be useful for purifying synthetic siderophores, sideromycins, and siderophore conjugates that often require final purification by tedious chromatographic steps.\textsuperscript{108} Immobilized SBPs might also be useful for studying siderophore membrane transport paradigms and probing important structure-binding relationships for SBPs and siderophores that might lead to an improved understanding of microbial siderophore utilization.\textsuperscript{31, 100, 101} In theory, SBP-resin can be applied as a general affinity-based immobilization strategy for a variety of chemical biology applications in much the same ways as established technologies such as MBP-maltose and avidin-biotin methods,\textsuperscript{117} although proof-of-principle studies will be needed to validate this claim.

SBP-resin has several advantages over covalent capture approaches to immobilize siderophores and other natural products.\textsuperscript{75} SBP-resin is non-covalent and reversible allowing for mild recovery of immobilized siderophores of interest without structural perturbation through simple displacement with a competing sacrificial siderophore. SBP-resin is highly specific and enables for targeting specific structural classes of siderophores in complex mixtures containing a variety of siderophore types. SBP-resin could be adapted for virtually any siderophore with a soluble, cognate SBP. SBPs are typically selective for specific structural classes of siderophores including hydroxamates, catecholates, and $\alpha$-hydroxycarboxylates.\textsuperscript{4} Presumably some non-specific siderophore binding might take place when trying to sequester target molecules from mixtures containing diverse siderophore structures. Our successful purification of the salmycins from crude \textit{S. violaceus} DSM 8286 cultures using immobilized FhuD2A24 suggests that selective siderophore binding can be achieved using a carefully chosen immobilized SBP with
the proper structural selectivity. In theory, siderophore BGCs could be mined for SBPs to construct siderophore affinity resins that might enable rapid isolation of new and even cryptic siderophores from microbial cultures. Genes encoding for SBPs from metagenomic studies could be converted to siderophore affinity resins to prospect dilute metagenomic samples, including human microbiome samples. Siderophores from probiotic and pathogenic microbes play important roles in human health. Detection of siderophores in patient-derived samples from the human microbiome is required for linking these microbial metabolites to biological processes relevant to mutualistic and pathogenic relationships. SBP-resins might be useful for concentrating siderophores from the human microbiome with potential applications in probiotics, pathogen diagnostics, and elucidation of important host-microbe and microbe-microbe interactions that depend on siderophores.

2.6 Materials and Methods

Strains, materials, and instrumentation.

*Staphylococcus aureus* ATCC 11632 and *Streptomyces violaceus* DSM 8286 were obtained from the ATCC and DSMZ collections, respectively ([Table 2.4]). *E. coli* BL21-Gold(DE3) and *E. coli* TOP10 cells were obtained from Agilent and Invitrogen, respectively. *E. coli* cells were made electrocompetent by standard methods. A Bio-Rad MicroPulser electroporator and 0.2 cm gap sterile electroporation cuvettes were used for electroporation. Bacteria were stored as frozen glycerol stocks at -80 °C. Codon-optimized *fhuD2Δ24* was purchased from GenScript in a pET28a vector for heterologous expression in *E. coli* BL21(DE3) with an N-terminal hexahistidine tag ([Table 2.1, 2.5]). N-His6-FhuD2Δ24 was overexpressed and purified as described previously ([Figure 2.3, 2.4]). DNA purification was performed with kits from
Qiagen. Plasmid sequencing was performed by Genewiz. Nickel-nitriiloacetic acid (Ni-NTA) agarose was purchased from ThermoFisher Scientific (catalog # R90115). Any kD SDS-PAGE gels were purchased from Bio-Rad. Proteins were dialyzed using 10K MWCO SnakeSkin dialysis tubing purchased from Thermo Fisher Scientific. Proteins were concentrated by centrifugal filtrations using 30K MWCO filters from Millipore. All aqueous solutions were prepared with water purified using a Milli-Q system and sterilized by filtration through a 0.2 µm filter. Media was sterilized using an autoclave unless otherwise stated. pH measurements were recorded using an Orion Star A111 pH meter and a PerpHecT ROSS micro combination pH electrode from Thermo Fisher. All buffers, salts, media, solvents, and chemical reagents were purchased from Sigma Aldrich unless otherwise stated. All media was sterilized in an autoclave prior to growing bacteria. Siderophore samples FO, SFO, AcFO, and Dan were prepared as described previously (Figure 2.18).31

LC-MS was performed using an Agilent 6130 quadrupole with G1313 autosampler, G1315 diode array detector, and 1200 series solvent module. Samples were prepared in 0.45 µ PTFE mini-UniPrep vials from Agilent. Separations were achieved using a 5 µ Gemini C18 column (50 x 2 mm) from Phenomenex fit with a guard column. Mobile phases were 0.1% formic acid in (A) H2O and (B) ACN. Data were processed using G2710 ChemStation software. 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 Å column (250 x 21.2 mm) from Phenomenex fit with a guard column (15 x 21.2 mm). Mobile phases for RP-C18 prep-HPLC were 5 mM ammonium acetate in (A) H2O and (B) ACN. Analytical HPLC was performed using a Beckman Coulter SYSTEM GOLD 127P solvent module and 168 detector with a Phenomenex Luna 10u C18(2) 100 Å column, 250 x 21.20 mm, 10 µm with guard column. HPLC data were processed...
using 32 Karat software, version 7.0. 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 spectrophotometer. High-resolution LC-MS/MS spectra were collected using a Q-Exactive (Thermo-Fisher Scientific) equipped with a custom built Eksigent microLC at the Donald Danforth Plant Science Center, St. Louis, MO. Mobile phases were 0.1% formic acid in (A) H2O and (B) ACN and a Supelco C8 column (0.5 x 150 mm) was used for chromatographic separations. 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 200–500 at a resolution setting of 70,000 (at m/z 200) for MS1 and a resolution of 17,500 for MS2. Proteins were analyzed by ESI-MS using a QTOF Bruker Maxis equipped with a microLC at the WUSTL NIH/NIGMS-supported Biomedical Mass Spectrometry Research Resource. Mobile phases were 0.1% formic acid in (A) H2O and (B) 80% H2O/20% ACN and an Eclipse XDB-C8 (3.5 μm) column was used for chromatographic separations. A flow rate of 200 μL/min was held constant while a solvent gradient (5% B ramped to 20% B over 2 min, then ramped to 70% B over 2.5 min, then ramped to 80% B over 0.5 min and held for 1 min, then ramped to 5% B over 0.5 min, and finally ramped to 0% B over 1.5 min. The mass spectrometer was operated with a drying temperature of 180 °C, a capillary voltage of 4,000 V, and a mass range of 250–2500 m/z. Data was processed using Intact Mass-Protein Metrics.
**FhuD2 fluorescence quenching.**

FhuD2 fluorescence quenching was performed as described previously by our group.\textsuperscript{31} Increasing concentrations of siderophore solutions in 100 nM \textit{N}-His\textsubscript{6}-FhuD2\textDelta24 in TBS buffer (25 mM Tris-HCl, 8 g/L NaCl, 0.2 g/L KCl, pH 7.4) were added over 2 min intervals to a 100 nM FhuD2 solution in TBS buffer, maintaining constant volume of 300 μL. Increments of siderophore concentrations were 0 (buffer control), 26.7, 53.2, 79.5, 105.6, 171, 299, 422, and 881 nM, or until maximum fluorescence no longer decreased. A PerkinElmer LS 55 Luminescence Spectrometer was used for all measurements. Emissions were analyzed from 300–400 nm with the following settings: the excitation wavelength was 280 nm; excitation and emission slits were both set to 10 nm; scan speed was set to 400 nm/min. Maximum fluorescence, usually around 340 nm, was taken for each siderophore concentration and dose dependent fluorescence quenching data were processed using GraphPad Prism v7.0b to calculate the apparent \textit{K}_d of \textit{N}-His\textsubscript{6}-FhuD2\textDelta24 for each siderophore using a nonlinear fit to a one binding site model. A HellmaAnalytics High Precision Cell cuvette made of Quartz SUPRASIL, light path 10x2 mm was used for all experiments. Experiments were performed in triplicate as independent trials.

**Siderophore purification with SBP-resin.**

A fritted glass column was loaded with fresh Ni-NTA agarose resin in 1:1 EtOH:H\textsubscript{2}O to give a working resin volume of 2.3 cm x 1 cm. The resin was washed with H\textsubscript{2}O and equilibrated with SBP buffer (50 mM K\textsubscript{2}HPO\textsubscript{4}, 150 mM NaCl, 1 mM DTT, pH 8) at 4 °C. \textit{N}-His\textsubscript{6}-FhuD2\textDelta24 was thawed from a frozen stock (500 μL of 1.5 mM in SBP buffer; this is enough protein to fully saturate the Ni-NTA agarose resin), diluted to 3 mL final volume with SBP buffer, and added to
the Ni-NTA agarose resin. After rocking at 4 °C for 30 min excess SBP buffer was eluted and the column was washed with SBP buffer until no N-His$_6$-FhuD2Δ24 was detected by SDS-PAGE analysis. The N-His$_6$-FhuD2Δ24-saturated Ni-NTA agarose resin is referred to as SBP-resin.

Three siderophores (FO, SFO, and AcFO) were used in pairs as the siderophore of interest (S$_1$) or sacrificial siderophore (S$_2$). Siderophore S$_1$ is first loaded to the SBP-resin by addition of 5 mL of a 0.1 mg/mL solution of S$_1$ in SBP buffer followed by 20 min of rocking at 4 °C. Excess SBP buffer is eluted and the SBP resin is washed five times with 15 mL of SBP buffer until LC-MS analysis shows no detectable ions for siderophore S$_1$. Next, 5 mL of a 0.1 mg/mL solution siderophore S$_2$ in SBP buffer is added and the SBP resin is rocked at 4 °C for 20 min. The column eluent is analyzed by LC-MS for the presence of siderophore S$_1$ ions to confirm displacement from the SBP resin by competitive binding of excess siderophore S$_2$. The SBP resin can now be used in a second cycle using the now resin bound siderophore S$_2$ as the siderophore of interest S$_1$. For LC-MS analysis of samples a gradient was formed from 0% B to 100% B over 10 min, followed by a 10 min hold at 100% B, and re-equilibration to 0% B over 5 min. Caution: using DTT in SBP buffer can lead to reduced Ni-NTA resin as indicated by a blue to orange color change during the procedure. BME can be used as an alternative to DTT to prevent this from taking place. Each experiment was performed in duplicate as independent trials.

**Separation of siderophores by ion exchange chromatography.**

A fritted glass column was packed by gravity with DEAE or Cellex P resin giving a working resin volume of 2 cm x 7 cm. Resins were washed with 50 mL of 10 mM ammonium acetate solution in H$_2$O (pH 7) followed by 50 mL of pure H$_2$O. For proof-of-principle studies, 1:1
mixtures of iron(III)-bound siderophores (\(\text{FO} + \text{SFO}; \text{SFO} + \text{AcFO}; \text{FO} + \text{AcFO}\)) at 0.1 mg/mL for each siderophore were prepared in pure H\(_2\)O. Samples from SBP-resin elutions containing combinations of siderophores at ~0.1 mg/mL each were first desalted and then reconstituted in pure H\(_2\)O. Desalting was accomplished by either lyophilization followed by trituration with MeOH or C18 chromatography. A 5 mL aliquot of \(\text{FO} + \text{SFO}\) solution was passed through DEAE resin. The flowthrough was collected (contains \(\text{FO}\)) and the column was washed with 2 x 5 mL pure H\(_2\)O. \(\text{SFO}\) was eluted from the DEAE resin using 5 mL of 4% aqueous NH\(_4\)OH. A 5 mL aliquot of \(\text{SFO} + \text{AcFO}\) solution was passed through DEAE resin. The flow-through was collected (contains \(\text{AcFO}\)) and the column was washed with 2 x 5 mL pure H\(_2\)O. \(\text{SFO}\) was eluted from the DEAE resin using 5 mL of 4% aqueous NH\(_4\)OH. A 5 mL aliquot of \(\text{FO} + \text{AcFO}\) was passed through Cellex P resin. The flow-through was collected (contains \(\text{AcFO}\)) and the column was washed with 2 x 5 mL pure H\(_2\)O. \(\text{FO}\) was eluted from the Cellex P resin using 5 mL of 4% aqueous NH\(_4\)OH. For each separation, the column load, flowthrough, washes, and elution were analyzed by LC-MS. Each separation was performed in duplicate as independent trials.

**Purification of salmyns from \textit{S. violaceus} DSM 8286.**

Salmyns A–D (\(\text{Sal A–D}\)) were purified from cultures of \textit{Streptomyces violaceus} DSM 8286 (Figure 2.9).\(^{103, 112, 113}\) Spore stocks of \textit{S. violaceus} DSM 8286 were grown on malt extract agar plates (10 g/L starch, 1 g/L casein, 1 g/L peptone, 1 g/L yeast extract, 10 g/L malt extract, 0.5 g/L K\(_2\)HPO\(_4\), 15 g/L agar) at 28 °C for 7–10 days, or until spores were formed. A liquid culture was started by adding a 1 cm\(^2\) slice from the agar plate to 250 mL of liquid growth media (15 g/L glucose, 15 g/L soy bean flour, 5 mL/L corn steep liquor, 2 g/L CaCO\(_3\), and 5 g/L NaCl, pH adjusted to 7.2) in a 1 L baffled flask. The starter culture was incubated at 28 °C with shaking at
225 r.p.m for 48 h. 50 mL of starter culture was transferred to a 3 L baffled flask containing 1 L of salmycin production media (20 g/L soy bean flour and 20 g/L mannitol, pH adjusted to 7.5). Salmycin production cultures were incubated with shaking at 225 r.p.m. at 28 °C for 4 days. Salmycin production was monitored by antibacterial susceptibility testing against *S. aureus* ATCC 11632, as described below. Cultures (10 L total volume) were vacuum filtered through celite and concentrated by rotary evaporation. The resulting solids were washed with a 90% MeOH/10% H$_2$O solution (v/v) several times. The combined MeOH washings were concentrated by rotary evaporation and dissolved in 10 mL of pure H$_2$O. A pure mixture of salmycins A–D can be obtained using N-His$_6$-FhuD2Δ24 SBP-resin as described previously using the salmycins as siderophore S$_1$ and FO, SFO, AcFO, or Dan as siderophore S$_2$. Dan is advantageous as siderophore S$_2$ because it offers the greatest separation from the salmycins by RP-C18 HPLC (Figure 2.13).

For preparative scale purification of salmycins A–D we employed the use of centrifugal filtration with soluble N-His$_6$-FhuD2Δ24 rather than Ni-NTA-immobilized protein. Culture supernatant (5 L total volume) from *S. violaceus* DSM 8286 production cultures was passed through a column of hydrophobic HP-20 resin (6 cm wide x 25 cm high) to absorb the salmycins. The HP-20 resin was washed with H$_2$O and the crude salmycins were eluted from the HP-20 resin by the addition of a 20% IPA/80% H$_2$O. The IPA/H$_2$O were removed via rotary evaporation to give a dark brown solid. The solid was suspended in a minimal volume of 90% MeOH/10% H$_2$O and then centrifuged at 5,000 r.p.m. at 4 °C for 20 min. The supernatant was collected and the solid was treated with 90% MeOH/10% H$_2$O a second time. The supernatants were combined and concentrated by rotary evaporation to give an orange solid. The solid was dissolved in H$_2$O and filtered through a DEAE anion exchange column (2 cm wide x 7 cm high). The flowthrough
from the DEAE resin was concentrated and dissolved in SBP buffer. This solution is suitable for siderophore-affinity chromatography using immobilized SBP columns (Figure 2.15), as described previously, or soluble SBP centrifugal filtration units. For the centrifugal filtration protocol using soluble SBP, eight Amicon Ultra 30K MWCO centrifugal filters were filled with 250 μL of 800 μM N-His$_6$-FhuD2Δ24 diluted to 1 mL total volume in SBP buffer. Approximately 15 mL of crude salmycins in SBP buffer was added to each centrifugal filter and rocked at 4 °C for 30 min. Each centrifugal filter was then centrifuged at 5,000 r.p.m. at 4 °C for 15 min or until ~1-2 mL of volume remained in the filtration unit. An additional 15 mL of crude salmycin solution in SBP buffer was added to each tube and the process was repeated until all of the crude salmycin solution was passed through the N-His$_6$-FhuD2Δ24 centrifugal filters. To elute the pure salmycins, a 5 mL solution of 0.2 mg/L Dan in SBP buffer was added to each centrifugal filter. After rocking for 30 min at 4 °C, the solutions were centrifuged at 5,000 r.p.m. and the flowthrough was collected. An additional 5 mL of SBP buffer was added to each centrifugal filter and the flowthrough after centrifugation was combined with the previous flowthrough to give ~10 mL total volume from each centrifugal filter. The flowthroughs from all centrifugal filtration units were combined and concentrated via rotary evaporation to yield an orange solid. To decrease the salt content, the solid was suspended in 90% MeOH/10% H$_2$O and filtered. The remaining solid was washed several times with 90% MeOH/10% H$_2$O and the pooled MeOH/H$_2$O washings were concentrated via rotary evaporation and redissolved in H$_2$O. HPLC and LC-MS analysis revealed a mixture of Dan and salmycins A–D that was separated using preparative RP-C18 HPLC using a gradient of 5% B held for 2 min, ramped to 25% B over 5 min, then 45% B over 20 min, and 100% over 3 min followed by re-equilibration to 0% B over 2 min (Figure 2.13). Danoxamine eluted from 15–16 min and salmycins A–D were collected
from 16–20 min. Fractions containing the salmycins were confirmed by bioactivity, analytical RP-C18 HPLC, LC-MS, and high-resolution LC-MS/MS (Figure 2.11, 2.14, 2.16). This resulted in a solution of analytically pure salmycins A–D with a total volume of 500 μL and a total concentration of 81 μM (42 μg), which enabled the determination of apparent $K_{Fe}$, apparent $N$-His$_6$-FhuD2Δ24 $K_d$, and S. aureus MIC values.

