A Mechanism of Antimicrobial Resistance and a Mitigation Strategy

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Christopher Bulow

Washington University in St. Louis

August 2018
To my wife and family

Viditque Deus cuncta quæ fecerat, et errant valde bona.
The ability to treat infections, perform surgery, and administer immunosuppressants and chemotherapy depends on effective antibiotics. The emergence and spread of antimicrobial resistance is far outpacing the development of new therapies\(^1\)\(^-\)\(^3\) threatening to thrust medicine into a post-antibiotic era\(^4\). Many mechanisms of antimicrobial action and of antimicrobial resistance remain poorly understood as drug development struggles to keep pace. As resistance develops, the human gut serves as a reservoir and provides ample opportunities for resistance gene transmission between commensal and pathogenic bacteria\(^5\)\(^-\)\(^11\). Once resistant organisms colonize the gut, they can persist for extended durations even without continued antimicrobial exposure\(^6\)\(^-\)\(^12\). New approaches are necessary to prevent or reverse colonization with resistant organisms. This work takes two important steps to addressing the antimicrobial resistance crisis: 1) Understanding an antimicrobial mechanism of action and a corresponding mechanism of resistance and 2) Developing an approach to prevent or reverse colonization of human hosts with resistant organisms.
Daptomycin, a broad spectrum antibiotic used for treating multi-drug resistant Gram-positive infections, is experiencing clinical failure against important infectious agents including *Corynebacterium striatum*, an opportunistic pathogen and skin commensal. The recent transition of daptomycin to generic status is projected to dramatically increase availability, use, and clinical failure. Here we confirm the genetic mechanism of high-level daptomycin resistance (HLDR, MIC > 256 µg/mL) in *C. striatum*, which evolved within a patient during daptomycin therapy. This work demonstrates that loss of function mutation in *pgsA2* and the loss of membrane PG is necessary and sufficient to produce high-level resistance to daptomycin in *C. striatum*. This elimination of PG and the absence of additional compensatory changes support the conclusion that PG is the target of daptomycin. This work highlights the importance of understanding how different bacterial species respond to lipopeptide antibiotics. Drugs that target membrane components may vary in efficacy by species due to differing abilities of species to alter or remove various membrane components.

Strategies to prevent infection by multidrug-resistant organisms (MDROs) are scarce; however, autologous fecal microbiota transplantation (autoFMT) may limit gastrointestinal MDRO expansion. AutoFMT involves banking one’s feces during a healthy state for later use in restoring gut microbiota following perturbation. In this pilot clinical trial involving 10 healthy participants, autoFMT was safe and well tolerated in the ten participants evaluated. The trial also evaluated the effects of amoxicillin-clavulanic acid (amox/clav) exposure and the ability of autoFMT to restore the microbiome. Both autoFMT and saline control restored metabolic capacity and resistance gene levels, but additional work is necessary to determine its ability to restore phylogeny. Importantly, metabolic capacity was perturbed following amox/clav even in cases where gross phylogeny remained unchanged.
Chapter 1
Introduction

1.1 Antimicrobial Resistance

The emergence and spread of antimicrobial resistance is far outpacing the development of new therapies\(^1\text{-}^3\). An ever growing number of bacterial infections are resistant to clinical antimicrobials of choice\(^2\text{-}^3\). Every year resistant infections are responsible for over 700,000 deaths globally\(^4\). This figure is predicted to reach 10 million deaths per year by 2050 and the World Health Organization warns that medicine is approaching a post-antibiotic era\(^4\). This will undermine the very foundations of modern medicine by limiting our ability to treat infections, perform surgery, and administer immunosuppressants and chemotherapy.

Antimicrobial resistance genes and the pathogenic or commensal organisms that carry them colonize body sites and environmental surfaces. This allows continued spread to new hosts\(^14\). The human gut is one important reservoir of such resistance genes and provides ample opportunities for resistance gene transmission between commensal and pathogenic bacteria\(^5\text{-}^11\). Once resistant organisms colonize the gut, they can persist for extended durations even without continued antimicrobial exposure\(^6\text{-}^12\). New approaches are necessary to prevent or reverse colonization with resistant organisms.

In an effort to mitigate the impending antimicrobial resistance crisis, two steps can be taken: 1) Understanding antimicrobial mechanisms of action and corresponding mechanisms of resistance and 2) Developing approaches that prevent or reverse colonization of human hosts with resistant organisms.
1.2 Mechanisms of Daptomycin Action and Resistance

Understanding mechanisms of antimicrobial resistance can aid in designing more effective therapies. An important first step is clarifying the mechanisms by which current antimicrobials kill or inhibit bacteria. This work brings much needed clarity to the daptomycin’s antimicrobial mechanism of action and provides a detailed explanation of high-level resistance observed in the clinic.

1.2.1 Daptomycin

Daptomycin is a last-resort antibiotic reserved for treatment of severe gram-positive infections. This non-lytic lipopeptide antibiotic has activity against both stationary and log phase Gram-positive bacteria such as Staphylococcus aureus, Enterococcus faecium, and Corynebacterium striatum. It is also known that daptomycin exhibits off target binding to eukaryotic cells, leading to toxic effects in humans. For this reason, daptomycin was originally abandoned by during the development phase. As the clinical need for effective antimicrobials grew, developers revisited daptomycin despite the risks of toxicity. Furthermore, the maximum approved dose of daptomycin has been steadily increased to treat more infections.

Much work has been performed to elucidate daptomycin’s mechanism of action since its approval for clinical use in 2003 but some key questions remain. It is known that daptomycin forms a complex with Ca$^{2+}$ and integrates into bacterial membranes leading to K$^+$, Mg$^{2+}$, and ATP leakage and cell death. Although this body of work and recent reports of resistance implicate phosphatidylglycerol (PG) as the target of daptomycin, the drug’s precise molecular target and mechanism of action has remained largely unproven. These aspects of mechanistic activity are critical to understanding resistance and eventually designing the next generation of
lipopeptide antibiotics. Conversely, resistance provides a tool for understanding mechanism of action.