**Determination of salmycin $K_{Fe}$.**

The apparent $K_{Fe}$ value for the purified mixture of salmycins A–D was measured using an EDTA competition assay for 1:1 siderophore:Fe(III) complexes described previously by our group (Figure 2.17c). A solution of pure salmycins at 0.1 μM was mixed with 1.2 equivalents of EDTA in HEPES buffer (10 mM HEPES, 600 mM NaCl, 100 mM KCl, adjusted to pH 7.4) to a total volume of 1 mL. The solution was transferred to a polypropylene cuvette and scanned continuously at 430 nm over 90 min at 1 s/scan. The experiment was performed in duplicate as independent trials. Apparent $K_{Fe}$ was calculated from the change in absorbance at 430 nm as described previously.

**Antibacterial susceptibility testing.**

Antibacterial activity of the salmycins was determined by an agar diffusion assay or liquid broth microdilution assay. For both assays, overnight cultures of S. aureus ATCC 11632 were grown in LB broth for 18–24 h starting from a frozen glycerol stock. For the agar diffusion assay, 40 μL of this culture was added to 34 mL of sterile, melted, and tempered (~47 °C) Mueller-Hinton No. 2 agar (HiMedia Laboratories) supplemented with 100 μM 2,2'-bipyridine (final concentration). After gentle mixing, the inoculated melted agar was poured into a sterile petri
dish (145 mm x 20 mm, Greiner Bio-One) and allowed to solidify at rt. Wells of 9 mm diameter were cut from the petri dish agar and filled with 50 μL of the test sample solution. All pure test samples (FO, salmcyins, and Dan) were tested at concentrations of 0.1 mg/mL. For testing of crude salmcyins during purification the concentration was unknown and antibacterial activity against *S. aureus* was used as a qualitative measurement for the presence of the salmcyins. The petri dish was incubated at 37 °C in ambient air for 18–24 h and the inhibition zone diameters were measured (mm) with an electronic caliper. MIC values were determined using the broth microdilution strategy following CLSI guidelines in Mueller-Hinton No. 2 broth supplemented with 100 μM 2,2′-bipyridine, as described previously by our group. A concentration gradient of 1–0.0002 μM salmcyins was used and an MIC value of 7.8 nM was recorded for halting growth of *S. aureus* ATCC 11632 after 18 h incubation at 37 °C in ambient air. The MIC was judged visually as the lowest salmcyin concentration to completely inhibit the growth of *S. aureus* ATCC 11632.

**Abbreviations**

AcFO, acetyl ferrioxamine; ACN, acetonitrile; ASMS, affinity selection-mass spectrometry; BME, β-mercaptoethanol; CLSI, Clinical and Laboratory Standards Institute; DTT, dithiothreitol; ESI, electrospray ionization; FO, ferrioxamine; FPLC, fast protein liquid chromatography; His6, hexahistidine; HPLC, high-performance liquid chromatography; IPA, isopropyl alcohol; LB, Luria Broth; LC-MS, liquid chromatography-mass spectrometry; MIC, minimum inhibitory concentration; MWCO, molecular weight cut-off; MTA, material transfer agreement; Ni-NTA, nickel nitrilotriacetic acid agarose; r.p.m., rotations per minute; rt, room temperature; S1, siderophore 1; S2 siderophore 2; SAC, siderophore-antibiotic conjugate; Sal,
salmycin; SBP, siderophore-binding protein; SDS, sodium dodecyl sulfate; SFO, succinoyl ferrioxamine.
2.7 Figures and Tables

Table 2.1 Primary protein sequences of wilde type (WT) FhuD2 and N-His$_6$-FhuD2Δ24 variants used in this work.$^a$

<table>
<thead>
<tr>
<th>WT-FhuD2$^b$ from S. aureus (GenBank Accession # AAK92086.1):</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKKLLLPLIIMLVLACGNQGEKNNKAETKSYKMDDGKTVDIPKDPKRIAVVAPTYAGG</td>
</tr>
<tr>
<td>GGLKKLGANIVAVNQVQVDQSKVLKDFFGVTKIGDGVEKVAKEKPDILIVYSTDKDIK</td>
</tr>
<tr>
<td>KKYQKVAPTVVDYNKHKYLEEQQEMLKGKIVGEKDKVEKAKWKKDWEETTAKDGKEIKK</td>
</tr>
<tr>
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</tr>
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<td>EEIEKYAGDYIVSTSEGKTPGYESTNMWKNLAKTEGHIVKVDAQTYWYNDPYTLDFMRKDLKELKIAAK</td>
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</table>

<table>
<thead>
<tr>
<th>N-His$_6$-FhuD2Δ24$^c$ encoded on pET28a plasmid:</th>
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<tbody>
<tr>
<td>MGSSHHHHHHSSGLVPRGSMMNKAETKSYKMDDGKTVDIPKDPKRIAVVAPTYAGG</td>
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<tr>
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<td>YQQKVAPTVVDYNKHKYLEEQQEMLKGKIVGEKDKVEKAKWKKDWEETTAKDGKEIKK</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>N-His$_6$-FhuD2Δ24$^d$ purified from E. coli BL21 and confirmed by ESI-MS:</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSSHHHHHHSSGLVPRGSMMNKAETKSYKMDDGKTVDIPKDPKRIAVVAPTYAGG</td>
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</tr>
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<tr>
<td>EEIEKYAGDYIVSTSEGKTPGYESTNMWKNLAKTEGHIVKVDAQTYWYNDPYTLDFMRKDLKELKIAAK</td>
</tr>
</tbody>
</table>

$^a$Adapted with permission from the supporting information file associated with Endicott, N. P.; Lee, E.; Wencewicz, T. A. “Structural basis for xenosiderophore utilization by the human pathogen Staphylococcus aureus” ACS Infectious Diseases, 2017, 3, 542-553. Copyright 2017 American Chemical Society/Timothy A. Wencewicz. $^b$Pre-lipoprotein signal sequence is highlighted in magenta. Soluble siderophore-binding domain highlighted in teal. $^c$Hexahistidine motif with thrombin cleavage site highlighted in yellow. Soluble siderophore-binding domain highlighted in teal. $^d$ESI-MS indicated loss of the N-terminal methionine residue (Figure 2.4).
Table 2.2 Molecular formulas, structures, and exact masses ([M+2+H]^+) of siderophores used in this work.

<table>
<thead>
<tr>
<th>Siderophore</th>
<th>Structure</th>
<th>Molecular Formula ([M+2+H]^+)</th>
<th>Exact Mass ([M+2+H]^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrioxamine (FO)</td>
<td><img src="image" alt="Structure" /></td>
<td>C_{25}H_{46}FeN_{6}O_{8}^+</td>
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</tr>
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<td>Succinyl Ferrioxamine (SFO)</td>
<td><img src="image" alt="Structure" /></td>
<td>C_{20}H_{50}FeN_{6}O_{11}^+</td>
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</tr>
<tr>
<td>Acetyl Ferrioxamine (AcFO)</td>
<td><img src="image" alt="Structure" /></td>
<td>C_{27}H_{48}FeN_{6}O_{9}^+</td>
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</tr>
<tr>
<td>Danoxamine</td>
<td><img src="image" alt="Structure" /></td>
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<td>673.2600</td>
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<tr>
<td>Salmycin A (Sal A)</td>
<td><img src="image" alt="Structure" /></td>
<td>C_{41}H_{71}FeN_{7}O_{21}^+</td>
<td>1053.4031</td>
</tr>
<tr>
<td>Salmycin B (Sal B)</td>
<td><img src="image" alt="Structure" /></td>
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<td>1038.3922</td>
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<td>Salmycin C (Sal C)</td>
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<td>1024.3765</td>
</tr>
<tr>
<td>Salmycin D (Sal D)</td>
<td><img src="image" alt="Structure" /></td>
<td>C_{40}H_{69}FeN_{7}O_{21}^+</td>
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Table 2.3 Extracted ion counts (EICs) for FO, SFO, and AcFO [M+H]+ ions after treating an equimolar mixture of the three siderophores with FhuD2 SBP-resin followed by elution with danoxamine.

<table>
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<tr>
<th>Siderophore</th>
<th>Load EIC</th>
<th>EIC(\text{Std}/\text{EIC}_{\text{Tot}})</th>
<th>Flowthrough EIC</th>
<th>EIC(\text{Std}/\text{EIC}_{\text{Tot}})</th>
<th>Elution EIC</th>
<th>EIC(\text{Std}/\text{EIC}_{\text{Tot}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>FO</td>
<td>362432</td>
<td>45%</td>
<td>5000</td>
<td>33%</td>
<td>159552</td>
<td>33%</td>
</tr>
<tr>
<td>SFO</td>
<td>246976</td>
<td>30%</td>
<td>5500</td>
<td>36%</td>
<td>229363</td>
<td>47%</td>
</tr>
<tr>
<td>AcFO</td>
<td>195712</td>
<td>24%</td>
<td>4500</td>
<td>30%</td>
<td>99232</td>
<td>20%</td>
</tr>
</tbody>
</table>

*a* Experiments using mixtures of three siderophores of interest (S1) was carried out as described for single siderophore (S1) experiments in the materials and methods section of the manuscript. Corresponding LC-MS traces are shown in Figure 2.7.

Table 2.4 Strains and plasmids used in this work.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Inducible Gene/Marker</th>
<th>Origin/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces violaceus</em> DSM 8286</td>
<td>None</td>
<td>Wild Type</td>
<td>DSMZ¹</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 11632</td>
<td>None</td>
<td>Wild Type</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>None</td>
<td>Cloning strain</td>
<td>Agilent</td>
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<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>None</td>
<td>Protein expression strain</td>
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<tr>
<td><em>E. coli</em> Top10</td>
<td>pET28a</td>
<td>N-\text{His}_{80}-\text{FhuD2\Delta24}</td>
<td>Wencewicz Lab²</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>pET28a</td>
<td>N-\text{His}_{80}-\text{FhuD2\Delta24}</td>
<td>Wencewicz Lab²</td>
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</table>

Table 2.5 Codon optimized nucleotide sequence of N-\text{His}_{80}-\text{FhuD2\Delta24} from S. aureus that was cloned into a pET28 vector for protein expression in E. coli BL21 (DE3). Adapted with permission from the supporting information file associated with Endicott, N. P.; Lee, E.; Wencewicz, T. A. “Structural basis for xenosiderophore utilization by the human pathogen *Staphylococcus aureus*” ACS Infectious Diseases, 2017, 3, 542-553. Copyright 2017 American Chemical Society/Timothy A. Wencewicz.

```
ATGAACAACAAGGCCGAGACCAAAAGCTACAAGATGGACGATGGTAAACCGTTGACATCCCGAAAAGATCGCAGGTATTGGCGTTGCGTTGCGCAACATCGGCGTTGGGCAACTGACACAGCAAGTGGATGCGGGCACCTACTGGTATAACGACG
CTGAAGAAACTGGGCTGCCAACCTGGTGGTCGTTGGCGTTGGGCAACTGACACAGCAAGTGGATGCGGGCACCTACTGGTATAACGACG
AGGTCATGGAAGGAAAGCGGCGGCACCGTTGGCAGCTGGATGCGGGCACCTACTGGTATAACGACG
CTGAAGAAACTGGGCTGCCAACCTGGTGGTCGTTGGCGTTGGGCAACTGACACAGCAAGTGGATGCGGGCACCTACTGGTATAACGACG
AGGTCATGGAAGGAAAGCGGCGGCACCGTTGGCAGCTGGATGCGGGCACCTACTGGTATAACGACG
```

66
Figure 2.1 Structures of ferrioxamine siderophores and sideromycins used in this study.
Figure 2.2 (A) General strategy for receptor-mediated purification of bacterial siderophores using SBP resin. An N-His$_6$ tagged siderophore-binding protein (FhuD2 from S. aureus) is adhered to a Ni-NTA agarose resin to form the SBP resin. The siderophore of interest (S$_1$) is loaded to the SBP resin and is bound by FhuD2. A sacrificial siderophore (S$_2$) is then added to displace siderophore S$_1$ from the SBP resin. FhuD2 is selective for binding trihydroxamate siderophores. (B) Unbiased and biased affinity selection-mass spectrometry (ASMS) strategies for integrating SBP-resins into siderophore natural product discovery platforms.

Figure 2.3 SDS-PAGE analysis of N-His$_6$-FhuD2 (~33.6 kDa) purified by Ni-NTA affinity chromatography after heterologous expression in E. coli BL21(DE3) cells. Image on left was not adjusted for contrast. Image on right was adjusted for contrast using Adobe Photoshop. Reprinted with permission from the supporting information file associated with Endicott, N. P.; Lee, E.; Wencewicz, T. A. “Structural basis for xenosiderophore utilization by the human pathogen *Staphylococcus aureus*” ACS Infectious Diseases, 2017, 3, 542-553. Copyright 2017 American Chemical Society/Timothy A. Wencewicz.
Figure 2.4 ESI MS analysis of N-His$_6$-FhuD2 expressed from E. coli BL21 and purified by NiNTA chromatography. Predicted mass with loss of N-terminal methionine: 33,582 Da, found: 33,582 Da.

Figure 2.5 Fluorescence quenching of N-His$_6$-FhuD2 by ferrioxamine siderophores. FO, SFO, and AcFO bind with nanomolar affinity to N-His$_6$-FhuD2. Graph depicts intrinsic tryptophan fluorescence quenching ($\lambda_{\text{excitation}} = 280$ nm; $\lambda_{\text{emission}} = 340$ nm) of N-His$_6$-FhuD2 and shows dose-dependent binding to FO, SFO, and AcFO. Boxed insert shows apparent $K_d$ values calculated using a single-binding mode model in GraphPad Prism version 7.0b.
Figure 2.6 Loading and displacing of siderophores on FhuD2 SBP resin. (A) Siderophore $S_1$ was loaded to the FhuD2 resin and then eluted with siderophore $S_2$. (B–G) All possible combinations of FO, SFO, and AcFO as the loading siderophore ($S_1$) and eluting siderophore ($S_2$) were successful in providing $S_1$ in the final elution from the SBP resin. EIC traces are off-set by 1 minute on the x-axis and a 10% off-set is used on the y-axis. EIC traces are representative of experiments performed in duplicated as independent trials.
Figure 2.7 Equimolar mixtures of FO, SFO, and AcFO competitively bind and elute from FhuD2 SBP-resin. EIC ion counts are provided in Table 2.3. Traces represent EICs for FO (blue trace; m/z 614 for [M+H]+), AcFO (red trace; m/z 656 for [M+H]+), and SFO (green trace; m/z 714 for [M+H]+) from the initial SBP-resin load (A), flow-through (B), buffer wash (C), and elution with danoxamine (E). This experiment was carried out exactly as described in the experimental section of the main text for with siderophore S1 being the equimolar mixture of FO, AcFO, and SFO.
Figure 2.8 Separation of the siderophore of interest (S₁) and the sacrificial siderophore (S₂) based on net charge using ion exchange chromatography. (A) Siderophores S₁ and S₂ were loaded to the ion exchange column. Siderophore S₁ washed through the column and siderophore S₂ was retained on the column and subsequently eluted. (B–G) All possible combinations of charge positive (FO), charge negative (SFO), and charge neutral (AcFO) were successfully separated as S₁ and S₂. EIC traces are off-set by 0.5 minute on the x-axis and a 10% off-set is used on the y-axis. EIC traces are representative of experiments performed in duplicated as independent trials.
Figure 2.9 Work-flow for the purification of salmycins from S. violaceus DSM 8286 cultures. Path A follows the procedure outlined in US Patents 5,475,094 and 5,519,123 until treatment with FhuD2 SBP-resin\(^1\),\(^2\). Path B is a simplified pathway that bypasses the HP-20 and DEAE resin treatments.

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\(^1\) FhuD2 SBP-resin

\(^2\) DEAE resin
Figure 2.10 Relative abundance total ion count for high-resolution LC-MS analysis of crude salmycins from S. violaceus DSM 8286 cultures reveals the presence of an impurity with m/z 1039.0129 (major peak) that co-elutes with the salmycins on the RP-C18 analytical HPLC column. Due to the concentration and similar molecular weight of this impurity, it is difficult to directly detect the salmycins in crude preparations and low resolution LC-MS analysis cannot differentiate the impurity from salmycin D (predicted m/z = 1039.3873 for [M+H]+).
Figure 2.11 Extracted ion chromatograms (EICs) from high-resolution LC-MS analysis of purified salmycins. Predicted m/z values for salmycin [M+H]⁺ ions are as follows: **Sal A**, 1053.4031; **Sal B**, 1038.3922; **Sal C**, 1024.3765; **Sal D**, 1039.3874.
Figure 2.12 Isolation of pure salmycins from S. violaceus DSM8286 culture supernatant by treatment with FhuD2 SBP-resin using danoxamine (Dan) as the eluting siderophore. Ion counts for the Sal A [M+H]^+ ion (m/z 1053) were tracked via LC-MS in the column load, flowthrough, wash, elution, and preparative HPLC fractions (Prep-C18 purified) along with growth inhibitory activity in an antibacterial agar diffusion assay using S. aureus ATCC 11632. EIC traces are offset by 1 minute on the x-axis and a 10% off-set is used on the y-axis. EIC traces are representative of experiments performed in duplicated as independent trial.
Figure 2.13 RP-C18 prep-HPLC chromatogram of salmcyins after elution from FhuD2 resin with FO. The y-axis represents absorbance units (AU) from optical absorbance at 427 nm.

Figure 2.14 Analytical HPLC analysis of salmcyins after elution from FhuD2 resin and purification by RP-C18 prep-HPLC. The y-axis represents milli-absorbance units (mAU) from optical absorbance at 427 nm.
Figure 2.15 Isolation of pure salmycins from S. violaceus DSM 8286 culture supernatant after treatment with HP20 resin, DEAE resin, and FhuD2 SBP-resin using FO as the eluting siderophore. Culture supernatant was treated as shown in path A of Figure 2.7. Ion counts for the Sal A [M+H]+ ion (m/z 1053) were tracked via LC-MS in the column load, flowthrough, wash, and elution along with growth inhibitory activity in an antibacterial agar diffusion assay using S. aureus ATCC 11632. As expected, the salmycins adhered to the FhuD2 SBP-resin and no bioactivity was detected in the column washings. Elution with FO generated a strong peak for Sal A in the m/z 1053 EIC. Further purification by RP-C18 prep-HPLC gave pure salmycins and removed excess FO, which antagonizes the bioactivity of the salmycins. EIC traces are off-set on the x-axis by 1 minute and a 25% off-set is used on the y-axis.
**Figure 2.16** High-resolution MS reveals Fe(III) isotopes for the salmycin isomers matching the predicted isotope patterns. The -2 iron isotope peaks labels are highlighted in bold for clarity. Refer to Table 2.2 for the parent ions.
Figure 2.17 Characterization of purified salmycins isolated from FhuD2 SBP-resin affinity chromatography and subsequent RP-C18 prep-HPLC. (A) UV-vis absorbance spectrum from 200–800 nm showing a characteristic absorbance band at 427 nm associated with the trihydroxamate-iron(III) coordination sphere. (B) Fluorescence quenching of N-His$_6$-FhuD2 by purified salmycins. Graph depicts intrinsic fluorescence quenching ($\lambda_{\text{excitation}} = 280$ nm; $\lambda_{\text{emission}} = 340$ nm) of N-His$_6$-FhuD2 and shows dose-dependent binding to iron(III)-bound salmycins with an apparent $K_d = 43 \pm 6$ nM. (C) Determination of the salmycins apparent $K_{Fe}$ ($\log K_{Fe} = 25.5 \pm 0.1$) using an EDTA competition assay. Graph represents the percentage of iron(III)-bound salmycins over time in the presence of excess EDTA as judged by optical absorbance at 427 nm. Apparent $K_{Fe}$ was calculated as described previously for 1:1 siderophore:iron(III) complexes. (D) Growth inhibitory activity of iron(III)-bound salmycins against *Staphylococcus aureus* ATCC 11632. Image shows an agar diffusion assay for antibiotic susceptibility with 9 mm wells treated with the salmycins (Sal; 0.1 mg/mL), an equimolar mixture of salmycins and danoxamine (Sal + Dan; both at 0.1 mg/mL), and an equimolar mixture of salmycins and ferrioxamine B (Sal + FO; both at 0.1 mg/mL). The growth inhibitory activity of the salmycins is more strongly inhibited by FO relative to Dan.
Figure 2.18 Analytical HPLC analysis of pure FO, SFO, AcFO, and Dan used in this work. The y-axis represents milli-absorbance units (mAU) from optical absorbance at (A) 427 nm or (B) 220 nm. The chromatograms in panel (C) represent blank MeOH injections to show the background baseline absorbance at 427 nm and 220 nm.
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2.8 References


58. Challis, G. L., and Ravel, J. (2000) Coelichelin, a new peptide siderophore encoded by the *Streptomyces coelicolor* genome: structure prediction from the sequence of its non-


Chapter 3: Biochemical Investigation of Nonribosomal Independent Siderophore Synthetases Reveals Missing Enzymes in the Ferrioxamine Siderophore Biosynthetic Pathway
3.1 Preface
This chapter was written by Gerry Sann Rivera (GSR) with feedback provided by Prof. Tim Wencewicz (TAW). GSR synthesized all compounds and purified all enzymes in these studies and performed all substrate screening assays. GSR performed high-resolution mass spectrometry isotope labeling studies with the help of Dr. Conghui Yao and Prof. Gary Patti in the WUSTL Dept. of Chemistry.

3.2 Abstract
Siderophore biosynthesis is varied amongst the structurally diverse siderophores. Many NRPS-dependent pathways have been well studied in contrast to the NRPS-independent pathways. Here we present studies on the Des gene cluster, responsible for producing DFO-E, and was presumed to also produce similar trihydroxamate siderophores. In vitro studies were done on the enzymes, DesA-D, that make up the Des cluster. A variety of substrates were fed into these enzymes, with particular interest in DesD, the siderophore biosynthetic enzyme responsible for oligomerizing the monohydroxamate molecules together to form the fully intact siderophore. Our results conclude that the Des cluster has a very specific substrate tolerance and has shown to only produce DFO-E under multiple conditions that should otherwise support the production of other siderophores. Our results support the hypothesis of a secondary cluster present in these organisms that allow for the complete biosynthesis of other trihydroxamate siderophores such as DFO-B.

3.3 Introduction
The biosynthetic pathway of siderophores varies based on the structure of the siderophore and the organism in which it is produced from. In general, these biosynthetic pathways can be
classified into two main categories which are the NRPS-dependent pathway and the NRPS-independent pathways.\textsuperscript{1} NRPS enzymes are modular and have multiple domains including the adenylation, condensation, and thiolation domains. They are typically selective towards their substrates, mainly consisting of amino acids.\textsuperscript{2} The NRPS-dependent siderophore biosynthetic pathways are generally better understood than those of the NRPS-independent siderophores (NIS).\textsuperscript{2} NIS synthetases have been found to be responsible for producing a wide variety of siderophores.\textsuperscript{3, 4} In particular, there is a biosynthetic gene cluster found in \textit{S. coelicolor}, shown in \textbf{Figure 3.1}, which is responsible for the biosynthesis of desferrioxamine E (DFO-E).\textsuperscript{5, 6} It is also evident that this same cluster of genes is present in a number of other bacteria that produce either DFO-E or other variants of trihydroxamate siderophores similar to that of DFO-E.\textsuperscript{7, 8} The biosynthesis of these trihydroxamate siderophores begins with the decarboxylation of lysine catalyzed by DesA.\textsuperscript{9, 10} DesB is proposed to sequentially hydroxylate to form \textit{N}-hydroxycadaverine.\textsuperscript{11} DesC shows similarity with acyl CoA-dependent acyl transferases and is proposed to catalyze the acylation of \textit{N}-hydroxycadaverine.\textsuperscript{6} DesD is the final biosynthetic enzyme in the cluster that is considered to be an enzyme that catalyzes the oligomerization of \textit{N}-hydroxy succinyl cadaverine into DFO-G, which cyclizes to form DFO-E.\textsuperscript{12} A DFO-E biosynthetic cluster found in \textit{Erwinia amylovora} has three enzymes that are homologous to that of the four enzymes in the Des gene cluster. Crystal structures have been obtained from these three enzymes that provide insight into the substrate shuttling that occurs within each enzyme.\textsuperscript{9}

\textit{In vivo} feeding studies have shown that the addition of select lysine derivatives into the producing organism can promote the production of other siderophore analogs.\textsuperscript{7, 13, 14} This suggests the broad scope of substrates that DesA-D are able to utilize to form a wide variety of trihydroxamate siderophores. The notion that DesA-D are responsible for the presence of a
multitude of trihydroxamate siderophores is further justified by the feature of most bacterial genomes in which genes in the same biosynthetic pathway are clustered in an operon.\textsuperscript{15}

In this work, we explore the Des cluster through in vitro enzyme screening studies. We propose that the Des cluster has a limited substrate scope that is only able to produce DFO-E. We also propose evidence for the presence of a secondary cluster responsible for modifying DFO-E, and producing related trihydroxamate siderophores including DFO-B. The secondary cluster is hypothesized to reside on a different operon than the Des cluster, revealing an early example of a biosynthetic compound that is the product of two different gene clusters. We also elucidate the interactions between the Des enzymes and how they complement each other to enhance product formation and efficiency.

### 3.4 Results and Discussion

**DesD Substrate Specificity**

In vitro enzyme assays were done by the Challis group on DesD produced from *S. coelicolor*, a known producer of DFO-E, by feeding substrates necessary to produce either DFO-E or DFO-B\textsuperscript{18}. DesD was shown to take these substrates to form DFO-E and DFO-B to a lesser extent. Since *S. coelicolor* is responsible for producing DFO-E and not DFO-B, it is possible that the substrate specificity and binding pockets of DesD differ between organisms. In order to compare substrate specificity of DesD between different organisms, we purified DesD from producing organisms, *S. griseoflavus* and *S. violaceus*, producers of sideromycins salmycin and ferrimycin, respectively (Figure 3.2).\textsuperscript{16,17} The siderophore component of salmycin is identical to that of danoxamine, whereas the siderophore component of ferrimycin is identical to that of DFO-B. We presume that the two siderophores are made prior to the covalent bond formation with the corresponding antibiotic. Three substrates for DesD were synthesized to show the
substrate scope of DesD (Figure 3.3). The synthetic routes for Compound 13 and 15 were designed similarly to that from Challis.\textsuperscript{12} Notable exceptions to the published synthesis include the use of different protecting groups, tert-Butyloxycarbonyl (Boc), benzyl, and 2,2,2-Trichloroethoxycarbonyl (Troc) groups. In addition, the coupling reagent used in this synthesis was diethyl azodicarboxylate (DEAD). The synthetic route for Compound 17 was synthesized by previously reported methods.\textsuperscript{25} Synthetic routes and experimental procedures are provided in Section 3.6.

A retrosynthetic analysis concludes that a 2:1 ratio of Compound 13:15 or 13:17 is necessary to produce either Danoxamine or DFO-B, respectively. Therefore, in order to replicate these conditions, the enzymes were fed to recombinant DesD in the same ratio. Figure 3.4 shows the in vitro assays for DesD, across all three organisms, exclusively produces DFO-E in the presence of not just 13 alone, but also with 13 and 15. Feeding in 17 seemed to inhibit activity and production of DesD entirely as no siderophore product was observed. This observation will be further discussed later in this chapter.

Although the retrosynthetic analysis predicts a 2:1 ratio of 13:15/17 as the most logical to obtain danoxamine or DFO-B, the concentrations in the cell would most likely not be the same. Therefore, we expanded upon the existing ratios to find the right balance of substrates that is necessary to produce the other siderophores. As shown in Figures 3.5-3.7, assays with varying substrate concentrations were performed, but all exclusively making either DFO-E or nothing regardless.

All three enzymes from the differing organisms performed similarly in terms of their substrate preferentiality. We hypothesize that the emergence of these different trihydroxamate siderophores in nature are either because of a synergistic effect between the four enzymes in the
biosynthetic gene cluster or because of a secondary cluster that can post-translationally modify DFO-E once it is produced by the Des cluster. The independent enzyme studies utilizing DesD aren’t an accurate reconstruction of what the cell environment would be like, and it is not surprising that we see a difference in concluding data between in vitro enzyme assays and in vivo feeding studies.

**DesD Substrate Inhibition**

As previously discussed, compound 17 has been shown to inhibit activity of DesD based on product formation analysis via mass spectrometry. We decided to delve further into this piece of data and determine if the enzyme is fully inhibited. We performed a coupled NADH assay to the DesD substrate assay to conclude whether compound 17 lowered ATP consumption within the enzyme. Figure 3.8 data show that the assay with compound 17 also showed consumption of ATP. In addition, the control group with no substrate also consumed ATP. The data was inconclusive because no-substrate control was indiscernible from the compound 13 control. Crystal structure analysis on DfoC, similar to that of DesD, contains a channel that would be able to recycle ATP without a release of any major substrate. Regardless, the product analysis shows no evidence that either of the three DesD enzymes are able to produce danoxamine when given compound 13 and 17 substrate together.

**Reconstitution of DesABCD**

The results in the previous DesD experiments showed a lack of substrate specificity, suggesting something else at play within the genes necessary for trihydroxamate siderophores. We purified out enzymes DesA-C from *S. violaceus* and performed the enzymatic biosynthesis
of DFO-E utilizing all four enzymes with lysine as the substrate. Figure 3.9 reveals that reconstituting the Des cluster leads to higher production of DFO-E than when using DesD alone, leading us to believe that there is a synergistic effect when all four enzymes are present. In addition, incorporation of substrates that are needed to make danoxamine and FO-B yields only FO-E, providing information that this synergistic effect between the four enzymes does not aid in the product diversity.

As shown in Figure 3.1, DesC is presumably responsible for transferring the acyl CoA substrate to N-hydroxy cadaverine. As such, succinyl CoA was used here as it was the CoA necessary for the production of DFO-E. Retrosynthetic analysis shows that a 2:1 ratio of succinyl CoA to acetyl CoA incorporation is essential for DFO-B to form rather than DFO-E. Similarly to the studies performed on DesD alone, we varied the acyl CoA substrate ratios, as shown on Figure 3.10, to determine what concentrations are needed for the Des enzymes to preferentially produce DFO-B over DFO-E. Our data show that within the observed concentration range, DFO-E was exclusively produced over DFO-B. This continues to contrast with existing natural product isolations that various strains produce varying trihydroxamate siderophores using the Des gene cluster, leading us to believe that there must be a secondary gene cluster that allows for the modification of DFO-E.

**S. pilosus Isotopic Feeding Studies**

We propose a secondary cluster that modifies the sole product of the Des gene cluster, DFO-E. In order to test this, we produced universally $^{13}$C labeled lysine into DesA-D in vitro to produce $^{13}$C DFO-E. This was isolated from the remaining substrates and protein by flowing through a DOWEX 50WX8 resin which crashed the protein and bound the remaining lysine. The
purified $^{13}$C labeled DFO-E was fed to cultures of *S. pilosus*, the organism responsible for making a surplus of DFO-B.\textsuperscript{18, 19} The siderophore was fed in at varying time points following inoculation of the media: t=0d, 2d, 4d to ensure any growth complications or changes to growth through feeding of $^{13}$C DFO-E. After each time point, the culture supernatant was isolated and analyzed via a high-resolution LC-MS/MS for the presence of isotopically labeled siderophore products. In Figure 3.11, we see that there was no incorporation of DFO-E into formation of DFO-B in any culture. The organism was found to produce orders of magnitude more DFO-E than the labeled version, which is a possible reason for the lack of incorporation of our labeled compound. We believe that the isotopically labeled DFO-E was outcompeted for cell entry by the naturally produced DFO-E and DFO-B. Nevertheless, this did not show any evidence towards the presence of a secondary cluster. Further feeding studies should include a higher concentration of $^{13}$C-DFO-E for a higher incorporation into the cell and a possibility in producing $^{13}$C DFO-B. A different strain that produces less DFO-B or DFO-E could also be helpful in reducing competition into the cell and for incorporation into post-NIS modifications.

**DesA-D Substrate Specificity**

Although the initial results showed a lack of product diversity from in vitro reconstitution of DesA-D, we decided to test the efficacy and tolerance of each enzyme through either individual enzyme assays or smaller combinatorial assays featuring different combinations of the four enzymes. A number of control reactions were done with the exclusion of each enzyme separately along with the exclusion of various cofactors. Figure 3.12 shows the absence of DesA still leads to a production of DFO-E, while the absence of both DesA and PLP halts production of DFO-E. We concluded from this data that PLP is capable of performing the decarboxylation
mechanism of lysine non-enzymatically without DesA. PLP has been found to be capable of doing this in several cases.\textsuperscript{19} The other enzymes and cofactors were found to be crucial in the formation of DFO-E, confirming previous reports describing the Des cluster.\textsuperscript{7,9}

Previous studies have shown the broad substrate specificity of DesC in \textit{S. coelicolor}, and we decided to expand on this result by incorporating a select number of acyl CoA.\textsuperscript{20} We tested the acyl CoA in the presence of DesA-C and investigated the production of these acyl products through addition of Fmoc and analysis through mass spectrometry. In contrast to both the DesD and fully reconstituted DesA-D studies previously shown, DesA-C showed low substrate specificity, leading to a production of various acyl products. As shown in Figure 3.13, the longer carbon chain acyl groups tend to lead to more product formation than even the native substrate, succinyl CoA. We believe that the lack of product diversity in the reconstituted Des gene cluster is due to the substrate specificity employed in DesD.

DesB is considered to be a monooxygenase enzyme which catalyzes the formation of \textit{N}-hydroxy cadaverine from cadaverine.\textsuperscript{12} A panel of substrates similar to that of cadaverine was tested as shown in Figure 3.14. In contrast to that of DesC, DesB was ineffective at catalyzing the incorporation of a hydroxyl group to the various other substrates. The lack of incorporation by DesB is consistent with our results of DesD and its unsuccessfulness in producing danoxamine, the siderophore portion of sideromycin, salmycin.\textsuperscript{21} Furthermore, DesB was found to be completely ineffective when tested without any other enzyme present. The presence of DesA or DesCD was necessary for DesB catalysis. We propose that although the gene cluster is considered an NIS cluster, it acts as a hybrid between NRPS-independent and NRPS-dependent clusters in which the four enzymes together form a synergistic effect that promotes effective enzyme activity. This idea is supported by another gene cluster from \textit{Erwinia amlyovora} that
produces DFO-E but only consists of three enzymes due to the fact that the enzyme DfoC is a fusion of both DesC and DesD. Further evidence is shown in Figure 3.9, in which we add compound 17, a proposed inhibitor of DesD, to the enzyme reaction of DesA-D, and show that the full gene cluster is still capable of forming DFO-E.