Unfortunately, even careful stewardship of daptomycin has not prevented resistance from emerging. Most commonly, low-level resistance is seen in \textit{Staphylococcus aureus}, which is not unexpected given that daptomycin is commonly used to treat Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) infections\textsuperscript{27}. Additionally, there have been reports of high-level resistance in other gram-positive bacteria, most notably the skin commensal \textit{C. striatum}\textsuperscript{28}.

Stepwise accumulation of mutations has been responsible for low (Minimum Inhibitory Concentration, MIC: 2-4 µg/ml) and intermediate (MIC: 4-8 µg/ml) daptomycin resistance in \textit{Staphylococcus aureus} over weeks of treatment\textsuperscript{29-37}. For example, in \textit{Staphylococcus aureus} single nucleotide polymorphisms (SNPs) in the \textit{yycFGHI} operon have resulted in 2-6-fold increases in daptomycin resistance through cell wall thickening and alteration of membrane charge\textsuperscript{30-35}. Membrane charge alteration occurs by increased lysinlyation of PG resulting in more positive surface charge\textsuperscript{27,38,39}. It has been suggested that this increased surface charge repels the positively charges Ca\textsuperscript{2+} daptomycin complex\textsuperscript{27,38,39}. Additional mutations that alter lipid translocation, decrease membrane fluidity, and thicken the cell wall have led to low-level daptomycin resistance\textsuperscript{32,36}. Importantly, these low and intermediate resistance mutations are lost when daptomycin selective pressure is removed\textsuperscript{37}.

While low and intermediate levels of daptomycin resistance have been observed in \textit{Staphylococcus aureus}, high levels of resistance have been observed in \textit{Bacillus subtilis} and more recently in \textit{Corynebacterium}. While resistance in \textit{Staphylococcus} can occur through increased lysinlyation this process has not been reported in \textit{Bacillus or Corynebacterium}. In
2011, *Bacillus subtilis* nearly 30 times more resistant (MIC = 27.5 ug/mL) to daptomycin than wild type was selectively evolved *in vitro* by serial passage through increasing concentrations of daptomycin. Alarmingly, this strain also displayed resistance to vancomycin, moenomycin, and bacitracin\textsuperscript{40}. Even so, *Bacillus subtilis* is a low-pathogenicity model organism and this level of resistance were observed *in vitro*\textsuperscript{40}. *B. subtilis* was found to harbor SNPs in 44 genes, including predicted reduction/loss of function mutations in phosphatidylglycerol synthase A (*pgsA*), transmembrane protein responsible for phosphatidylglycerol (PG) synthesis\textsuperscript{40,41}. Characterization of the lipid membrane revealed a reduction in PG content from 30% in the wild-type to 10% in the resistant mutant. This is an important parallel to the loss of PG through lysinlyation in low-level *Staphylococcus aureus* resistance. *In vitro* attempts to knock out *pgsA* alone were unsuccessful perhaps due to essentiality of some level of PG in *B. subtilis*\textsuperscript{40}.

Aside from reports of *Streptomyces* species capable of inactivating daptomycin enzymatically\textsuperscript{42,43}, clinical isolates have become resistant to daptomycin through membrane alteration\textsuperscript{26,40,41}. Specifically, studies of daptomycin’s target to date highlight the importance of PG for daptomycin activity\textsuperscript{26,40,41}. A recent comparative genomic and lipidomic study of *Staphylococcus aureus*, *Corynebacterium striatum*, and *Enterococcus faecalis* indicated mutations in PG synthase and subsequent lack of PG synthesis confers daptomycin resistance\textsuperscript{26}.

Recently, there have been increased reports of even higher-level daptomycin resistance (≥4000 fold increase in resistance) in a number of clinical pathogens, including viridians group *Streptococci*\textsuperscript{44}, *Enterococcus faecium*\textsuperscript{19}, and *Corynebacterium striatum*\textsuperscript{28}. This high-level daptomycin resistance (HLDR)—defined here as a minimum inhibitory concentration (MIC) ≥ 256 in *Corynebacterium striatum* is the subject of Chapter 2. The findings presented here are consistent with a complete loss of PG from the membrane without additional compensatory
changes. Understanding of the mechanism underlying such high-level resistance are expected to aid in design of more effective lipopeptide antibiotics. Daptomycin resistance and accompanying clinical failures are expected to expand as daptomycin transitions to generic status and clinical use increases⁴⁵.

### 1.2.2 Corynebacterium striatum

*Corynebacterium striatum* is a Gram-positive bacterium which can reside as a commensal organism on the skin⁴⁶. However, it has become a growing threat to hospital systems and patients as an opportunistic pathogen with the ability to rapidly evolve high-level daptomycin resistance⁴⁷. Left unchecked, *Corynebacterium striatum* has the potential to become an epidemiological scourge. *Corynebacterium striatum* has been associated with bacteremia, endocarditis, urinary tract, wound, respiratory, central line, medical device and hardware infections²⁸,⁴⁸-⁵⁴. HLDR was first observed in *C. striatum*, in a patient with native valve endocarditis in 2012⁵⁵. Again in 2014, a clinical laboratory reported evolution of HLDR *C. striatum* in a patient with an infected left ventricular assist device, during 17 days of daptomycin therapy²⁸.

*C. striatum* poses such a serious threat in part due to its ability to rapidly and completely remove PG from its outer membrane, demonstrated here. This work establishes a genetic, transcriptomic, lipidomic and biochemical understanding of how *C. striatum* rapidly evolves high-level daptomycin resistance. It demonstrates that loss of function mutations in *pgsA2* result in reduced membrane PG content from ~45% to <1%. This mechanism is notably distinct from previously described resistance in *S. aureus* and *Streptomyces spp*²⁹-³⁶ in that PG can be nearly eliminated without apparent compensatory mechanisms. This elimination of PG and the absence of additional compensatory changes support the conclusion that PG is the target of daptomycin.
This work highlights the importance of understanding how different bacterial species respond to lipopeptide antibiotics. Drugs that target membrane components may vary in efficacy by species due to differing abilities of species to alter or remove various membrane components.

1.2.3 Autologous Fecal Microbiota Transplantation

The spread of multidrug resistance among pathogenic organisms threatens the efficacy of antimicrobial treatment options\textsuperscript{56}. The human gut serves as a reservoir for many drug resistant organisms and their resistance genes and perturbation of the gut microbiome by antimicrobial exposure can open metabolic niches to resistant pathogens. Once established in the gut, antimicrobial resistant bacteria can persist even after antimicrobial exposure ceases.

Despite the growing antimicrobial resistance threat, the factors that allow resistant pathogens to colonize the gut remain largely unknown. Furthermore, there are no established methods to effectively reverse the effects of antimicrobial exposure on commensal or pathogenic bacteria.

Several strategies have been proposed for mitigating the threat of resistance but each carries risk. These strategies include targeted antimicrobial therapy, single species probiotics, and allogeneic fecal microbiota transplantation (alloFMT). This work proposes and evaluates autologous fecal microbiota transplantation (autoFMT) as a method of limiting gastrointestinal multidrug resistant organism (MDRO) expansion. AutoFMT involves banking one’s feces during a healthy state for later use in restoring gut microbiota following perturbation (Figure 1-1).
Figure 1-1: AutoFMT Banking a healthy participant’s feces for later use in restoring the fecal microbiome to a healthy and balanced state after perturbation such as antimicrobial treatment.

Targeted delivery of antimicrobials has been proposed as a method of limiting resistance gene selection. For example, using non-absorbable broad-spectrum antimicrobials such as rifaximin confines the drug to the gut and may prevent systemic resistance selection. However, this practice still leads to resistance selection in the gut and allows opportunistic pathogens to gain access in this critical body habitat. From there, they can spread to the rest of the body microbiome and rapidly to other human hosts.

The ingestion or delivery of microbial species as probiotics to confer health benefit dates back to ancient times and has been refined gradually to the probiotic approaches familiar today. Strains of microbes largely belonging to the genera of *Lactobacillus*, *Bifidobacterium*,...
Saccharomyces, Enterococcus, and Escherichia are administered with the intention of displacing or preventing colonization with pathogens\textsuperscript{61}. Due to the wide variation in microbial community structure between individuals\textsuperscript{62}, the safety and efficacy of these probiotics varies greatly\textsuperscript{63,64}. Furthermore, treatments limited to one or several species cannot protect the many diverse niches in the gut from pathogen or MDRO colonization\textsuperscript{65}.

A healthy fecal microbiome defends against pathogen and MDRO invasion through colonization resistance\textsuperscript{66}. Fecal microbiota transplantation (FMT) has been proposed as a method of restoring the microbiome to a healthy state after perturbation\textsuperscript{67}. Unlike single species probiotics, FMT can fill many niches of the gut microbiome. By displacing infectious or resistant microbes, the FMT can restore species diversity, antimicrobial susceptibility, and colonization resistance\textsuperscript{67}. FMT from healthy allogeneic donors (alloFMT) has been successful in treating Clostridium difficile infection (CDI)\textsuperscript{67,68}. Some studies indicate patients who receive alloFMT for CDI may have a reduction in MDROs in feces and experience less risk of infection\textsuperscript{69-75}. However, this approach has been found to allow transmission of resistance genes from donor to recipient\textsuperscript{76}. Donor feces may also transmit pathogens which are being asymptptomatically harbored by the allogeneic donor. Additionally, microbiota structure varies significantly between individuals and a poor donor-recipient match may lead to dysbiosis or FMT failure\textsuperscript{77-80}.

AutoFMT has the potential to restore the gut to a healthy state while avoiding the risks of donor resistance genes and donor-recipient mismatch. AutoFMT involves storage of a healthy person’s fecal material for later use to restore their own gut microbiota after perturbation, such as antimicrobial use. Bolstering the commensal microbiome by autoFMT following exposure to antimicrobials may be effective at combating colonization with pathogens and MDROs\textsuperscript{71}. 
AutoFMT is predicted to have a more desirable safety profile compared to alloFMT because the feces originated from the participant and was collected during a healthy state.

The pilot clinical trial described here evaluated the effect of amoxicillin-clavulanic acid (amox/clav) exposure and autoFMT on gastrointestinal microbiome taxonomic composition, resistance gene content, and metabolic capacity. AutoFMT was safe and well tolerated in the ten participants evaluated. Shotgun metagenomic sequencing allowed for analysis of genes beyond the taxonomic data available through 16S sequencing. Importantly, metabolic capacity was perturbed even in cases where gross phylogeny remained unchanged. This underscores the need to understand the effects of antimicrobial therapy on the microbiome in greater detail.

These findings highlight the gaps in current knowledge regarding the effects of antimicrobials on the gut microbiome and the efficacy of mitigation strategies. Due to cost and sequencing constraints microbiome studies have primarily relied on 16S rRNA analyses of bacterial taxonomic composition and diversity. While such studies have been transformative in demonstrating how well taxonomic compositions match between FMT donors and recipients over time, they are generally not designed to illuminate functional changes in the microbiome. Accordingly, complementary approaches are required to enable a higher resolution understanding of the impact of FMTs on the composition, dynamics, and transmission of resistance genes and metabolic capacity encoded by the microbiome. Shotgun sequencing provides the ability to identify resistance and metabolically linked genes. Functional metagenomic selection and sequencing will allow for even more detailed analysis of functional shifts in the microbiome.
To prevent the arrival of a post-antibiotic era we must better understand the mechanisms of action and collateral effects of antimicrobials. The work described here makes progress toward understanding mechanisms of action and high-level resistance of daptomycin, a key clinical antimicrobial. Furthermore, this work identifies important post-antimicrobial shifts in gut microbiome metabolic capacity that would have been overlooked by traditional 16S rRNA analyses of the microbiome. The struggle against antimicrobial resistance predates humans and finds its origins in the ongoing war between fungi and bacteria. Our strategic advantage in this conflict lies in better understanding our microbial foes and our antimicrobial tools.
Chapter 2
Mechanism of High-Level Daptomycin Resistance in *Corynebacterium striatum*

Collaboration Statement

This work was performed a collaboration between Christopher Bulow, Nicholas K. Goldner, Kevin Cho, Meghan Wallace, Fong-Fu Hsu, Gary Patti, Carey-Ann Burnham, Paul Schlesinger, and Gautam Dantas. C.B. and N.K.G designed, performed experiments, analyzed data and wrote the manuscript. K.C. and M.W. performed experiments and analyzed data. FF.H, G.P., CA.B., P.H.S. and G.D. designed experiments, analyzed data, and wrote the manuscript.