### 3.5 Conclusions

Previous studies have proposed that the Des cluster is responsible for making a variety of trihydroxamate siderophores because it is present in organisms that are known to produce these trihydroxamate siderophores.\(^8\),\(^22\),\(^23\) Our current studies propose that the four enzymes in the Des cluster are not the only factors at play to producing the different linear analogs of DFO-E. Although the earlier Des enzymes in the pathway have a broader substrate specificity, the whole Des cluster as a whole is unable to produce anything but DFO-E in vitro. In Figure 3.15 we outline potential pathways that DFO-E can take to modify its existing structure into other similar trihydroxamate siderophores such as DFO-B. One such pathway is hydrolysis of DFO-E and opening of the ring to form DFO-G, allowing for further chemistry to occur at either end of the siderophore. We base our hypothesis of this hydrolysis mechanism on past examples elucidating a few hydrolase enzymes responsible for hydrolyzing siderophores.\(^24\) In addition, sideromycins may also have a separate gene cluster responsible for producing the antibiotic that is conjugated sometime after DFO-E production. Examples include that of ferrimycin, in which the siderophore structure matches that of DFO-B, and salmycin, in which the siderophore structure matches that of danoxamine (Figure 3.2).\(^16\),\(^17\)

We also propose that the four enzymes work in a synergistic and regulatory effect rather than independently. This is not surprising as there are a number of other siderophores that have been classified into the NRPS-dependent gene clusters, which are much more understood than
the NIS counterparts. Our data suggest that there are interactions that cause for the increased productivity of the enzymes when all are present in contrast to the experiments with just one enzyme. Figure 3.17 presents crystal structures of the three enzymes in *Erwinia amylovora* that are able to produce desferrioxamine E. The crystal structure of DfoC represents an enzyme that performs both the mechanisms of DesC and DesD, which is further evidence that there is a specific substrate channling mechanism that incorporates each enzyme to allow for more efficient siderophore biosynthesis. Figure 3.17C illustrates each domain that is responsible for either the acyltransfer or the oligomerization, as well as a potential channel in between the dimers that could be used for the acyltransfer product to be channeled down to the second binding pocket of the enzyme for oligomerization.

It remains unclear which enzymes might be responsible for the hydrolysis and post-NIS modification of DFO-E. This work makes it evident that there is a gap in knowledge and a discrepancy between in vitro and in vivo studies. The mechanism of DesD leading to biased formation of macrocyclic ferrioxamine siderophores remains to be investigated. The current working model does not account for the formation of linear siderophores, supporting our hypothesis that other enzymes are involved in post-NIS tailoring reactions. Our fully reconstituted Des gene cluster studies set the stage for further elucidating the biosynthesis of trihydroxamate siderophores and sideromycins.

### 3.6 Materials and Methods

*Strains, materials, and instrumentation.*

*Staphylococcus aureus* ATCC 11632, *Streptomyces pilosus* ISP 5097, *Streptomyces griseoflavus* DSM 40698 and *Streptomyces violaceus* DSM 8286 were obtained from the ATCC and DSMZ collections, as noted in Table 3.1. *E. coli* BL21-Gold(DE3) and *E. coli* TOP10 cells
were obtained from Agilent and Invitrogen, respectively. *E. coli* cells were made electrocompetent by standard methods. A Bio-Rad MicroPulser electroporator and 0.2 cm gap sterile electroporation cuvettes were used for electroporation. Bacteria were stored as frozen glycerol stocks at -80 °C. Codon-optimized *fhuD2Δ24*, *desA*, *desB*, *desC*, and *desD* from the strains *S. violaceus* DSM 8286, *S. griseoflavus* DSM 30698, and *S. coelicolor* A3(2) was purchased from GenScript in a pET28a vector for heterologous expression in *E. coli* BL21(DE3) with an N-terminal hexahistidine tag (Table 3.2). All N-His<sub>6</sub> proteins were overexpressed and purified as described. DNA purification was performed with kits from Qiagen. Plasmid sequencing was performed by Genewiz. Nickel-nitriloacetic acid (Ni-NTA) agarose was purchased from ThermoFisher Scientific (catalog # R90115). Any kD SDS-PAGE gels were purchased from Bio-Rad. Proteins were dialyzed using 10K MWCO SnakeSkin dialysis tubing purchased from Thermo Fisher Scientific. Proteins were concentrated by centrifugal filtrations using 10K MWCO filters from Millipore. All aqueous solutions were prepared with water purified using a Milli-Q system and sterilized by filtration through a 0.2 μm filter. Media was sterilized using an autoclave unless otherwise stated. pH measurements were recorded using an Orion Star A111 pH meter and a PerpHecT ROSS micro combination pH electrode from Thermo Fisher. All buffers, salts, media, solvents, and chemical reagents were purchased from Sigma Aldrich unless otherwise stated. All media was sterilized in an autoclave prior to growing bacteria.

LC-MS was performed using an Agilent 6130 quadrupole with G1313 autosampler, G1315 diode array detector, and 1200 series solvent module. Samples were prepared in 0.45 μ PTFE mini-UniPrep vials from Agilent. Separations were achieved using a 5 μ Gemini C18 column (50 x 2 mm) from Phenomenex fit with a guard column. Mobile phases were 0.1%
formic acid in (A) H$_2$O and (B) ACN. Data were processed using G2710 ChemStation software. 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 Å column (250 x 21.2 mm) from Phenomenex fit with a guard column (15 x 21.2 mm). Mobile phases for RP-C18 prep-HPLC were 5 mM ammonium acetate in (A) H$_2$O and (B) ACN. Analytical HPLC was performed using a Beckman Coulter SYSTEM GOLD 127P solvent module and 168 detector with a Phenomenex Luna 10u C18(2) 100 Å column, 250 x 21.20 mm, 10 μm with guard column. HPLC data were processed using 32 Karat software, version 7.0. 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 spectrophotometer. High-resolution LC-MS/MS spectra were collected using a Q-Exactive (Thermo-Fisher Scientific) equipped with a custom built Eksigent microLC at the Donald Danforth Plant Science Center, St. Louis, MO. Mobile phases were 0.1% formic acid in (A) H$_2$O and (B) ACN and a Supelco C8 column (0.5 x 150 mm) was used for chromatographic separations. 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 $m/z$ 200–500 at a resolution setting of 70,000 (at $m/z$ 200) for MS$_1$ and a resolution of 17,500 for MS$_2$. NMR was performed on a Varian Unity Plus-300 MHz instrument. Optical absorption spectroscopy was performed on a Cary 50 fit with an auto sampler and water Peltier thermostat system using 1 cm quartz cuvettes.

For protein expression, a 5 mL culture of *E. coli* BL21 harboring the appropriate plasmid was grown overnight in LB containing 50 μg/mL kanamycin with agitation at 37 °C. A 200 μL aliquot of this culture was used to inoculate 500 mL of terrific broth (12 g/L tryptone, 24 g/L yeast extract, 5 g/L glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄) containing 50 μg/mL kanamycin. Culture was grown at 37 °C with agitation until OD₆₀₀ reached approximately 0.7. Culture was cooled in an ice bath for 20 min, then 500 μL of a sterile 0.5 M IPTG solution was added. Culture was then incubated with agitation for 16 hours at 20 °C. From this point, all protein purification steps were performed at 4 °C. Cells were harvested by centrifugation at 5,000 rpm for 20 min. Supernatant was discarded, and cell pellets were each suspended in 40 mL cold lysis buffer (50 mM K₂HPO₄ pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, 20 mM imidazole, 10% glycerol). Cell suspensions were transferred to 50 mL Falcon tubes and flash frozen in liquid nitrogen. Frozen cells were thawed and gently rocked for 30 min before being mechanically lysed using an Avestin EmulsiFlex-C5 cell disruptor. Cell lysate was centrifuged at 45,000 rpm for 35 min and supernatant was incubated with pre-washed Ni-NTA resin for 30 min. Resin was washed twice with 40 mL lysis buffer then eluted five times with 10 mL elution buffer (50 mM K₂HPO₄ pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, 300 mM imidazole, 10% glycerol). Fractions containing the majority of protein, as judged by SDS-PAGE with Coomassie blue visualization, were combined in 10,000 MWCO SnakeSkin dialysis tubing from Thermo Scientific and soaked overnight in 1.8 L phosphate buffer (5 mM K₂HPO₄ pH 8.0, 150 mM NaCl, 1 mM DTT). Dialyzed protein solution was concentrated using an appropriately sized spin filter (EMD Millipore Amicon® Ultra 15 mL Centrifugal Filters). Concentrated protein
solutions were flash frozen in liquid nitrogen and stored at -80 °C. SDS Page gel was run as shown in Figure 3.15.

**LC-MS Substrate Screen of DesD**

For substrate screens, 1.8mM of **Compound 13**, 3 mM ATP, 15 mM MgCl₂, 25 mM Tris-HCl (pH 8.0) and 25 μM His₆-DesD were added in ddi water for a final volume of 200 μL and incubated for 3 hours at 37 °C. The reaction was quenched by adding 10 μL of 0.5 M FeCl₃. After incubation, solution was mixed and centrifuged at 5000 rpm for 30 seconds. Supernatant was taken and analyzed via LC-MS. His₆-Sal_DesD and His₆-Ferri_DesD were also tested at the same concentration as His₆-DesD.

**LC-MS Substrate Screen of DesABCD**

For substrate screens, 25 mM lysine, 25 μM pyridoxal phosphate (PLP), 30 μM Flavin adenine dinucleotide (FAD), 150 μM nicotinamide adenine dinucleotide phosphate (NADPH), 0.1 M phosphate buffer (0.72 M K₂HPO₄, 0.17 M KH₂PO₄), 1.8 mM succinyl CoA, 3 mM ATP, 15 mM MgCl₂, 25 μM His₆-DesA, 25 μM His₆-DesB, 25 μM His₆-DesC, 25 μM His₆-DesD, and 20 μL glycerol were added in ddi water for a final volume of 200 μL and incubated for 3 h at 37°C. After incubation, solution was mixed and centrifuged at 5000 rpm for 30 seconds. Supernatant was taken and analyzed via LC-MS. Succinyl CoA was replaced with acetyl CoA in the ratios as indicated in Figure 3.10 for a total CoA concentration of 1.8 mM. Reactions performed in Figure 3.9c included 1.2mM succinyl CoA and 0.6mM of **Compound 17**. In addition, the reactions performed in Figure 3.9a, c contain a 2:1 ratio of succinyl CoA to either acetyl CoA or **Compound 17** respectively.
Controls run on Figure 3.12 included the same concentrations and total volume as the normal assay with the exclusion of the stated Des enzyme while still containing its corresponding cofactor i.e. a DesB control assay excludes DesB but includes NADPH and FAD.

**LC-MS Substrate Screen of DesABC**

For substrate screens, 25 mM lysine, 25 μM pyridoxal phosphate (PLP), 30 μM FAD, 150 μM NADPH, 0.1 M phosphate buffer (0.72 M K₂HPO₄, 0.17 M KH₂PO₄), 1.8 mM succinyl, acetyl, octanoyl, or decanoyl CoA, 25 μM His₆-DesA, 25 μM His₆-DesB, 25 μM His₆-DesC and 20 μL glycerol were added in ddi water for a final volume of 200 μL for 3 h at 37 °C. The reaction was quenched by adding 200 μL acetonitrile to afford a 50/50 mixture of water/acetonitrile. Solution was mixed and centrifuged at 5000 rpm for 30 seconds, yielding a precipitate and a bilayer. Supernatant was isolated and mixed. 150 μL of mixed supernatant was added to a solution containing 100 μL sodium borate, 210 μL acetonitrile, and 40 μL Fmoc (20 mM) and incubated for 30 min at r.t. Two layers formed, and the acetonitrile layer was isolated and analyzed via LC-MS.

**LC-MS Substrate Screen of DesAB**

For substrate screens, 25 mM lysine, 25 μM pyridoxal phosphate (PLP), 30 μM FAD, 150 μM NADPH, 0.1 M phosphate buffer (0.72 M K₂HPO₄, 0.17 M KH₂PO₄), 25 μM His₆-DesA, 25 μM His₆-DesB, and 20 μL glycerol were added in ddi water for a final volume of 200 μL for 3 h at 37 °C. The reaction was quenched by adding 200 μL acetonitrile to afford a 50/50 mixture of water/acetonitrile. Solution was mixed and centrifuged at 5000 rpm for 30 seconds, yielding a precipitate and a bilayer. Supernatant was isolated and mixed. 150 μL of mixed supernatant was
added to a solution containing 100 μL sodium borate, 210 μL acetonitrile, and 40 μL Fmoc (20mM) and incubated for 30 min at r.t. Two layers formed, and the acetonitrile layer was isolated and analyzed via LC-MS. Substrates used for DesAB included 5-hydroxylysine, cadaverine, 1,5-diaminobutane, 1,5-aminopentanol, and 1,4-aminobutanol. These substrates replaced lysine for a final concentration of 25 mM.

UV-Vis Activity Analysis of DesD
We added 25 mM Tris-HCl (pH8.0), 3 mM ATP, 15 mM MgCl₂, 5 mM PEP, 0.5 mM NADH, 5 μM His₆-Sal_DesD, 12.6 units lactate dehydrogenase (LDH), 8.4 units pyruvate kinase (PK), 4 units myokinase (MK), and 100 μM Compound 13 were added in ddi water for a final volume of 500 μL in 1 cm quartz cuvette. Testing of Compound 17 was done with 66 μM Compound 13 and 33 μM Compound 17. Absorption spectroscopy was carried out on a Cary 50 fit monitoring at 340 nm wavelength over 40 min at 37 °C. Controls were done without adding the enzyme, and without adding the substrate.

Streptomyces pilosus ¹³C DFO-E Feeding Studies
Production of ¹³C DFO-E was carried out using ¹³C lysine and following the previous protocol on 4x scale under “LC-MS Substrate Screen of DesABCD” with the exception of quenching with 10 μL 10% acetic acid for a final concentration of 0.5% acetic acid. Supernatant was taken and flowed through a Dowex 50 WX8 hydrogen form 100-200 mesh resin to remove any excess ¹³C lysine from the solution. Flowthrough fractions were collected, and the fractions containing ¹³C DFO-E were pooled and concentrated with a rotary evaporator. Resulting solid was dissolved in 1.5 mL ddi water. S. pilosus spore stock was streaked onto SFM agar and grown at 28 °C for 5
days. Six SFM cultures were added to six 50mL conical flasks, and each flask was inoculated with a 1x1 cm *S. pilosus* culture from SFM agar. $^{13}$C DFO-E was spiked into the sample immediately after inoculation, after 48 hours, and after 96 hours. Cultures were grown at 28 °C at 225 rpm for 4 days. Time points for each culture were taken by taking 1 mL of culture immediately after spiking of $^{13}$C DFO-E at 0 hours, 48 hours, and 96 hours. The time point sample was then filtered through celite and analyzed via High-resolution mass spectrometry.

*Synthesis of DesD analogs*

5-amino-1-pentanol (5g, 48 mmol) was dissolved in 20 mL THF and 25 mL H$_2$O. Et$_3$N (6.75 mL, 48 mmol) was added to solution. Boc$_2$O (10.58g, 48 mmol) was dissolved in 15 mL THF and added dropwise to solution and the reaction was stirred at rt for 14 hours. THF was removed by rotary evaporation, and the product was extracted with 3x EtOAc, and washed with 5% citric acid, H$_2$O, and brine. Rotary evaporation led to a clear and colorless oil which was mixed in with hexanes at -20 °C for 14 hours. Hexane was decanted off and solvent was removed by rotary evaporation to yield **Compound 8**. $^1$H NMR (300 MHz, Chloroform-d) $\delta$ 3.63 (t, $J = 6.4$ Hz, 2H), 3.11 (q, $J = 6.5$ Hz, 2H), 1.43 (s, 18H).
O-benzyl hydroxylamine (5g, 31 mmol) was added to 25 mL THF and 25 mL H₂O. 2,2,2-trichloroethylchloroformate (4.31 mL, 31 mmol) added to NEt₃ (4.40 mL, 31 mmol) added dropwise. The reaction was stirred for 3 hours at rt. THF was removed with rotary evaporation. Compound was extracted in EtOAc, and washed with 1 M HCl, H₂O, and brine. Removed solvent to yield **Compound 10**. ¹H NMR (300 MHz, Chloroform-d) δ 7.46 – 7.30 (m, 5H), 4.92 (s, 2H), 4.80 (s, 2H).

**Compound 8** (2.02g, 10 mmol) and 3.98g **Compound 10** (3.98g, 13 mmol) dissolved in 70 mL THF. Triphenylphosphine (3.46g, 13 mmol) was added. DEAD 40% in toluene (2.76 mL) added drop wise. Reaction stirred at rt for 18 hours. Solvent removed by rotary evaporation to yield a yellow oil. Product was purified using a silica column with 20:80 EtOAc:Hexane to yield **Compound 11**. ¹H NMR (300 MHz, Chloroform-d) δ 7.47 – 7.32 (m, 5H), 4.92 (s, 2H), 4.83 (s, 2H), 3.50 (t, J = 7.1 Hz, 2H), 3.08 (dd, J = 6.6 Hz, 2H), 1.72 – 1.56 (m, 4H), 1.43 (s, 9H), 1.39 – 1.29 (m, 2H).
**Compound 11** (0.7g, 1.5 mmol) was added to 4.75 mL THF, 4 mL AcOH, 0.3g succinic anhydride, and 3.3g activated Zn. Let reaction run until starting material was not present in TLC. Zn was removed by vacuum filtration. Solution was concentrated and redissolved in minimal benzene. Hexane was added to a 1:1 ratio until precipitate formed. Remaining solution was concentrated down to yield **Compound 12**. $^1$H NMR (300 MHz, Chloroform-d) $\delta$ 7.44 – 7.30 (m, 5H), 4.82 (s, 2H), 3.75 – 3.54 (m, 1H), 3.15 – 2.99 (m, 3H), 2.75 – 2.56 (m, 3H), 1.63 (p, J = 6.8 Hz, 2H), 1.43 (m, 13H).

**Compound 12** (80mg, 0.196 mmol) was dissolved in methanol. Added 10 wt% Pd/C and added H$_2$ gas, letting the reaction run for 11 hours at rt. Solution was syringe filtered and concentrated down to yield the Benzyl-protected **Compound 13**. Benzyl-protected **Compound 13** was added to TFA and let stir for 1 hour at rt. Solution was concentrated down and redissolved in methanol. Added methyl sulfonic acid to form mesylate salt. Concentrated solution and redissolved in methanol. Product was purified using preparatory HPLC using water/acetonitrile with 0.1% TFA to yield **Compound 13**. $^1$H NMR (300 MHz, Methanol-d4) $\delta$ 3.75 – 3.62 (m, 4H), 2.94 (t, J = 7.6 Hz, 2H), 2.87 – 2.74 (m, 1H), 2.67 – 2.55 (m, 2H), 1.79 – 1.62 (m, 2H), 1.52 – 1.34 (m, 2H).
Compound 11 (0.3g, 0.622 mmol) was dissolved in 1.6 mL THF, 0.4 mL AcOH. 0.2g activated Zn and 123mg acetic anhydride was added. Stirred reaction for 90 minutes at rt. Extracted in EtOH and washed with water and brine and dried on MgSO₄ to yield Compound 14. ¹H NMR (300 MHz, Methanol-d₄) δ 7.47 – 7.33 (m, 4H), 4.88 (s, 2H), 4.86 – 4.81 (m, 2H), 3.66 (t, J = 7.1 Hz, 2H), 3.00 (td, J = 6.9, 1.7 Hz, 2H), 2.04 (s, 3H), 1.53 – 1.21 (m, 14H).