2.1 Abstract

Daptomycin, a last line of defense antibiotic for treating Gram-positive infections, is experiencing clinical failure against important infectious agents including *Corynebacterium striatum*. The recent transition of daptomycin to generic status is projected to dramatically increase availability, use, and clinical failure. Here we confirm the genetic mechanism of high-level daptomycin resistance (HLDR, MIC > 256 µg/mL) in *C. striatum*, which evolved within a patient during daptomycin therapy, a phenotype recapitulated *in vitro*. In all 8 independent cases tested, loss of function mutations in phosphatidylglycerol synthase (*pgsA2*) were necessary and sufficient for high-level daptomycin resistance. Through lipidomic and biochemical analysis we demonstrate that daptomycin’s activity is dependent on membrane phosphatidylglycerol (PG) concentration. Until now, the verification of PG as the *in vivo* target of daptomycin has proven
difficult since tested cell model systems were not viable without membrane PG. *C. striatum* becomes high-level daptomycin resistant by removing PG from the membrane and changing membrane composition to maintain viability. This work demonstrates that loss of function mutation in *pgsA2* and the loss of membrane PG is necessary and sufficient to produce high-level resistance to daptomycin in *C. striatum*.

### 2.2 Introduction

Current trends in rapidly emerging antimicrobial resistance and decreasing drug development require mitigation\(^1^\)\(^-^\)\(^3\). Antibiotic resistant infections claim over 700,000 lives globally, and this annual toll is predicted to swell to 10 million deaths a year by 2050 without significant intervention. A growing number of bacterial infections are already resistant to nearly all first-line antibiotics\(^2,^13\). Physicians are forced to use “last-resort”, broad-spectrum antibiotics more frequently, and resistance to even these carefully safeguarded drugs has emerged\(^86,^87\). Daptomycin is one such last-resort non-lytic\(^15\) lipopeptide antibiotic, effective against both stationary and log phase Gram-positive bacterial pathogens\(^16\) including *Staphylococcus aureus*, *Enterococcus faecium*, and *Corynebacterium striatum*\(^17^-^19\). Daptomycin integrates into the bacterial cell membrane in a Ca\(^{2+}\) dependent manner, causing membrane dysfunction that leads to K\(^+\), Mg\(^{2+}\), and ATP leakage and cell death\(^24,^25\). Very low levels of resistance were observed during the early phase of daptomycin’s clinical use. Regrettably, recent clinical reports of treatment failures have emerged of pathogens exhibiting >2000-fold increases in daptomycin resistance\(^28,^44,^88\), often over rapid time-scales (hours to a few days of treatment), which are beginning to challenge daptomycin’s efficacy. These failures are expected to expand as daptomycin transitions to generic status and clinical use increases\(^45\).
C. striatum is an emerging opportunistic pathogen that colonizes the skin much like S. aureus. C. striatum’s ability to rapidly transition from daptomycin susceptible to resistant poses a serious epidemiological threat to our healthcare system. This work establishes a genetic, transcriptomic, lipidomic and biochemical understanding of how C. striatum rapidly evolves high-level daptomycin resistance. This mechanism is notably distinct from previously described resistance in S. aureus and Streptomyces spp.

In S. aureus, low-level, step-wise accumulations in resistance phenotypes are responsible for low (Minimum Inhibitory Concentration, MIC: 2-4 µg/ml) and intermediate (MIC: 4-8 µg/ml) daptomycin resistance. The majority of these observations come from pathogenic S. aureus, which was the first approved therapeutic target for daptomycin. Accumulation of multiple single nucleotide polymorphisms (SNPs) in the yycFGHI operon in S. aureus has resulted in 2-6-fold increases in daptomycin resistance through cell wall thickening and alteration of membrane charge. Increases in positively charged membrane phospholipids, which reduces the affinity of the Ca2+-conjugated daptomycin for the surface membrane increased the MIC. Additionally, mutations that alter lipid translocation, decrease membrane fluidity, and thickening of the cell wall have led to low-level (3-6 fold) increases in daptomycin resistance. Mutations associated with the above physiological changes in pathogenic S. aureus have all led to small stepwise increases (2-6 fold) in resistance over long periods of time (weeks of treatment) and lose resistance to daptomycin when no longer under daptomycin’s selection pressure. In contrast, some environmental Streptomyces species have been shown to inactivate daptomycin enzymatically; clinical isolates have not used this mechanism to date.

The first report of higher levels (~20-fold over wild-type) of daptomycin resistance come from laboratory adaptive evolution experiments with the non-pathogenic, soil bacterium,
Bacillus subtilis. Daptomycin resistant B. subtilis was found to harbor SNPs in 44 genes, including predicted reduction/loss of function mutations in phosphatidylglycerol synthase A (pgsA), an essential enzyme for phosphatidylglycerol (PG) synthesis. Characterization of the lipid membrane revealed a reduction in PG content from 30% in the wild-type to 10% in the resistant mutant. Consistent with the lack of complete ablation of PG in the membrane, attempts to knock out pgsA alone genetically were not successful due to presumed essentiality of PG in B. subtilis. Nevertheless, studies of daptomycin’s target to date corroborate in vivo the importance of PG in daptomycin activity. Indeed, a recent comparative genomic and lipidomic study of S. aureus, C. striatum, and Enterococcus faecalis indicated mutations in PG synthase and subsequent lack of PG synthesis confers daptomycin resistance.

Over the past few years, there has been a steady increase in reports of even higher-level daptomycin resistance (≥4000 fold increase in resistance) in a number of clinical pathogens, including viridians group Streptococcus, Enterococcus faecium, and C. striatum. This high-level daptomycin resistance (HLDR)—defined here as a minimum inhibitory concentration (MIC) ≥ 256 µg/ml daptomycin—was first observed in C. striatum, in a patient with native valve endocarditis in 2012. In 2014, a clinical laboratory reported in vivo evolution of HLDR C. striatum in a patient with an infected left ventricular assist device, during 17 days of daptomycin therapy. Evolution of HLDR was recapitulated in vitro in 100% of tested C. striatum isolates (n=50) after 24 hours of daptomycin exposure. C. striatum is a Gram-positive bacterium which typically resides as a commensal organism on the skin. However, it has become a growing threat to hospital systems and patients as an opportunistic pathogen. Indeed, C. striatum has been associated with a plethora of infection types over the past 20 years including bacteremia, endocarditis, urinary tract, wound, respiratory, central line, medical device and hardware.
infections. Here we use a combination of comparative genomics, transcriptomics, lipidomics, electron microscopy, and biochemical lipid and liposome characterization to elucidate the mechanism of HLDR evolved in *C. striatum* both within patients and *in vitro*. We demonstrate that loss of function mutations in *pgsA2* result in reduced membrane PG content from ~45% to <1%. This shift and the absence of additional compensatory changes support the conclusion that PG is the target of daptomycin. This work highlights the ability of bacterial cells to manipulate membrane lipid composition to effectively evade lipid targeted lipopeptides.

### 2.3 Results

#### 2.3.1 PG Synthase Mutations in HLDR *C. striatum* Strains

All cases of evolved high-level daptomycin resistance (HLDR) in *C. striatum* that were tested (Figure 2-1 and Supplemental Table 2-1) had a predicted loss of function mutation in PG synthase. We performed whole genome sequencing of eight pairs of *in vivo* and *in vitro* evolved HLDR *C. striatum* isolates (n = 16), and found *pgsA2* to be the only gene mutated consistently in all HLDR mutants. The affected gene encodes PG synthase, responsible for converting CDP-DAG to PG (Figure 2-2a). The mutations observed include coding changes at universally conserved sites (Figure 2-1b), the dimer interface (Figure 2-1c), the active site (Figure 2-1d), and those leading to premature stop codons (Figure 2-1e). Each of these mutations is predicted by snpEFF and PHYREII homology modeling to result in loss of PG synthase activity.
Figure 2-1: All HLDR isolates have predicted nonfunctional mutations in *pgsA2*. a, Structure of PG synthase monomer with mutations in conserved sites overlaid. b, mutation in CDP alcohol phosphatidyl transferase active site conserved across species. c, mutation in the dimer interface domain. d, mutation in substrate binding pocket. e, premature stop mutations predicted to produce truncated products.

We found no additional SNPs in the *C. striatum* genomes predicted to alter cellular biosynthetic processes in potential compensation for PG synthase loss of function. In *B. subtilis*, loss of just *pgsA2* (PG synthase) was lethal and compensatory mutations were necessary for cell survival \(^{40}\) leading us to consider whether additional mutations may also be required for HLDR in *C. striatum*. 
A total of 8 additional non-synonymous SNPs in biosynthetic pathways were detected in the in vivo evolved isolate (Supplemental Figure 2-1). Aside from the pgsA2 mutation, no SNPs in biosynthetic pathways were detected in the in vitro evolved HLDR isolates. This is consistent with the longer time between susceptible and resistant isolate collection in vivo (17 days) versus in vitro (24 hours). The remaining SNPs in genes not related to biosynthesis did not cluster in similar pathways. No consistent genetic change besides the loss of function mutation in pgsA is predicted to result in compensatory changes that would contribute to membrane viability or HLDR in C. striatum.

Each of the parent daptomycin susceptible C. striatum isolates were derived from different patients, and the in vivo and in vitro HLDR phenotypes were evolved independently. This breadth of evolution events in C. striatum isolates obtained from infected patients provides a robust, clinically relevant cohort for assessing mutations necessary to daptomycin resistance. Accordingly, comparative genomics indicates that loss of function SNPs in pgsA2 encoding PG synthase are the only genomic change necessary for HLDR and no additional mutations are required to maintain resistant cell viability. In a recent report of evolved xenobiotic resistance in Corynebacterineae (the suborder which includes Corynebacterium) minimal genetic mutations were observed between susceptible and resistant pairs, but large-scale transcriptomic changes were found to explain the change in phenotype\textsuperscript{26,94}. Accordingly, we tested whether whole cell transcriptional changes were potentially responsible for compensating the loss of PG synthase function and stabilization of the membrane in HLDR C. striatum.
2.3.2 Minimal Transcriptional Changes in HLDR C. striatum