Compound 14 was dissolved in methanol. Added 10 wt% Pd/C and added H₂ gas, letting the reaction run for 11 hours at r.t. Solution was syringe filtered and concentrated down to yield Benzyl-protected Compound 15. Benzyl-protected Compound 15 was added to TFA and let stir for 1 hour at r.t. Solution was concentrated down and redissolved in methanol. Added methyl sulfonic acid to form mesylate salt. Concentrated solution and redissolved in methanol. Product was purified using preparatory HPLC using water/acetonitrile with 0.1% TFA to yield Compound 15. ¹H NMR (300 MHz, Methanol-d₄) δ 3.66 (s, 2H), 2.95 (s, 2H), 2.14 (s, 3H), 1.73 (q, J = 7.2 Hz, 4H), 1.43 (d, J = 7.3 Hz, 2H).
Compound 16 (100mg, 0.25 mmol) was dissolved in methanol. Added 10 wt% Pd/C and added H₂ gas, letting the reaction run for 11 hours at rt. Solution was syringe filtered and concentrated down to yield Compound 17. ¹H NMR (300 MHz, Methanol-d4) δ 3.65 – 3.48 (m, 5H), 2.77 (t, J = 6.6 Hz, 2H), 2.64 – 2.49 (m, 4H), 1.71 – 1.48 (m, 4H), 1.46 – 1.27 (m, 2H).
### 3.7 Figures and Tables

**Table 3.1** Strains and plasmids used in this work.

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<tr>
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<th>Plasmid</th>
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<td>Wild Type</td>
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<td>Protein expression strain</td>
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<td>N-His$_6$-Coel_DesD</td>
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<td>pET28a</td>
<td>N-His$_6$-Sal_DesC</td>
<td>Wencewicz Lab$^2$</td>
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Table 3.2 Primary protein sequences of wilde type (WT) FhuD2, N-His$_6$-FhuD2Δ24 variants, DesA, DesB, DesC, and DesD and its variants used in this work.$^a$

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<th>Protein</th>
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</tr>
<tr>
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WT-DesC from *S. violaceus*

MTFTFRTLDPKAELLeHHGWVTHPKAAFWMQMDDAKLEDVERAYMEIAADEHHHALLGLQDGEPAFLMEKYDPARRELVGLYEARPGDVGMHFLTPTDKPVHGFTKSVTAVMAHLFEDPATERVVVEPDRVNRKAVHALNAAVGFVPEREQKPEKALLSFCTREQFLAATG

WT-DesD from *S. violaceus*

MSLADSVAHLSPERWEQANRLRIRKLATEAEFAHERLITPERDGDVVVRSDDLGTYRFTAAVRILDWQIDADSIHRDGEELPLAALDFIELKDSLGLDSEILPVYLEEISSTLSGTYKLTKPRITAALAAAGGFQAIETGMEGHPCFVANNRLGFGIHELYSYAPETASPVRLVWLAAHRSRAAFTAGVQIDYESVRQELGGEVTDDERVFHVGLRERGLDPADYLIEIPVHPWQWNNKLSTVFSAEVARQHLVCLGEGDFDELYAQQSITFFNTSHPEKHYVTALSVINM

WT-DesD from *S. griseoflavus*

MSLTDAAHLSPERWEQANRLVRKALAEFHERLTPEPADGQDDYVVRSDDLGTSYRFTAVRRALDHQVDAGSITRTRDGAELPLAALDFIELHRTLGLSDEILPVYLEEISSTLSGTCYLTKPQVTAAGLLEGFGQAETGMEGHPCFVANNRLGFGIDELAYAPETHPVRVLWLAAHRSRAAFTAGGIDYASVQRQELGEETVERFDGVLRGRGLDPADYLHPPWQWNNKLSTVFSAEVARQNLVCLGEGDFDELYAQQSITFFNATHPEKHYVKTASLVLNMGFMGRGLSAYMEATPAINWDLRLIDNDPVKLSTGLSIRERAAVGYRHLEYEATDROYSPYRKLMAALWREPVPVSPLRDGESLTTMAALVHVDHEGRSVAGELIARSLAPTAWRHRLRAYYTPLLHSFYAYDLVMYPMGENVILVLDGVERAIYKDIAEICVMDPDAVLPPEVQRIARVEDPTKLSVTDVFDFCFRFLAVNLASEGVLSEDFWRTVAETREYQESMPEDAKFRQYDMFAPFASLCLNLQLNRNKNQMVMDLADPSGALQLVGTLRNPIAGF

WT-DesD from *S. coelicolor*

MSLADAVAHLSPERWEQANRLVRKALAEFHERLTPEPADGQDDYVVRSDDLGTSYRFTAVRRALDHQVDAGSITRTRDGAELPLAALDFIELHRTLGLSDEILPVYLEEISSTLSGTCYLTKPQVTAAGLLEGFGQAETGMEGHPCFVANNRLGFGIHELYSYAPETASPVRLVWLAAHRSRAAFTAGVQIDYESVRQELGGEVTDDERVFHVGLRERGLDPADYLHPPWQWNNKLSTVFSAEVARQNLVCLGEGDFDELYAQQSITFFNATHPEKHYVKTASLVLNMGFMGRGLSAYMEATPAINWDLRLIDNDPVKLSTGLSIRERAAVGYRHLEYEATDROYSPYRKLMAALWREPVPVSPLRDGESLTTMAALVHVDHEGRSVAGELIARSLAPTAWRHRLRAYYTPLLHSFYAYDLVMYPMGENVILVLDGVERAIYKDIAEICVMDPDAVLPPEVQRIARVEDPTKLSVTDVFDFCFRFLAVNLASEGVLSEDFWRTVAETREYQESMPEDAKFRQYDMFAPFASLCLNLQLNRNKNQMVMDLADPSGALQLVGTLRNPIAGF
Adapted with permission from the supporting information file associated with Endicott, N. P.; Lee, E.; Wencewicz, T. A. “Structural basis for xenosiderophore utilization by the human pathogen Staphylococcus aureus” ACS Infectious Diseases, 2017, 3, 542-553. Copyright 2017 American Chemical Society/Timothy A. Wencewicz. Pre-lipoprotein signal sequence is highlighted in magenta. Soluble siderophore-binding domain highlighted in teal. Hexahistidine motif with thrombin cleavage site highlighted in yellow. Soluble siderophore-binding domain highlighted in teal. ESI-MS indicated loss of the N-terminal methionine residue (Figure 2.4).
Figure 3.1 Biosynthetic pathway for DFO-E utilizing the Des gene cluster found in *S. coelicolor* A3 (2) and other trihydroxamate siderophore producing organisms.

Figure 3.2 Sideromycins salmycin and ferrimycin A1. Salmycin’s siderophore structure is from danoxamine. The antibiotic is believed to act similar to an aminoglycoside with ribosome inhibitory properties. Ferrimycin A1’s siderophore structure is from FO-B. The antibiotic mechanism of action is not known.
Figure 3.3 Synthetic route taken to obtain the three substrates used in the DesD studies. These monomers are believed to be the building blocks of the trihydroxamate siderophores FO-B, FO-E, and danoxamine.
DesD substrate studies performed in vitro utilizing the three substrates from Figure 3.3. (A) Reactions were performed using the DesD enzyme in *S. coelicolor*. (B) Reactions were performed using the DesD enzyme in *S. violaceus*. (C) Reactions were performed using the DesD enzyme in *S. griseoflavus*. The leftmost column utilizes Compound 13 in the efforts of producing FO-E. The middle column utilizes Compounds 13 and 15 to attempt to produce danoxamine. The rightmost column utilizes Compounds 13 and 17 to attempt to produce FO-B. This data shows exclusively FO-E production regardless of the substrates present.
Figure 3.5 DesD reactions performed with varying ratios of the three substrates, Compounds 13, 15, and 17. FO-E production is found to be the sole product of all combinations, with a retention time of 4 minutes.
Figure 3.6 Ferri DesD reactions performed with varying ratios of the three substrates, Compounds 13, 15, and 17. FO-E production is found to be the sole product of all combinations, with a retention time of 4 minutes.
Figure 3.7 Sal DesD reactions performed with varying ratios of the three substrates, Compounds 13, 15, and 17. FO-E production is found to be the sole product of all combinations, with a retention time of 4 minutes.
Figure 3.8 Analysis of AMP and ADP formation with the reaction involving Compound 17. (C) A typical curve representing the consumption of ATP from DesD. (B) A non-specific ATP consumption without the presence of any substrate. The curve on (A) is very similar to that of (B), providing a clue that Compound 17 inhibits production. (D) A control without any substrate or enzyme, resulting in no consumption of ATP.
Figure 3.9 Sal DesABCD reactions performed utilizing a variety of substrates. Traces in blue correspond to FO-E production, traces in red correspond to danoxamine production, and traces in green correspond to FO-B production. The same trend is observed when all Des enzymes are present when compared to just Sal DesD. (A) Reactions performed using substrates needed to make FO-E. (B) Reactions performed using substrates needed to make Danoxamine. (C) Reactions performed using substrates needed to make FO-B.
Figure 3.10 Sal DesABCD reactions performed under varying concentrations of succinyl CoA and acetyl CoA. Traces in blue, red, green, and purple correspond to FO-E, DFO-E, FO-B, and DFO-B, respectively. FO-B production is not observed under any of these picked ratios.
Figure 3.11 High-resolution MS traces representing the various isotopically labeled versions of FO-E and FO-B. The traces labeled $^{13}$C FO-B and $^{13}$C FO-E correspond to fully incorporated $^{13}$C molecules. The traces labeled FO-B and FO-E correspond to no incorporation of the isotopically labeled molecules. There is production of FO-B but no incorporation of $^{13}$C FO-E resulting in formation of $^{13}$C FO-B.
Figure 3.12 Control groups of the Des enzymes with one or more enzymes/cofactors missing. The traces show the amount of FO-E produced. Based on these traces, all enzymes are necessary except for Sal DesA. In the absence of Sal DesA, PLP can perform the decarboxylation of lysine.

Figure 3.13 Sal DesABC reactions involving different acyl CoA derivatives consisting of acetyl CoA (A), succinyl CoA (D), octanoyl CoA (B), and decanoyl CoA (C). Fmoc was added to the enzymatic product to increase retention time on the column and improve ionization. Sal DesC is readily capable of performing the acyl transferase reaction to all CoA’s listed except succinyl CoA. This is surprising since the product of 3.8d is needed to make FO-E.
Figure 3.14 Various combinations testing the substrate scope of both Sal DesA and Sal DesB. Similarly to Figure 3.13, all enzymatic products were introduced to Fmoc to increase retention time and improve ionization. Sal DesA is capable of performing the decarboxylation step by itself. Sal DesAB is also able to take in different substrates such as hydroxyl-lysine. Sal DesAB is not capable of making N-hydroxy cadaverine without the presence of Sal DesCD. It is also not capable of taking other derivatives of cadaverine.
Figure 3.15 SDS-PAGE analysis of N-His-Sal DesA (~52.5 kDa), Sal DesB (~48.3 kDa), Sal DesC (~19.7 kDa), Ferri DesD (~66.5 kDa), Coel DesD (~66.5 kDa), and Sal DesD (~66.5 kDa) purified by Ni-NTA affinity chromatography after heterologous expression in E. coli BL21(DE3) cells.

Figure 3.16 Potential pathways that can exist to convert DFO-E into the other trihydroxamate siderophores found in nature. A hydrolase is present in order to uncyclize the siderophore to allow for modifications to occur at both ends.
Figure 3. Crystal structures of DfoJ, DfoA, and DfoC from *Erwinia amylovora*, which are responsible for producing DFO-E. (A) Crystal structure of DfoJ, which catalyzes the decarboxylation of lysine to cadaverine. PDB#: 5O8P (B) Crystal structure of DfoA, which catalyzes the monooxygenation of cadaverine to N-hydroxy-cadaverine. PDB#: 5O7O (C) Image taken from Benini et al. Crystal structure of DfoC, which catalyzes two reactions: succinyl transfer and trimerization of the monohydroxamates. Pink and cyan domains are the acyltransferase domains, while the purple and blue domains are the oligomerization domains. PDB#: 5O5C.
3.8 References


Chapter 4: Cell Entry and Drug Release
Mechanism of Siderophores
4.1 Preface
This chapter was written by Gerry Sann Rivera (GSR) with feedback provided by Prof. Tim Wencewicz (TAW). Synthetic ester analogs were synthesized by TAW. Synthetic DFO-NBD was synthesized and characterized by Dr. Nathaniel Endicott (NPE). All Fe exchange were performed by NPE. All siderophore displacement studies were performed by GSR. All hydrolysis studies were performed by GSR. GSR performed NMR studies with the help of Dr. Jeff Kao in the WUSTL Dept. of Chemistry. Figure 4.1-4.3 were adapted from a manuscript in preparation and NPE’s dissertation.

4.2 Abstract
This chapter covers two main topics: FhuD2 interactions with other metallophores and siderophore-drug conjugate hydrolysis mechanisms. In regards to the first topic covering FhuD2 interactions, we screen a number of FhuD2 mutations to determine which residues are important in facilitating the displacement mechanism between siderophores. We have also probed the interaction of FhuD2 with another metallophore, transferrin. Recent studies have shown that FhuD2 can catalyze the iron transfer between siderophore and transferrin. Here, we have probed its ability to facilitate the displacement mechanism between siderophore and transferrin and provide evidence of stable protein-protein interactions between FhuD2 and transferrin. In the second topic covering siderophore hydrolysis mechanisms, we test a number of apo- and holo-siderophore esters to provide mechanistic insight in the process of hydrolysis and its relationship with iron release once inside the cell. This has proven useful in studying sideromycins and their overall pathway once inside the target cell.
4.3 Introduction

With the rise of antibiotic resistance, it is imperative that new and innovative strategies are discovered. A typical resistance mechanism that bacteria employ on a wide variety of antibiotics is the exclusion or efflux of the compound, thereby reducing the intracellular concentration to levels below the MIC that allows the bacteria to continue to proliferate.\(^1\) As previously discussed in Chapter 1, a potential strategy to combat this type of resistance is to exploit other biological pathways that allow the drug to enter the intracellular cytoplasm of multi-drug resistant bacteria.\(^2\) Biological pathways used for this strategy should involve specificity towards bacterial cells over eukaryotic cells, and a broad substrate scope.\(^3\) Bacterial iron-acquisition pathways utilizing siderophores will be the focus of this study because it is an excellent candidate pathway that nature has already designed methods for exploitation. Bacteria have evolved to employ a “Trojan horse” strategy to sneak antibiotics into competing bacteria through the use of siderophore-drug conjugates, sideromycins.\(^4\) Sideromycins show great potential for new drug design because they have the possibility to incorporate old antibiotics rendered useless from resistance, and recycle them into a new entry pathway with the intent of bypassing membrane resistance mechanisms. Another advantage to using sideromycins is to take existing potent antibiotics that have poor membrane permeability and facilitate membrane translocation via the siderophore pathway.\(^5,6\)

Currently, there are only a handful of sideromycins found in nature, which include albomycins and salmycins.\(^4\) Sideromycins follow the same entry pathway as siderophores. In the case with albomycin, it crosses the outer membrane through the FhuA and Ton-dependent proteins, and subsequently binds to FhuD in the periplasm.\(^7,8\) It is shuttled to FhuB and is translocated into the cytoplasm by FhuC.\(^9\) Salmycin is selectively active against gram-positive strains by utilizing a different pathway from albomycin, and instead enters the cytoplasm via a
siderophore binding protein FhuD2.\textsuperscript{10, 11} FhuD2 is known to uptake hydroxamate-containing xenosiderophores.\textsuperscript{12, 13} \textit{S. violaceus}, the salmycin producing strain, has developed resistance to its own sideromycin by mutating FhuD2 and excluding the antibiotic from the cytoplasm.\textsuperscript{14} Part of our work is to elucidate the mechanistic intricacies of FhuD2 in order to determine the substrate specificity. We identified a few active site mutants of FhuD2 that were essential for siderophore displacement, while others affected iron-shuttle between holo- and apo- siderophore. We also probed the protein-protein interaction between FhuD2 and holo-transferrin, determining whether displacement can occur between siderophores and transferrin in the active site of FhuD2.

Once inside the cytoplasm, sideromycins are free to interact with its designated biological target. In the case for albomycins, they have been found to dissociate the siderophore and seryl-t-RNA synthetase inhibitor before the antibiotic reaches its biological target; a knockout of \textit{pepN}, the enzyme responsible for cleaving off the antibiotic via amide hydrolysis, results in abolished activity.\textsuperscript{14, 15} Not much is known about the drug-release mechanism of salmycins. With this information, it is reasonable to hypothesize that the other sideromycins have the potential to also hydrolyze off their antibiotic region before reaching the biological target. Although salmycin has very potent in vitro activity against gram-positive bacteria, mouse studies have shown drastically reduced in vivo potency. The proposed cause of the reduced activity is the extracellular hydrolysis of the drug before entry into the cell. In order to design new siderophore-drug conjugates that overcome problems similar to that of extracellular hydrolysis, it is imperative that we determine and compare a variety of drug-release mechanisms to provide insight on what linker region is best for each compound. The focus of this study is to elucidate the naturally occurring sideromycin drug-release mechanism in salmycins and potentially others as well. We delve into a panel of ester analogs to probe the relationship between linker length, presence of
iron, and rate of hydrolysis. Based on this information, we propose a mechanism of drug release that can be modeled onto the salmycins.

4.4 Metallophore Interactions with Siderophore-Binding Protein FhuD2

As previously discussed in Chapter 1, the uptake pathway of siderophores is better characterized in gram-negative bacteria than in gram-positive bacteria. There is a great emphasis on exploring the siderophore uptake pathway in gram-positive bacteria. Many of them, such as MRSA, depend on virulence factors that are connected to nutrient acquisition pathways.\textsuperscript{16, 17} One of these nutrient acquisition pathways is the siderophore pathway with the incorporation of siderophore-binding protein (SBP) FhuD2.\textsuperscript{18} FhuD2 is expressed to recognize and aid in shuttling xenosiderophores, primarily FO-B, into the cytoplasm.\textsuperscript{19} FhuD2 is an important protein to study because of its classification as a virulence factor which is used to counteract nutritional immunity within the host. Interestingly, it has been found that a number of SBPs have similar binding affinities towards both the apo- and holo- siderophores.\textsuperscript{20} Figure 4.1 displays a potential scenario that gives reason to the high binding of apo-siderophores which include two uptake mechanisms, displacement and shuttle. In this work, we propose a mechanism hypothesizing which binding pocket residues are important in carrying both the shuttle and displacement cycles specifically in the Fhu pathway in \textit{S. aureus}.

\textit{FhuD2 Mutant Displacement Studies}

These sets of experiments probe the binding pocket of FhuD2, and attempt to elucidate the overall mechanism and the residues involved for both displacement and shuttle mechanisms. In order to do this, we expressed the following FhuD2 mutations in Figure 4.2a and compared
these mutant FhuD2 proteins to WT. Residue mutations of FhuD2 were chosen based on the crystal structure of FhuD2 bound to FOB in two conformations, open and closed. Our mechanism proposes that FhuD2, upon coming into contact with two siderophores (one pre-bound and another within close proximity to the protein), will adopt two main conformations; a closed and open conformation. The different conformations can either increase or decrease the siderophore iron affinity by interacting with the hydroxamate ligands which can facilitate faster iron exchange. **Figure 4.3** illustrates our proposed mechanism for switching between closed and open conformations; W173 and R175 have direct interactions with the hydroxomates on the siderophore backbone, facilitating the stretching and contraction of the siderophore and leading to a change in the iron binding affinity. Residues Y167, Y169, and Y254 are involved in binding the iron from the external iron-source (whether it be from a siderophore or other iron-scavenging protein such as transferrin). We propose that these three residues are important in catalyzing exchange of metal between a neighboring iron source and the pre-bound apo-siderophore. Residue W255 seems to adopt the largest positional change between open and closed conformations. Y106 and Y256

Although the main focus of this mutation study was to probe the shuttle mechanism, the displacement efficiency of the mutants were compared to that of the wild type as well. **Figure 4.2** illustrates the relative counts of eluted siderophore due to displacement, revealing that most of the proposed residues that are involved in the shuttle mechanism do not affect the displacement mechanism. Mutating residues R175, W255, and W173 resulted in a near-abolishment of displacement activity. Residues R175 and W173 are proposed to interact with two hydroxamate ligands of the siderophore, while W255 is one of the residues that moves between the N-terminal and the center of the siderophore during the change between open and
closed conformations. Mutation W173A’s reduced displacement efficiency is due to a drop in $k_{\text{cat}}$. Mutation R175A’s drop in efficiency is due to its overall decreased substrate affinity. W173 is also found on a cleft of FhuD2 near the binding pocket that moves away (open) or towards (closed) the siderophore depending on the conformation.

We compared the various mutant efficiencies in the shuttle and displacement mechanism, and concluded that the 8 residues play different roles in each mechanism. For example, Y167F, Y169F and Y254F show a significant drop in catalytic efficiency but perform displacement relatively similar to that of WT. This is not surprising since our model proposes that these three residues are important in binding the iron from the neighboring iron source and catalyzing iron exchange. In the displacement mechanism, the three residues do not need to bind to iron from the other iron-source and as such, we see little change in displacement efficiency. W173 and R175 are important in both FhuD2 mechanisms because of the direct interactions they have with the siderophore backbone.

**Transferrin-FhuD2 Interaction**

Previous work in this lab has shown that FhuD2 can catalyze the iron exchange of holo-transferrin with apo-siderophores, expanding the available iron source that this SBP can scavenge nutrients from. As a virulence factor, FhuD2 can be considered significant for nutrient acquisition due to its ability to scavenge iron from one other iron source in addition to siderophores. We further investigated this pathway by attempting to observe the displacement mechanism between transferrin and FO-B (**Figure 4.4**). We also attempted to capture and observe the protein-protein interaction between FhuD2 and transferrin. We first sought to determine whether the displacement mechanism could also involve FhuD2. **Figure 4.5** shows a
lack of displacement between holo-transferrin and FO-B despite kinetic data displaying a high FhuD2-transferrin complex affinity. We believe that although FhuD2 has similar binding affinities towards transferrin and FO-B, the protein-protein interaction is thermodynamically weak. The weak interaction leads to a transient binding and lack of retention after several rigorous buffer washes on the column. Transferrin also has shown the ability to bind to the Ni-NTA resin, complicating the overall binding event since transferrin can also displace FhuD2 off the column. Future studies to improve upon this would be to use FhuD2 agarose beads in order to eliminate Ni-NTA as a factor.

With this data, we decided to probe the interaction by attempting to observe it via size exclusion chromatography. Currently, our column conditions and detection methods seem, as shown on Figure 4.5b-d, are not sufficient enough to detect the protein-protein interaction. This data is consistent with the lack of displacement between transferrin and siderophore, which adds to the theory that the interaction between the two proteins is transient in nature. Further studies are needed to determine whether this interaction is stable enough to be isolated and observed. These future studies are explained in further detail in Chapter 5.

4.5 Relationship between Iron Reduction and Drug Release

The siderophore pathway is well characterized in both gram-positive and gram-negative bacteria, as illustrated in Figure 4.6. The difference between the siderophore pathway and sideromycin pathway in competing bacteria differ in that the sideromycin’s function is to reach its intracellular target to either negate growth or kill the bacteria completely. Consequently, if it is assumed that sideromycins require a drug-release mechanism to occur, it is important to determine where in the siderophore pathway this event happens.
There are two possible scenarios in which drug-release occurs, one when the sideromycin hydrolyzes off the antibiotic region before iron reduction and release, and another when hydrolysis occurs after iron reduction and release. Figure 4.7 illustrates a few mechanisms that differ in their timing with iron release. The mechanism in Figure 4.7 shows the process of hydrolysis occurring after iron reduction and release, utilizing the free oxygen on the neighboring hydroxamate as a nucleophile to attack the carbonyl carbon on the ester linker region. The intramolecular nucleophilic attack is favorable when a 5 or 6-membered ring intermediate is formed, and under slightly basic conditions (pH>9) when the hydroxamate oxygen is deprotonated. The ringed intermediate is then cleaved by a nucleophilic attack of H₂O, leading to the hydrolyzed carboxylic acid version of the siderophore and the free antibiotic.

The other two possible mechanisms as shown occur before iron reduction, utilizing the metal center either as a Lewis acid or an H₂O activator. In the iron bound form, the siderophore’s interaction with the metal center slightly polarizes the terminal ester, enhancing its electrophilicity and promoting a nucleophilic attack from water. The metal center can also interact with the water, bringing it into closer proximity and promoting a semi-intramolecular attack on the ester. Both mechanisms require a ligand displacement of the metal, which would require a high amount of energy or a possible enzyme that catalyzes this event.

In order to test which mechanism is at play, a panel of both holo- and apo-desferrioxamine siderophore ester analogs were synthesized (Figure 4.8). The siderophores vary in carbon chain length between the hydroxamate and the ester. A couple of other siderophores were also synthesized to restrict bond rotation in Compound 5, and to introduce a Thorpe-Ingold conformational effect in Compound 6. The panel of siderophore ester analogs were dissolved in pH 7 buffer and incubated at room temperature and periodically observed.
either through analytical HPLC or H-NMR to calculate the relative rates of hydrolysis as shown in Figure 4.9.

Two striking results were discovered, the first of which being that the carbon chain length greatly affects the rate of hydrolysis of both the apo- and holo-siderophores. In Figure 4.9a-b, moving from one carbon to two carbons in the holo- forms of both Compound 1 and Compound 2 respectively, increases the stability of the holo-siderophore ester over ten-fold; Compound 1 almost completely hydrolyzes to its acid form within 24 hours whereas Compound 2 doesn’t begin to hydrolyze even after 4 days. We see the same effect in the apo-siderophore ester Compound 2 where hydrolysis is not observed after 57 days while the apo-siderophore ester Compound 1 hydrolyzes fully to its acid form in 24 hours (Figure 4.10). Increasing the carbon chain length of the holo-siderophore esters further establishes the trend that the farther away the ester is from the hydroxamate, the slower the rate of hydrolysis becomes. We see that after 73 days in Compound 4 that hydrolysis does not begin due to the stability of the ester complex (Figure 4.9d). This trend gives us an indication that there is some interaction between the ester and the neighboring hydroxamate or metal center, leading us to believe that the mechanism in Figure 4.7a could be the dominant mechanism at play.

Compound 5 rate of hydrolysis is shown on Figure 4.9e. The intended effect of the cyclopropyl group is to hinder rotational bond movement between the hydroxamate and the terminal ester. We see that after 73 days hydrolysis does not occur. Comparing the hydrolysis rates of holo-siderophores of Compound 2 and 5, they both contain two carbons between the hydroxamate and the ester, and yet the hydrolysis rates differ greatly. This gives evidence to the idea that the steric play an important role in hydrolysis of the ester when iron is not present, further supporting mechanism 1 in Figure 4.7. The introduction of the gem-dimethyl moiety in
**Compound 6** should theoretically stabilize the cyclic tetrahedral intermediate and enhancing the rate of hydrolysis. We do not see this trend occur, and the stability of the ester remains strong due to steric effects. This contrasts to the previously reported data and presents data not supporting mechanism 1. Further studies behind these compounds and their hydrolysis is necessary to determine the mechanism of hydrolysis.

**Sideromycin Hydrolysis**

Our previous experiments in **Chapter 2** provided the necessary sideromycin, salmcyin, to test hydrolysis and monitor the rate via an agar diffusion assay. A semi-pure sample of salmcyin was diluted a number of times to generate a standard curve in **Figure 4.11** that we compared the following experimental results, in order to quantify the rate of hydrolysis. In contrast to the previous siderophore ester hydrolysis experiments, this experiment was done at 37° C to speed up the hydrolysis process. We hypothesize that the hydrolysis rate of salmcyin would be similar to that of the holo siderophore ester **Compound 2**, which had a half-life of about 50 days. As shown in **Figure 4.11**, salmcyin potency decreases over the course of a couple weeks but maintains some activity. We suspect that the slow hydrolysis rate of the antibiotic is to aid in the stability of the compound while in the extracellular environment, and that enzymes inside the cell are necessary for hydrolysis and potency of salmcyin.

**4.6 Conclusions**

**Metallophore-Siderophore Interactions**

It was previously thought that FhuD2 along with other siderophore-binding proteins were used to sequester siderophores and facilitate the shuttle of siderophores into the intracellular environment. Part of this work has shown that it also has the ability to bind other proteins such as
transferrin, which allow it to obtain iron from other sources other than the free floating iron in solution. This may give reason to why FhuD2 is considered to be a virulence factor in *S. aureus*; it has the ability to pull iron from other sources that are present in the human body. Previous studies done in this lab have shown the ability of FhuD2 to catalyze iron exchange between transferrin and siderophore. This work sought to determine whether FhuD2 could also facilitate displacement between transferrin and siderophore within the binding pocket. In an effort to observe this event, we determined that although FhuD2 is able to catalyze iron shuttling with transferrin, the protein-protein interaction between the two is not long-lived enough to observe displacement. Further work can be done to probe the protein-protein interaction between FhuD2 and transferrin. Currently, there isn’t enough evidence to support an observable interaction between transferrin and FhuD2. This may lead to a deeper understanding as to the mechanistic details of FhuD2 and its ability to obtain iron from a variety of sources.

This work has done some preliminary work to determine the mechanism that occurs within the binding pocket to facilitate either displacement or iron shuttle mechanisms. Our data suggests that there are key residues that are needed to facilitate both transport paradigms. Further studies would need to be done to elucidate the mechanism. We propose that the rate of displacement and shuttle may involve the binding affinity of the siderophore to the binding pocket of FhuD2. This work can lead to a better understanding of what is necessary in a siderophore to bind and interact with the specific residues, thus tailoring the binding affinity and therefore the displacement or shuttle rate.
**Relationship between Iron Reduction and Drug Release**

We have screened a variety of siderophore ester analogs that help elucidate the mechanism and timing of drug release with respect to iron reduction. Our data strongly suggest there is an interaction between the terminal ester and the neighboring hydroxamate due to the fact that the rate of hydrolysis significantly decreases as they are brought farther apart. Based on this data, it is possible that some variation of the mechanism in Figure 4.7a is occurring. We do not have conclusive data suggesting whether or not there is a connection between iron reduction and drug release since the rates between apo- and holo- siderophore Compound 1 are comparable. Further studies for this project would include the incorporation of iron reductase enzymes to see whether it has an effect on the hydrolysis rates. It would also be beneficial if the set of experiments were done at a higher temperature to speed up the hydrolysis rates of some of the slower siderophore esters. If salmycin’s hydrolysis rate is comparable to that of Compound 2, then an increase in temperature to 37 °C would result in much faster and more observable rates. Other siderophore analogs that may include protection of the neighboring hydroxamate may show more direct evidence as to the necessary interactions in the hydrolysis mechanism. Determining this mechanism may aid in future siderophore-drug candidates.

### 4.7 Materials and Methods

*Strains, materials, and instrumentation.*

*Staphylococcus aureus* ATCC 11632 and *Streptomyces violaceus* DSM 8286 were obtained from the ATCC and DSMZ collections, noted in Table 4.1. *E. coli* BL21-Gold(DE3) and *E. coli* TOP10 cells were obtained from Agilent and Invitrogen, respectively. *E. coli* cells were made electrocompetent by standard methods. A Bio-Rad MicroPulser electroporator and 0.2 cm gap sterile electroporation cuvettes were used for electroporation. Bacteria were stored as
frozen glycerol stocks at -80 °C. Codon-optimized *fhuD2Δ24* was purchased from GenScript in a pET28a vector for heterologous expression in *E. coli* BL21(DE3) with an *N*-terminal hexahistidine tag (*Table 4.2*). All *N*-His₆ proteins were overexpressed and purified as described previously (*Figure 4.11*).³¹ DNA purification was performed with kits from Qiagen. Plasmid sequencing was performed by Genewiz. Nickel-nitriloacetic acid (Ni-NTA) agarose was purchased from ThermoFisher Scientific (catalog # R90115). Any kD SDS-PAGE gels were purchased from Bio-Rad. Proteins were dialyzed using 10K MWCO SnakeSkin dialysis tubing purchased from Thermo Fisher Scientific. Proteins were concentrated by centrifugal filtrations using 10K MWCO filters from Millipore. All aqueous solutions were prepared with water purified using a Milli-Q system and sterilized by filtration through a 0.2 μm filter. Media was sterilized using an autoclave unless otherwise stated. pH measurements were recorded using an Orion Star A111 pH meter and a PerpHecT ROSS micro combination pH electrode from Thermo Fisher. All buffers, salts, media, solvents, and chemical reagents were purchased from Sigma Aldrich unless otherwise stated. All media was sterilized in an autoclave prior to growing bacteria.

LC-MS was performed using an Agilent 6130 quadrupole with G1313 autosampler, G1315 diode array detector, and 1200 series solvent module. Samples were prepared in 0.45 μ PTFE mini-UniPrep vials from Agilent. Separations were achieved using a 5 μ Gemini C18 column (50 x 2 mm) from Phenomenex fit with a guard column. Mobile phases were 0.1% formic acid in (A) H₂O and (B) ACN. Data were processed using G2710 ChemStation software. Analytical HPLC was performed using a Beckman Coulter SYSTEM GOLD 127P solvent module and 168 detector with a Phenomenex Luna 10u C18(2) 100 Å column, 250 x 21.20 mm, 10 μm with guard column. HPLC data were processed using 32 Karat software, version 7.0.
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 spectrophotometer. NMR was performed on a Varian Unity Plus-600 MHz and 500 MHz instrument.

**Siderophore displacement from immobilized FhuD2**

This procedure is adapted from a procedure reported in Rivera, G. S. M.; Beamish, C. R.; Wencewicz, T. A. ACS Infectious Diseases 2018, 4, 845-859. A fritted glass column was loaded with fresh Ni-NTA agarose resin in 1:1 EtOH:H2O to give a working resin volume of 2.3 cm x 1 cm. The resin was washed with H2O and equilibrated with SBP buffer (50 mM K2HPO4, 150 mM NaCl, 1 mM DTT, pH 8) at 4 °C. N-His6-FhuD2 was thawed from a frozen stock (100 μL of 3.8 mM in SBP buffer; this is enough protein to fully saturate the Ni-NTA agarose resin), diluted to 3 mL final volume with SBP buffer, and added to the Ni-NTA agarose resin. After rocking at 4 °C for 30 min excess SBP buffer was eluted and the column was washed with SBP buffer until no N-His6-FhuD2 was detected by SDS-PAGE analysis. The N-His6-FhuD2-saturated Ni-NTA agarose resin is referred to as SBP-resin. Five siderophores (FO-B, FO-NBD, FO-E, and Dan) were used in pairs as the siderophore of interest (S1) or sacrificial siderophore (S2). Siderophore S1 is first loaded to the SBP-resin by addition of 5 mL of a 0.02 mg/mL solution of S1 in SBP buffer followed by 20 min of rocking at 4 °C. Excess SBP buffer is eluted and the SBP resin is washed five times with 15 mL of SBP buffer until LC-MS analysis shows no detectable ions for siderophore S1. Next, 5 mL of a 0.02 mg/mL solution siderophore S2 in SBP buffer is added and the SBP resin is rocked at 4 °C for 20 min. The column eluent is
analyzed by LC-MS for the presence of siderophore S1ions to confirm displacement from the SBP resin by competitive binding of excess siderophore S2. The SBP resin can now be used in a second cycle using the now resin bound siderophore S2 as the siderophore of interest S1. For LC-MS analysis of samples a gradient was formed from 5% B to 95% B over 20 min, followed by a 3 min hold at 100% B, and re-equilibration to 5% B over 2 min. Caution: using DTT in SBP buffer can lead to reduced Ni-NTA resin as indicated by a blue to orange color change during the procedure. BME can be used as an alternative to DTT to prevent this from taking place. Each experiment was performed in duplicate as independent trials.