No significant transcriptional changes were detected in biosynthetically linked genes in clinically evolved HLDR in C. striatum (Figure 2-2c, Supplemental Figure 2-2b). We compared the transcriptomes of the WP1a (index, daptomycin susceptible) and RP1b (HLDR evolved in the patient) strains grown in cation-adjusted Mueller-Hinton broth (CAMHB) to exponential phase in biological triplicate. Even the largest magnitude transcriptional changes did not exceed +/- 85%, much smaller than transcriptomic changes typically associated with phenotypic alteration. Expression changes in genes related to phospholipid biosynthesis were small in magnitude (less than 25%) and not significant. Furthermore, most transcriptomic changes observed occurred in transposase genes and hypothetical proteins of viral origin. The greatest magnitude change in biosynthetically linked gene expression was the LGFP repeat protein transcript detected at levels 1.302-fold (~30%) greater in the HLDR strain. This small change is not predicted to contribute to resistance or viability. PgsA2 expression levels changed by only 0.981-fold (not significant) in the HLDR strain, further indicating that the HLDR phenotypic loss of PG is due to loss-of-function mutations rather than reduced expression. Lack of transcriptional alterations prompted us to interrogate the membrane composition of daptomycin susceptible and HLDR C. striatum strains using a comparative lipidomics approach, as we hypothesized that the HLDR phenotype resulted from the disruption of PG synthase activity, which effectively removes PG from the membrane (Supplemental Figure 2-3b).
Figure 2-2: Lipid metabolism pathway of phosphatidylglycerol, observed SNPs and relative abundance changes of key lipids between WT and HLDR isolates. The PG (a) and CL (b) lipid synthesis pathways were constructed with KEGG. 8 WT and HLDR isolate paired genomes were compared and nonsynonymous single nucleotide polymorphisms identified. The metabolites names and structures are on the left, with key lipids colored in red and green. R1 represents the 16:0 carbon chain and R2 represents the 18:1 carbon chain. The enzyme nomenclature for each enzymatic step and their corresponding genes are located next to the
appropriate synthesis arrow. SNP mutations for each enzyme/gene unit are to the far right with the number of mutations out of the 8 HLDR isolates, unless no mutations were present in any of the isolates. Except for pgsA2, none of the lipid synthesis genes for PG have SNPS. On the left side, black bars indicate the functional completeness of the PG synthesis pathway. WT proceeds through the entire synthesis pathway producing PG, while HLDR ends at the production of CDP-DAG. The green color of the lipid CDP-Dag indicates a 543-5946-fold buildup of that metabolite in the HLDR isolates compared to WT. The Red color of PG and cardiolipin indicates a reduction of that metabolite in the HLDR isolates compared to WT with a 369-1990-fold reduction in PG. c, Expression levels of genes involved in lipid synthesis pathways were not significantly altered in HLDR (p>0.05). Expression levels of the housekeeping genes rpoA and gyrA were also not significantly altered (p>0.05).

2.3.3 Lipidomics Reveals Loss of Phosphatidylglycerol in the Membrane

We found that loss of PG synthase function leads to removal or at least a > 360-fold reduction of membrane phosphatidylglycerol (PG) content in HLDR C. striatum isolates. Analysis by mass spectrometry of whole-cell membrane lipid content of four pairs of daptomycin susceptible and HLDR isolates, which represent each of the four types of predicted loss-of-function pgsA2 mutations (Figure 2-2 b-e and Supplemental Table 1) reveals loss of PG. In each isolate pair, PG detection was 369-1990-fold (p ≤ 0.0001) lower in the evolved HLDR isolates than in the daptomycin susceptible ancestor (Figure 2-3a), levels in the HLDR isolates which are indicative of complete removal of PG in the membrane, resulting in the HLDR phenotype. In addition to PG, cardiolipin (CL, a derivative of PG) was the other lipid absent in the HLDR isolates (Figure 2-3a, c). However, CL was also absent in the daptomycin susceptible WP1a isolate, and because of subsequent in vitro data, we posit it is not the primary target of
daptomycin. We also found no lipidomic evidence that the sn-1/sn-2 fatty acyl groups in PG is being modified to shield it from daptomycin binding hypothesized in low-level resistant S. aureus isolates. Conversion of PG to CL, a proposed mechanism of daptomycin resistance, also does not contribute to the C. striatum HLDR mechanism, since CL is absent in the HLDR strains (p ≤ 0.0001) (Figure 2-3c). PG synthase converts CDP-Dag, a biosynthetic precursor of PG, into phosphatidyl glycerol (Figure 2-2a). In the absence of PG synthase, we expected CDP-Dag to either be utilized in a secondary lipid synthesis pathway or to accumulate in the cell. In support of the latter hypothesis, we found that CDP-Dag levels are significantly (543-5946-fold; p ≤ 0.0001) higher in HLDR isolates over their WT counterparts (Figure 2-2a and Figure 2-3b). PG synthase in HLDR strains across mutation types is nonfunctional (Figure 2-1b-e and Figure 2-2a) and no compensatory changes are being made in the PG biosynthesis pathway (Figure 2-2a-c, Supplemental Figure 2-2), enabling high-level CDP-Dag accumulation (Figure 2-2a and Figure 2-3).
Figure 2-3: Lipidomic comparison of WT and resistant paired isolates across mutation types a-e, the y-axis represents the relative abundance of the important phospholipid between the WT and HLDR isolates. WT-HLDR pairs are associated by color with WT represented by solid block colors and HLDR represented by black striped colors. The lipid that is most abundant in the WT or HLDR isolate has been normalized to 100. Fold changes, where calculable, are listed above the WT and
HLDR comparisons; fold change was calculated using (b-a)/a, where ‘b’ is the largest value and ‘a’ is the smallest to maintain a positive number. The structures of the lipid are directly to the right of the graph with R₁ = 16:0 carbon chain and R₂ = 18:1 carbon chain. Statistical analysis was performed with 1-way anova and every column was means compared p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) , p ≤ 0.0001 (****).