**Siderophore mix displacement with SBP-resin**

This procedure is adapted from a procedure reported in Rivera, G. S. M.; Beamish, C. R.; Wencewicz, T. A. ACS Infectious Diseases 2018, 4, 845-859. A fritted glass column was loaded with fresh Ni-NTA agarose resin in 1:1 EtOH:H2O to give a working resin volume of 2.3 cm x 1 cm. The resin was washed with H2O and equilibrated with SBP buffer (50 mM K2HPO4, 150 mM NaCl, 1 mM DTT, pH 8) at 4 °C. N-His6-FhuD2 was thawed from a frozen stock (100 μL of 3.8 mM in SBP buffer; this is enough protein to fully saturate the Ni-NTA agarose resin), diluted to 3 mL final volume with SBP buffer, and added to the Ni-NTA agarose resin. After rocking at 4 °C for 30 min excess SBP buffer was eluted and the column was washed with SBP buffer until no N-His6-FhuD2 was detected by SDS-PAGE analysis. The N-His6-FhuD2-saturated Ni-NTA agarose resin is referred to as SBP-resin. A mixture of five siderophores (FO-B, FO-NBD, FO-E, and Dan) were used as the siderophores of interest, with the sixth siderophore (SFO) as the sacrificial siderophore. The mixture of five siderophores is first loaded to the SBP-resin by addition of 5 mL of a 0.005 mg/mL solution of each siderophore in SBP
buffer followed by 20 min of rocking at 4 °C. Excess SBP buffer is eluted and the SBP resin is washed five times with 15 mL of SBP buffer until LC-MS analysis shows no detectable ions for siderophore S1. Next, 5 mL of a 0.02 mg/mL solution SFO in SBP buffer is added and the SBP resin is rocked at 4 °C for 20 min. The column eluent is analyzed by LC-MS for the presence of each siderophore in the mixture (FO-B, FO-NBD, FO-E, and Dan) ions to confirm displacement from the SBP resin by competitive binding of excess siderophore SFO. For LC-MS analysis of samples a gradient was formed from 5% B to 95% B over 20 min, followed by a 3 min hold at 100% B, and re-equilibration to 5% B over 2 min. Caution: using DTT in SBP buffer can lead to reduced Ni-NTA resin as indicated by a blue to orange color change during the procedure. BME can be used as an alternative to DTT to prevent this from taking place. Each experiment was performed in duplicate as independent trials.

*Siderophore-transferrin displacement from immobilized FhuD2*

This procedure is adapted from a procedure reported in Rivera, G. S. M.; Beamish, C. R.; Wencewicz, T. A. ACS Infectious Diseases 2018, 4, 845-859. A fritted glass column was loaded with fresh Ni-NTA agarose resin in 1:1 EtOH:H2O to give a working resin volume of 2.3 cm x 1 cm. The resin was washed with H2O and equilibrated with SBP buffer (50 mM K2HPO4, 150 mM NaCl, 1 mM DTT, pH 8) at 4 °C. N-His6-FhuD2 was thawed from a frozen stock (100 μL of 3.8 mM in SBP buffer; this is enough protein to fully saturate the Ni-NTA agarose resin), diluted to 3 mL final volume with SBP buffer, and added to the Ni-NTA agarose resin. After rocking at 4 °C for 30 min excess SBP buffer was eluted and the column was washed with SBP buffer until no N-His6-FhuD2 was detected by SDS-PAGE analysis. The N-His6-FhuD2-saturated Ni-NTA agarose resin is referred to as SBP-resin. FO-B, SFO-B, and transferrin were
used in pairs as the siderophore/analyte of interest (S1) or sacrificial siderophore/analyte (S2). Transferrin is first loaded to the SBP-resin by addition of 5 mL of a 1 mg/mL solution of S1 in SBP buffer followed by 20 min of rocking at 4 °C. Excess SBP buffer is eluted and the SBP resin is washed five times with 15 mL of SBP buffer until SDS-PAGE gels show no transferrin. Next, 5 mL of a 0.02 mg/mL solution siderophore S2 in SBP buffer is added and the SBP resin is rocked at 4 °C for 20 min. The column eluent is analyzed by SDS-PAGE for the presence of transferrin to confirm displacement from the SBP resin by competitive binding of excess siderophore S2. The SBP resin can now be used in a second cycle using the now resin bound siderophore S2 as the siderophore of interest S1. For LC-MS analysis of samples a gradient was formed from 5% B to 95% B over 20 min, followed by a 3 min hold at 100% B, and re-equilibration to 5% B over 2 min. Caution: using DTT in SBP buffer can lead to reduced Ni-NTA resin as indicated by a blue to orange color change during the procedure. BME can be used as an alternative to DTT to prevent this from taking place. Following displacement, 10 mL elution buffer (50 mM K$_2$HPO$_4$, 500 mM NaCl, 300 mM Imidazole, 10% glycerol, 5 mM β-mercaptoethanol) was added twice, followed by a 10mL of a 1M solution of tetrasodium-EDTA. All solutions were analyzed by SDS-PAGE for the presence of transferrin. Elutions containing siderophore were analyzed by LC-MS.

**Siderophore Ester Hydrolysis**

Siderophore esters were synthesized by Dr. Timothy Wencewicz.$^{27}$ Siderophores were prepared in 0.1-0.5 mg/mL solutions in pH 7 phosphate buffer (0.1 M NaH$_2$PO$_4$, 0.12 M Na$_2$HPO$_4$·7 H$_2$O) in ddH$_2$O. Siderophore solutions were incubated at r.t. then analyzed. Iron bound siderophore esters were analyzed via analytical HPLC as noted under Strains, Materials, and Instrumentation.
Iron free siderophore esters were analyzed via H NMR also noted under the same section. For analytical HPLC analysis of samples a gradient was formed from 0% B to 100% B over 20 min, followed by a 20 min hold at 100% B, and re-equilibration to 0% B over 20 min. System solvents were buffered with 5 mM ammonium acetate.

*Salmycin Hydrolysis*

Salmycin sample was obtained by purification method explained in Chapter 2. For these qualitative studies, a crude salmycin mixture was used. Salmycin solution was lyophilized and brought up in pH 7 phosphate buffer, and incubated at 37 °C. Samples were removed from original solution and frozen and stored in -80 °C for time points. Antibacterial activity of the salmyncins was determined by an agar diffusion assay. Overnight cultures of *S. aureus* ATCC 11632 were grown in LB broth for 18–24 h starting from a frozen glycerol stock. For the agar diffusion assay, 40 μL of this culture was added to 34 mL of sterile, melted, and tempered (~47 °C) Mueller-Hinton No. 2 agar (HiMedia Laboratories) supplemented with 100 μM 2,2’-bipyridine (final concentration). After gentle mixing, the inoculated melted agar was poured into a sterile petri dish (145 mm x 20 mm, Greiner Bio-One) and allowed to solidify at rt. Wells of 9 mm diameter were cut from the petri dish agar and filled with 50 μL of the test sample solution. All test samples (salmycin hydrolysis at various time points) were tested at their undiluted concentrations. The petri dish was incubated at 37 °C in ambient air for 18–24 h and the inhibition zone diameters were measured (mm) with an electronic caliper.
4.8 Figures

Figure 4.1 Siderophore transport paradigms including siderophore displacement and iron shuttling via a siderophore or transferrin iron donor. (A) Iron shuttle mechanism occurs where an apo-siderophore is pre-bound to the SBP. The SBP catalyzes the iron transfer between the holo-siderophore and the pre-bound apo-siderophore, allowing the pre-bound siderophore to enter the cell. (B) Siderophore displacement mechanism occurs where the pre-bound apo-siderophore is displaced from the SBP binding pocket for a holo-siderophore, allowing the new holo-siderophore to enter the cell. (C) Iron shuttle mechanism between siderophore and transferrin occurs where the SBP catalyzes the iron transfer between the holo-transferrin and the pre-bound apo-siderophore.

Adapted from NPE dissertation.
Figure 4.2 Mutational scanning of FhuD2 reveals active site residues involved in substrate binding and catalysis. (a) Active site residues in FhuD2 that were mutated. Color-coding of residues matches data sets in all panels. (b) Michaelis-Menten plot for wild-type and mutant FhuD2 variants (100 nM) reveals saturation kinetics for ferric transferrin and apparent changes in $v_{\text{max}}$ for the exchange of ferric iron to 2 μM DFO-NBD. (c) Relative binding and displacement of FOB to wild type and mutant FhuD2 variants. N-His$_6$-FhuD2 variant was immobilized on Ni-NTA resin and loaded with FOB, washed with phosphate buffer, and eluted with SFO. Column elutions were analyzed by LC-MS for FOB (m/z = 614 for [M+H]$^+$). Extracted ion chromatograms (EIC) are shown for FOB and were normalized to a quinoline internal standard. EICs are representative for at least two independent trials. (d) Stacked state model of the transition from “open” (dark, opaque residues) to “closed” (transparent residues) for FhuD2 highlighting residue dynamics. Panels (e) and (f) show surface models for FhuD2 in the (e) “closed” and (f) “open” states revealing a cleft from the movement of Y167, Y169, and W173. Images in panels (a), and (d)–(f) were generated using PyMOL v2.2 (Schrödinger, Inc.). The stacked state model in panel (d) was generated using the morph function in PyMOL. The “closed” and “open” states of FhuD2 were generated from PDB entries 4fil and 4fna, respectively. Error bars in panel (b) represent standard deviations for at least two independent trials.

Adapted from NPE dissertation.
Figure 4.3 Mechanistic model for iron exchange from an octahedral ferric iron source (transferrin is shown here) to a siderophore cofactor (DFOB is shown here) bound to FhuD2.

Adapted from NPE dissertation.
Figure 4.4 Schematic showing the outline of a Ni-NTA column loaded with His$_6$-tagged FhuD2 that can facilitate the displacement mechanism between transferrin and FO-B. The transferrin will bind to FhuD2. After a few washes with buffer, a solution containing FO-B is introduced to the column to displace transferrin. The elution should contain a mixture of excess FO-B and the transferrin that was eluted off. After a few more washes with buffer, transferrin can be reintroduced to the column to displace the pre-bound FO-B, completing the cycle of displacement. Eluted FO-B can be observed through LC-MS, while eluted transferrin can be observed through SDS-PAGE.
Figure 4.5 (A) FhuD2 is loaded into the column in an excess amount. Lanes 1 and 2 show the flowthrough of FhuD2, revealing that the column is fully saturated. Lane 3 is the solution of transferrin that is loaded onto the column. The flowthrough and washes are collected as shown in Lanes 4-9. Lane 4 shows trace amounts of FhuD2, representing the displacement of FhuD2 by transferrin onto the Ni-NTA resin. Lanes 10-11 show the elutions with FO-B; there aren’t any protein bands that conclude any displacement occurs when FO-B is introduced. Lanes 12-16 contain the washes with buffer. Lanes 17-18 are an elution with a different siderophore, SFO in efforts to displace transferrin from the column. Lanes 19-20 are the elutions with imidazole, which displace both FhuD2 and the leftover transferrin from the column. We see little amounts of transferrin eluted, and proceed to elute with EDTA in Lanes 21-22. Lanes 23-24 are blank. (B-D) Size-exclusion chromatography results showing the elution times of (B) FhuD2: 15-23 minutes, (C) Transferrin: 12-19 minutes, and (D) FhuD2+Transferrin: 12-22 minutes. Red trace: 254nm. Blue Trace: 280nm.
Figure 4.6 General schematic of how siderophores are released into the environment and the possible scenarios that can occur. (A) A siderophore-producing bacteria effluxes out the biosynthetically produced siderophore to obtain the insoluble Fe$^{3+}$. It can ideally be transported back into the cell through various transport siderophore pathways, following iron release and recyclability of the siderophore. In competing environments, another bacteria can scavenge the siderophore and utilize the iron source for itself. (B) A defense mechanism introduced by attaching an antibiotic to the siderophore. When iron thievery occurs, the competing bacteria will uptake the siderophore-antibiotic conjugate where drug release supposedly occurs, allowing for the function of the antibiotic within the competing bacteria’s cell. Typically, there is a self-protection method in place for the siderophore-antibiotic producing bacteria.

Adapted from Wencewicz dissertation.
Figure 4.7 Three hypothesized mechanisms of how the antibiotic is hydrolyzed from the siderophore once inside the cell. (A) Hydrolysis occurs after iron-release, where the neighboring hydroxamate nucleophilically attacks the ester carbon and forming a 6-membered ring tetrahedral intermediate. OR$_2$ is removed and water attacks the carbonyl carbon to break open the ring and form the hydrolyzed product. (B) Hydrolysis occurs before iron-release, where lewis acid activation occurs with the iron, polarizing the CO bond of the ester. The resulting carbonyl carbon is more electrophilic and readily available for nucleophilic attack by water to form the hydrolyzed product. (C) Hydrolysis also occurs before iron-release, where water activation occurs, bringing a molecule of water within close proximity to the siderophore. The intramolecular-like hydrolysis mechanism can occur.
Figure 4.8 A panel of synthetically produced desferridoxamine and danoxamine ester analogs. These were used to test the effects of chain length of the ester, and the differences between apo- and holo-siderophores. Synthesis and characterization found in Wencewicz PhD Dissertation and NPE’s publication.
Figure 4.9 Siderophore ester hydrolysis rate studies performed using analytical HPLC. All experiments were done at pH 7 phosphate buffered ddi water. (A)-(F) panel of holo-siderophore esters and the rates of hydrolysis observed through analytical HPLC. The retention time of the methyl ester is different than that of the hydrolyzed carboxylic acid, allowing for the observation of the conversion between unhydrolyzed and hydrolyzed product. (A)-(D) panel of siderophore esters with differing carbon chain lengths between the hydroxamate and the terminal ester. (E)-(F) panel of siderophore esters utilizing either a gem-dimethyl or cyclopropyl moiety to probe formations of specific intermediates.
Figure 4.10 A panel of apo-siderophore esters and the rates of hydrolysis observed through $^1$H NMR. Peaks shown are the methyl hydrogens on the terminal ester. Disappearance of the peak represents hydrolysis of the siderophore ester.
Figure 4.11 Salmycin, a sideromycin produced by Streptomyces violaceus DSM 8286. The proposed mechanism of action of the antibiotic is that it is hydrolyzed off first before it interacts with the ribosome to inhibit protein synthesis. (A) Agar diffusion assays showing the decrease in potency of salmycin on S. aureus as the salmycin solution was left to incubate in pH7 phosphate buffered ddH2O at 37°C. (B) A series of dilutions made by an original salmycin solution to form a standardized curve and quantify the rate of hydrolysis of salmycin.
4.9 References

Chapter 5: Conclusions and Perspectives
5.1 Preface
This chapter was written by Gerry Sann Rivera (GSR) with feedback provided by Prof. Tim Wencewicz (TAW). GSR synthesized all compounds and purified all enzymes in these studies and performed all substrate screening assays. Mutant *S. violaceus* strains with gene knock-outs were produced by GSR, Spencer Stumpf and Prof. Joshua Blodgett in the Department of Biology at WUSTL. GSR performed high-resolution mass spectrometry isotope labeling studies with the help of Dr. Conghui Yao and Prof. Gary Patti in the WUSTL Dept. of Chemistry.

5.2 Summary of Dissertation
The pathway involved in the biosynthesis and utilization of trihydroxamate siderophores is an interesting point of study in which a large portion is elucidated in the work presented in this dissertation. The initial aim for the project was to delve into the specific sideromycin pathway of salmycin to elucidate how it is produced and how it is effective towards gram-positive bacteria. Initial experiments and results proved to be difficult as I realized that the knowledge of salmycins, along with other trihydroxamate siderophores and sideromycins, was sparse and left much to be explored. The first area that was explored was the expression and isolation of salmycin, a sideromycin produced in ug/L quantities due to its single digit nanomolar potency towards *S. aureus* and likely other gram-positive bacteria. The patented isolation was designed for a large industrial scale consisting of over 100L batches of culture; something not feasible in a graduate school lab.¹ The scale-down resulted in reduced yields compared to the patent, and isolation of such small quantities proved difficult. Studying the siderophore uptake pathway involving FhuD2, a siderophore-binding protein utilized in *S. aureus* to capture
xenosiderophores\textsuperscript{2,3}, allowed us to utilize the targeted specificity of this protein to our advantage in designing a trihydroxamate siderophore affinity chromatography. The results, as shown in Chapter 2, were promising, as the salmycins were purified and characterized. In addition, the applications for this affinity chromatography spanned the improvement of isolation techniques for known siderophores and unknown siderophores.

Siderophore biosynthesis has been widely studied and classified into NRPS-dependent and NRPS-independent siderophore gene clusters. The aim of this project was to probe the NIS clusters which are less studied than their counterparts. The project’s inspiration came from the salmycin structure and its interesting disaccharide antibiotic that was previously proposed to act as an aminoglycoside and inhibit protein synthesis.\textsuperscript{4} A quick look at the genome resulted in a siderophore gene cluster responsible for biosynthesizing desferrioxamines in other known organisms. Since bacteria tend to cluster their genes for the production of small molecules, we hypothesized a biosynthetic pathway involving the flanking genes of the Des cluster that would biosynthesize salmycin through the incorporation of sugars. Further investigation into the genome along with in vitro characterization of the individual Des genes within \textit{S. violaceus} concluded in a rejection of the current hypothesis and proposition of a new mechanism involving more than one gene cluster. Our work here has shown evidence that the four enzymes in the Des cluster are not only incapable of biosynthesizing salmycin, but also other trihydroxamate siderophores such as DFO-B. This data suggests that modification of DFO-E occurs in a separate gene cluster. Future work is necessary to determine whether or not this is true.