Until recently, determining the in vivo target of daptomycin had been challenging because it was biologically untenable to remove PG from the membrane of model Gram-positive bacteria without cell death. A recent study corroborates that C. striatum appears to uniquely compensate for the complete removal of PG in its membrane by increasing the proportion of two other lipids in the membrane: PI (Figure 2-3e and Supplemental Figure 2-3a, b) and glucuronosyl diacylglycerol (Glua-Dag) (Figure 2-3d). PI is 1.6-5.3-fold higher (p ≤ 0.0001 and p ≤ 0.001) in HLDR over WT (Figure 2-3e and Supplemental Figure 2-3a) and Glua-Dag is 5.5-23.0-fold higher (p ≤ 0.0001 and p ≤ 0.01) in HLDR over WT (Figure 2-3d). We demonstrate that the previously proposed mechanisms of daptomycin resistance which include altering membrane fluidity, leaflet organization, and morphology do not contribute to HLDR in C. striatum, as the lipid membrane composition changes in the HLDR isolates do not visibly alter the membrane (by transmission electron microscopy, Supplemental Figure 2-4a) or charge (by zeta potential, Supplemental Figure 2-4b) compared to daptomycin susceptible counterparts. These data affirm that PG is the in vivo target of daptomycin and loss of PG due to non-functional PG-synthase is necessary and sufficient for the HLDR phenotype.
2.3.4 Surface Plasmon Resonance Indicates PG is the Preferred Target of Daptomycin

In support of our genomic and lipidomic conclusions, we performed a structure-function analysis of PG, which we show is the target of daptomycin and is necessary and sufficient for daptomycin activity in vitro. By combining Surface Plasmon Resonance (SPR), which measures binding, and carboxyfluorescein liposome stability assays (CFLSA) studies, which measure activity, we are able to understand the structural interactions of daptomycin with PG. We use 200 nm artificial liposomes of relevant membrane lipid compositions to determine these relationships. Four lipid species—PG, CL, phosphatidic acid (PA) and phosphatidylcholine (PC) (Figure 2-4f and Supplemental Figure 2-3)—were tested for daptomycin binding affinity. PI and Glua-Dag are both found in WT and HLDR isolates, and we did not test them in the next set of experiments because our lipidomics analysis indicated they are not the in vivo targets of daptomycin. Three types of liposomes of defined composition were assembled, comprised of PG, CL, and PA combined at a 1:1 molar ratio with PC, and compared with homogeneous PC-only liposomes. Daptomycin showed a significantly higher affinity to PG liposomes (p ≤ 0.0001 and p ≤ 0.001) than any of the other lipids tested (Figure 2-4). PG has been hypothesized to be an in vitro target of daptomycin due to its charged phosphate, however, the lack of binding to PC and minimal binding to PA (Figure 2-4e) indicate that the negatively charged phosphate plays a subordinate role in daptomycin binding. Additionally, when the fatty acyl groups are restricted to the bilayer surface plane, as they are in CL, daptomycin binds with much lower affinity (Figure 2-4a-e). When the phosphatidyl-sn-glycerol-3-phosphate glycerol head group is accessible, as it is with PG, the daptomycin binds more efficiently (Figure 2-4a-e). Also, the daptomycin binding
to the 1:1 PG liposomes appears to saturate with daptomycin above 20 μg/ml indicating that PG is acting as a binding site for the daptomycin (Figure 2-4e). Accordingly, we would expect daptomycin activity to correlate with the binding of PG, CL, PA, and PC, and we tested this through a carboxyfluorescein liposome stability assay (CLFSA).
Figure 2-4: Daptomycin binding across concentrations and liposome content a-e. The y-axis plots the normalized binding of daptomycin at varying concentration ratios to liposomes. The x-axis shows the type of liposomes tested, which include 1:1 equimolar ratios of PG, PA or CL to PC and a control liposome made entirely of PC. e, demonstrates stepwise increases in daptomycin binding.
to PG. Statistical analysis was performed with 1-way anova and every column was means compared, $p \leq 0.05 (*)$, $p \leq 0.01 (**)$, $p \leq 0.001 (***)$, $p \leq 0.0001 (****)$.

2.3.5 CFLSA Indicates PG is Necessary and Sufficient for Daptomycin Activity

We found that presence of PG in the bacterial membrane correlates with daptomycin’s bactericidal activity. Carboxyfluorescein liposome stability assays (CLFSA) confirmed PG’s role in daptomycin activity in vitro. Liposomes were generated as above in the presence of self-quenching carboxyfluorescein. Daptomycin is added and interacts with the liposome membrane, releasing and diluting the carboxyfluorescein, which is then unquenched in the buffer producing dramatically increased fluorescence$^{101}$. Daptomycin had higher activity against PG containing membranes in all cases (Figure 2-5a-f) and acts in a concentration dependent manner against both PG and PA (Figure 2-5f). Even though PA had lower binding affinity to daptomycin than CL (Figure 2-4e), daptomycin was more active against PA than it was against liposomes containing CL where there are no available glycerol-3-phosphates extending from the membrane surface for daptomycin binding (Figure 2-5a, d). This is consistent with the observed PG to CL daptomycin activity relationship. Furthermore, this suggests to us that the larger (4 alkane chain CL) suppresses daptomycin’s integration with membrane structure that is necessary for increased permeability in the CFLSA assay inhibiting daptomycin activity. We observed a reduction in daptomycin activity in liposomes that contain CL even though they have a higher binding affinity than PA and PC (Figure 2-4e and Figure 2-5a-f). These findings indicate that the conversion of PG to CL can reduce activity of daptomycin in membranes providing low-level resistance against daptomycin in vivo.
Figure 2-5: Daptomycin activity across concentrations and liposome content a-f. The y-axis plots the % activity of varying concentrations of daptomycin based on absolute fluorescence that is
normalized to the fluorescence achieved by the addition of triton X-100. a-e, The x-axis shows the type of liposomes tested, which include 1:1 equimolar ratios of PG, PA, or CL to PC and a control liposome made entirely of PC. f, the x axis indicates the concentration of daptomycin added to the different liposome compositions. g, Relation of % activity of 35 µg/ml of daptomycin based on absolute fluorescence that is normalized to the fluorescence achieved by the addition of triton X-100 (y-axis) to the % PG content of the liposome tested with PC contributing the remainder of the required lipid to reach 100% composition (x-axis). Daptomycin activity against liposomes is correlated with MIC values for WT and daptomycin resistant bacterial isolates where the % PG content is known. Statistical analysis was performed with 1-way anova and every column was means compared, p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) p ≤ 0.0001 (****).