The mechanism of FhuD2 was further studied to determine that the efficacy of the siderophore affinity chromatography was due to the displacement mechanism that FhuD2 is able to do under certain conditions. Combining other work produced from this lab, we were able to
replicate the various mechanisms of entry into the cell via FhuD2; displacement and shuttle mechanism. The displacement mechanism, as explained previously in Chapter 4, requires an excess of one siderophore to displace off the current siderophore-FhuD2 complex. In contrast, the shuttle mechanism requires a catalytic amount of FhuD2 with relatively equal concentrations of each siderophore. Experiments determining the specific mechanism and residues involved in the mechanism have been performed, showing that there are residues in the binding pocket that affect displacement/shuttle more than others also in the binding pocket. A potential mechanism involving several residues has been proposed, but still needs more results to ensure its accuracy.

In addition, we have determined that FhuD2 may also obtain iron from other non-siderophore sources, notably transferrin. Kinetic studies have shown that iron transfer from transferrin is catalyzed with the presence of FhuD2, but a protein-protein interaction has not been thoroughly observed yet. This may be due to the weak and/or transient interactions between the proteins.

Lastly, we explored the drug-release mechanism that some sideromycins undergo after entering the cell. The various siderophore-ester analogs were used to compare hydrolysis rates and determine a mechanism and timing of release of the drug when associated with iron-release upon entry into the cell. We discovered that the carbon chain length of the terminal ester played an important role in hydrolysis rate since increasing the chain by one carbon drastically slowed the hydrolysis rate of both apo- and holo- siderophore esters. The exact mechanism of release is not certain, but Chapter 5.2 explains the potential avenues and future experiments to further study this.

As a whole, the project was set out to elucidate as many gaps of knowledge regarding salmycins in an effort to learn more about the pathways that naturally produced sideromycins undergo, and how it can also be applied broadly to siderophores in general. As shown in Figure
5.1, much has been studied, but as discussed below, there are a few experiments that can be done to fill in the remaining gaps.

5.2 Detection of Protein-Protein Interactions with FhuD2 and Transferrin

As previously discussed in Chapter 4, our future directions include new experiments with the hopes of observing the protein-protein interaction between FhuD2 and transferrin. The current SEC data suggests that there is a stable interaction between the two proteins, which supports the data exploring the iron shuttle mechanism between siderophore and transferrin. The current SEC data is just not clear enough to definitely say there is an interaction. Further SEC experiments to detect protein-protein interaction through size exclusion chromatography will need to be done to improve upon the existing data. It is also possible to run mass spectrometry on the two proteins using native spray mass spectrometry.

Other ways that could be done to detect the interaction between the two proteins is through far-western blot analysis which may solve the issue of the interaction being too weak to remain intact after multiple wash steps. In this assay, the conditions seem a lot more forgiving and may allow us to observe the weak interaction between the FhuD2 and transferrin.

5.3 Gene Deletion to Determine Salmycin Biosynthetic Gene Cluster

As of now, the elucidation of the Des cluster and determining the genes required for salmycin production is top priority. As discussed in Chapter 3 and briefly summarized in Chapter 1, our work suggests evidence that challenges the current dogma that the Des cluster is responsible for biosynthesizing a variety of trihydroxamate siderophores based on substrate
feeding studies. Despite this contradicting evidence in hand, we have yet to obtain results proving that a secondary cluster exists. In order to solidify our current hypothesis that there are additional clusters that take DFO-E as a substrate and modify its ends to yield differing trihydroxamate siderophores, we will need to find the genes responsible for this mechanism. Extensive genome mining has been done on *S. violaceus* in search for the required genes for salmycin production, and it would be a wise decision to continue the pursuit in discovering the biosynthetic pathway of salmycin.

A collaboration with the Blodgett Lab has begun and is in the process of producing mutant strains with gene deletions. Our aim is to determine the necessary genes required for salmycin and ferrimycin production in *S. violaceus* and *S. griseoflavus*, respectively. The first target sites for mutations will be the DesD gene independently, and the whole Des cluster, DesA-D which encode for the biosynthetic genes needed to make the siderophore. DesD deletion mutants were previously done and resulted in complete lack of siderophore production in *S. coelicolor*.\(^5\) We expect to see similar results in our gene deletion mutations for our strains.

A number of promising gene clusters have been hypothesized to be responsible for cleaving the amide bond of DFO-E, producing the antibiotic, and modifying both ends of the siderophore to biosynthesize salmycin. As shown in Figure 5.2, there are a few key functional groups and chemical moieties that we used to search for potential biosynthetic gene clusters for the antibiotic portion of salmycin. The first cluster is illustrated on Figure 5.3a and targets the flanking genes from the Des cluster. First, it is typical of bacteria to cluster their genes that are responsible for making one natural product into one area of the genome.\(^6\) It would be intuitive that the genes that encode the biosynthetic machinery to make the antibiotic portion of salmycin would be adjacent to the Des cluster that makes DFO-E. Our issue with this initial hypothesis is

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that the search for homologous gene clusters to the Des cluster in *S. violaceus* showed us that many other organisms have the same flanking genes despite not being able to produce salmycin (Figure 5.3b). Regardless, these flanking genes are found to be upregulated in strains that produce a wide variety of siderophores, providing them with a competitive advantage in nutrient-poor conditions.\(^7\)

Another gene cluster that we are actively interested in is the valanimycin gene cluster (Figure 5.4). Typically, when searching for gene clusters of natural products with elucidated structures, starting on rare and novel functional groups in nature can narrow the search. The oxime moiety in salmycin is a unique functional handle for enzyme hunting. We were able to find a few genes from the valanimycin gene cluster in *S. violaceus*; *vlmA*, *vlmH*, *vlmO*, and *vlmR*.\(^8\) In addition, in the same vicinity, we discovered an aminotransferase, methyltransferase, hydrolase and lipase which would all be enzymes needed to break open the DFO-E ring, and functionalize the terminal alcohol group and the N-methyl on the heptose ring.

Another proposed gene cluster is the septacidin gene cluster because of its similar heptose moiety it shares with salmycin.\(^9\) We suspect that the enzymes necessary for producing the heptose ring from D-sedoheptulose-7-phosphate (S-7-P) could be responsible for producing the heptose ring of salmycin (Figure 5.5). We were also able to discover a cluster of genes with high similarity to that of SepB and others, which are the main enzymes in the septacidin biosynthetic gene cluster that make the heptose ring.

Our last cluster of interest is that which contains HygM, an N-methyl transferase in the hygromycin B gene cluster (Figure 5.6).\(^8\) Hygromycin B is an interesting molecule when trying to determine the mechanism of action of salmycin because of the structural similarity between the two. There is literature precedence that believes salmycin is an aminoglycoside antibiotic that
inhibits protein synthesis in the ribosome.\textsuperscript{9} The issue with this hypothesis is that the typical aminoglycoside structure contains a 2-deoxystreptamine (2-DOS), which salmymins do not.\textsuperscript{10, 11} Nevertheless, we can still take some insight into the structural similarities between hygromycin B and salmymcin due to the fact that they are both derived from sugars. What’s most intriguing about hygM is that it acts on a similar methyl group to that of salmymcin, which is situated on a sugar, with the only difference being a hexose ring on hygromycin B, and a heptose ring on salmymcin. We discovered that S. violaceus contains a \textit{hygM}, along with a few other genes that are similar to that of hygromycin B’s gene cluster.

Out of these potential gene clusters, we decided to narrow in and target a few specific mutation sites. As shown in \textbf{Figure 5.7}, we propose to knockout DesD, DesA-D, the genes right of DesD, a gene cluster surrounding SepB, and a gene cluster surrounding vlmA, vlmH, vlmO, and vlmR. The process for this is ongoing as our collaborators attempt to produce the mutant strains for us. Once the strains are in hand, salmymcin production will begin on all mutant strains along with WT. There are two avenues to compare the salmymcin production of these mutant strains. The first being very quick and qualitative where we isolate the supernatant of each culture and test antibacterial activity via an agar diffusion assay against \textit{S. aureus} as previously done in Chapter 2. We suspect that this process will be sufficient because knocking out a gene in the salmymcin biosynthetic gene cluster should lead to complete lack of activity from the supernatant on \textit{S. aureus}. If more quantitative results are necessary, we can pursue a more rigorous purification that allows for a more accurate yield calculation and comparison via LCMS.

The gene mutation project has also given birth to a separate project that aims to produce a strain that expresses salmymcin in larger quantities than that of WT. The protocol for designing
and exclusively expressing these gene deletion mutant strains, \( rpsL \) mutants were made that were apramycin resistant and streptomycin susceptible. It has been shown that \( rpsL \) mutants have sometimes led to increased production in secondary metabolites.\(^{12}\) We tested the salmycin production of 5 of these \( rpsL \) mutants and compared it to WT. Figure 5.8 shows an agar diffusion assay with \( rpsL \) 25-6 containing more salmycin than that of WT. It would be interesting to pursue this part of the project with the goal of obtaining a strain that overexpresses salmycin. This leads to higher salmycin production and therefore more material that can be used for testing the antibacterial mechanism of action, which is still completely unknown.

### 5.4 Iron Reductase Assisted Siderophore Hydrolysis

The general consensus for iron release from siderophores is grouped into two mechanisms: iron release outside of the cell, and iron release inside of the cell. Both are considered to involve ferrisiderophore reductases siderophore based iron acquisition and pathogen control, which our current model for testing hydrolysis does not include.\(^{13}\) The next set of experiments that would supplement our understanding of the sideromycin hydrolysis mechanism is to include ferrisiderophore reductases into the mix to see if the presence of these reductases increases the rate of hydrolysis. The only issue with this currently is that there aren’t any reductases that possess specificity towards the Fe-siderophore complex amongst the pool of iron containing small molecules in the cell.

The hydrolysis studies were done at room temperature, revealing that the process is quite slow for most siderophore-esters. In order to do more substrate testing on a reasonable time-scale, it would be advantageous to increase the temperature to allow for a faster hydrolytic rate. The salmycin hydrolysis was observed at 37 °C, and showed dramatic increase in hydrolysis rates when compared to the similar compound, siderophore-ester **Compound 2** (Figure 4.8).
Some work has been done to test whether hydrolysis is affected by pH, but there was a lack of compound to test other pH ranges for most of the siderophore esters.

With reference to Chapter 2, ferrimycin, another naturally produced sideromycin, would be an interesting compound to test and compare the hydrolysis of. In order to do this, a larger scale-up can be done to purify out a batch of ferrimycin to perform characterization tests that include the hydrolysis rate. The scale-up is doable with the procedure we have set up for isolation of siderophores and sideromycins from culture supernatants. The linker between the siderophore and antibiotic of ferrimycin differ greatly from the linker in salmycin, so it would be interesting to compare the rates and determine a mechanism of release for ferrimycin.

5.5 Sideromycin Pathway Elucidation

Lastly, it would be beneficial to close the gaps left in the entire sideromycin pathway that were not covered in this PhD work. Figure 5.1 can be directly compared to Figure 1.7 to determine what is left. As previously described, a significant amount of work has been done to elucidate the biosynthesis, iron binding, cell uptake, iron release, and hydrolysis of sideromycins, normally salmycin. I have discussed the steps that are needed to further explore these areas to acquire a complete understanding of the steps in sideromycin pathway.

With that said, there are still two areas that have not been explored as extensively as the others. The first being the structure elucidation and full characterization of these sideromycins, salmycin and ferrimycin. Although characterization studies have been done for salmycin, there hasn’t been enough isolated compound of either salmycin or ferrimycin in real time to get NMR data and provide full structural characterization. Previous groups have reported NMR from salmycin isolation and from total synthesis.1, 4 As of now, there are still a couple hypothesized structures of ferrimycin floating around in literature, and no NMR characterization of either of
the isolated salmycin or ferrimycin. Mutant strains of *S. aureus* that are resistant to salmycin have been isolated. Experiments shown in Figure 5.9 include the addition of FhuD2 to the *S. aureus* ΔFhuD2 mutants have been done to probe the uptake pathway of salmycins and its importance in its potency.

The second area that has not been covered is the mechanism of action of the antibiotic. It is still not clear whether or not hydrolysis needs to occur in order for the antibiotic to reach its desired intracellular target. Acquisition of the hydrolyzed product would be useful in determining whether or not the active component is the whole sideromycin or just the cleaved antibiotic. We have confirmed that addition of a competing siderophore inhibits activity, so at the very least, the siderophore of salmycin is necessary for transfer into the cell. In addition, there has not been any definitive evidence showing that salmycin is an aminoglycoside that acts on the ribosome. Structurally, salmycin does not include the common 2-deoxyribonuclease that aminoglycosides share in common. It resembles more of a disaccharide that may be involved in preventing peptidoglycan formation.

Studies focused on these two areas would help complete our understanding of the salmycin pathway, and lead to similar studies on ferrimycins and other sideromycins. New pathways need to be discovered in the efforts to remedy the losing battle to antibiotic resistance. Sideromycins provide a unique pathway that isn’t fully utilized in the clinic, and may allow for the discovery or improvement of new drugs.
5.6 Figures

Figure 5.1 General schematic of the salmycin pathway utilized by both the producing organism and the competing and susceptible organism, in this case *S. aureus*. Areas boxed in red represent the points in the pathway that have been studied in this Ph.D.
**Figure 5.2** The structure of salmycin with the black area representing the siderophore portion, and the red are representing the antibiotic portion. Functional groups highlighted with a blue circle are points of interest that were taken into consideration when searching for the biosynthetic gene cluster of salmycin. In addition to the moieties highlighted in blue, another area of interest was the siderophore portion and the genes responsible for the biosynthesis of the trihydroxamate siderophore.
A. Figure 5.3 (A) is a representation of the Des cluster (red) in *S. violaceus* that is believed to be responsible for salmycin production. The flanking genes (blue) are shown in other organisms to be upregulated during production of a variety of siderophores.\(^7\) (B) is an antismash collection of homologous similar gene clusters found in organisms that do not produce salmycin despite having very similar flanking genes.

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**HE971709_c17**: *Streptomyces davawensis* strain ICM 4913 complete genome. (28% of genes show similarity)

**LGHN01000048_c1**: *Streptomyces* sp. XY332 P409contig14.1, whole genome shotgun... (28% of genes show similarity)

**CP011664_c9**: *Streptomyces* sp. Mgi1, complete genome. (28% of genes show similarity)

**LFML01000013_c1**: *Streptomyces* roseus strain ATCC 31245 contig 14, whole genome shotgun... (28% of genes show similarity)

**LJCM01000011_c1**: Actinobacteria bacterium OY450 contig 10, whole genome shotgun... (28% of genes show similarity)

**LGDB01000178_c2**: *Streptomyces* sp. WM6372 P400contig29.1, whole genome shotgun... (28% of genes show similarity)

**LGDL01000200_c1**: *Streptomyces* sp. XY533 P414contig5.1, whole genome shotgun... (28% of genes show similarity)

**LGDM01000081_c1**: *Streptomyces* sp. XY511 P413contig17.1, whole genome shotgun... (28% of genes show similarity)

**LGCZ01000047_c1**: *Streptomyces* sp. WM6349 P397contig14.1, whole genome shotgun... (28% of genes show similarity)

**LGDS01000135_c1**: *Streptomyces* sp. H036 P430contig37.1, whole genome shotgun... (28% of genes show similarity)
Figure 5.4 Valanimycin biosynthetic pathway utilizing the Vlm genes. An $N$-oxo-diazo moiety is present in the final structure, similar to the oxime in salmycin.
Figure 5.5 Septacidin biosynthetic pathway with the corresponding genes. SepB highlighted in red is the gene of interest because of its role in the formation of the heptose ring similar to that of salmycin.
Figure 5.6 Hygromycin B biosynthetic pathway. Hygromycin B is an aminoglycoside antibiotic, which is what salmycin’s antibiotic portion is classified as. HygM highlighted in red is responsible for methylating the nitrogen, which is also necessary in salmycin.
Figure 5.7 Compilation of the notable gene clusters of interest in *S. violaceus* DSM 8286. (A) Contains the Des gene cluster and the flanking genes around it. (B) Contains similar enzymes from the valanimycin gene cluster highlighted in red, in addition to a lipase and hydrolase enzyme that could be of interest. (C) Contains the genes that are similar to SepB of the septacidin gene cluster. (1) is the first gene knockout of interest, knocking out *desD*. (2) is designed to knockout the entire Des cluster. (3) knocks out the four genes flanking the right of the Des cluster. (4) knocks out the genes that are similar to the valanimycin gene cluster. (5) knocks out the two enzymes that are similar to SepB of the septacidin gene cluster.
Figure 5.8 Agar diffusion assay showing the increased production of salmycin in *rpsl* mutants 25-3 and 25-6 with respect to the wild type (WT). Supernatant solutions were tested for each culture against *S. aureus*. 
Figure 5.9 Agar diffusion assay showing salmycin activity on the wild type *S. aureus* strain vs the *S. aureus* ΔFhuD2 mutant strain. FhuD2 was spiked into the solution at varying concentrations to attempt to rescue activity of salmycin with the mutant strain, but the mutant remained resistant.
5.7 References

Appendix: NMR Data for Chapter 3
A.1 $^1$H NMR Data for Compounds
$\text{H-NMR (300 MHz, CDCl}_3\text{)}$

$\text{H-NMR (300 MHz, CDCl}_3\text{)}$
$^1$H NMR (300 MHz, CDCl$_3$)

$^1$H NMR (300 MHz, CD$_2$OD)

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