2.3.6 CFLSA Indicates PG Concentration Predicts Daptomycin Activity in vivo

We found that daptomycin bactericidal activity is correlated with the percent composition of PG in the membrane. Liposomes with 0%-50% PG were generated as above and daptomycin’s activity against those liposomes were tested at a consistent 35 µg /ml daptomycin. At or above 20% PG composition, daptomycin did not show a significant change in activity (Figure 2-5g). This observation directly maps to daptomycin’s in vivo bactericidal activity as measured by MIC across a number of bacterial species. B. subtilis, C. striatum, and S. aureus strains with 30-50% membrane PG content have MICs ≤ 1 µg/ml daptomycin. Below 20% PG content, daptomycin’s activity drops precipitously, both in vitro and in vivo. Daptomycin resistant B. subtilis with a PG content of 10% showed a 27-fold increase in MIC. This is recapitulated with our artificial liposomes where equivalent daptomycin reduction results in 3.66-fold decrease in carboxyfluorescein-based fluorescence (Figure 2-5g), indicative of a loss in daptomycin activity.
When membrane PG composition is ≤ 5%, daptomycin’s activity is indistinguishable from complete absence of PG in the artificial liposome. In HLDR *C. striatum*, which has 0% PG in its membrane we see a ≥4000 increase in MIC\textsuperscript{28,102} and our data shows a 13.5-fold decrease in carboxyfluorescein fluorescence when the liposomes have < 5% PG. Thus, the percentage composition of PG relative to other membrane lipids is predictive of daptomycin susceptibility and activity both in vivo and in vitro. It also suggests that the CFLSA in vitro model is an effective method of studying daptomycin’s interactions with membranes.

2.4 Conclusions

*C. striatum* is an emerging, commensal, opportunistic pathogen that has the potential to cause widespread drug-resistant infections. The rapid adaptive evolution of loss of function *pgsA2* (PG synthase) mutations which result in the significant loss or removal of membrane PG are necessary and sufficient for high-level daptomycin resistance in *C. striatum*, which lead to catastrophic daptomycin treatment failure in patients. No additional genomic or transcriptomic compensation mechanisms are evident in the evolved HLDR phenotype. The HLDR mutants also have no changes in cell wall thickness, cell surface charge, conversion of PG to cardiolipin, or membrane shape—mechanisms previously implicated in lower-level daptomycin resistance\textsuperscript{30,31,33-36,42,43,99,104-107}. Rebalancing of membrane composition to include more PI in the absence of PG as observed by lipidomic profiling likely results in membrane stability. *C. striatum*’s ability to completely remove PG from its membrane, with simple loss-of-function point mutations in PG synthase, further demonstrates that PG is the in vivo and in vitro target of daptomycin. The remarkable ability of *C. striatum* to remove a previously presumed necessary membrane phospholipid could make *C. striatum* an ideal model for developing new Gram-positive antibiotics like daptomycin and studying the potential for resistance to develop.
2.5 Methods

2.5.1 Whole Genome Sequencing and Comparison

We sequenced eight sets of *C. striatum* strains before and after emergence of high-level resistance to daptomycin. Isolates were sequenced using the Illumina Hi Seq 2500 platform, generating 101bp paired-end reads. One case of resistance emergence occurred in a patient bloodstream while the remaining strains evolved resistance during in vitro selection. We used the original susceptible patient isolate as our reference strain and assembled this genome de novo using SPADES\textsuperscript{108}. This reference genome was annotated using the Prokka v1.12 and the Pfam database\textsuperscript{109}. We assembled the remaining 15 genomes by mapping to this reference using bowtie2\textsuperscript{110}. We identified Single Nucleotide Polymorphisms (SNPs) between the resistant strains and their respective susceptible controls using Pilon.\textsuperscript{111} The effects of these mutations were predicted using SNPeff\textsuperscript{90}. All of the genes annotated in the reference genome were clustered by predicted metabolic function using Blast2Go\textsuperscript{112}. Genes with predicted loss of function mutation were annotated on this metabolic map. *pgsA2* was the only gene with biosynthetic function predicted to contain loss of function mutations in more than one case of HLDR (in fact, *pgsA2* loss of function mutations were found in all cases of resistance). 33 other non-synonymous SNPs were detected across all eight strain pairs. Only eight of these SNPs were in genes predicted to affect biosynthetic processes. After clustering by Gene Ontology (GO), *pgsa2* altering phospholipid biosynthesis was the only metabolic alteration predicted in more than one case of resistance. Phyre2 homology modeling\textsuperscript{92} supported predictions that the *pgsA2* mutation in every resistant strain was loss of function.
2.5.2 Transcriptomic Methods

We performed transcriptomic profiling of the susceptible and the in vivo evolved resistant isolate from the original patient in triplicate. Frozen culture was streaked onto CAMHB plus blood plates and grown overnight for single colony selection and then and inoculated into 50 ml CAMHB broth and grown overnight. The following day, the cultures were diluted to 0.5 McFarland standard and split into three 100 mL cultures per condition. The diluted cultures were incubated at 37 °C with shaking for 1 hour. The cells were collected by centrifugation at 200 g for 15 minutes. The pellets were resuspended in RNAlater (76104 Qiagen) and frozen at -80 until analysis.

We used bead beating and SDS treatment to disrupt the sample cells, phenol: chloroform extraction to remove proteins, and alcohol precipitation followed by DNAase treatment to isolate RNA from the frozen samples. Ribosomal RNA was removed with Ribo-Zero rRNA Removal Kit (Epicentre). cDNA libraries were generated from the isolated RNA and amplified as described in Yoneda, Henson et al.\textsuperscript{94} The double stranded cDNA libraries were sequenced using the Nextera\textsuperscript{113} platform to generate at least 7 million 75bp reads from each sample.

Reads from the triplicate susceptible and HLDR samples were aligned to the reference genome constructed from the index susceptible isolate using cufflinks\textsuperscript{114}. Differences in expression between susceptible and HLDR strains were calculated using cuffdiff\textsuperscript{115}. Fold change was calculated as resistant expression level divided by susceptible expression level. Expression levels of the control housekeeping genes rpoA and gyrA remained constant in resistant versus susceptible samples (Fold change 1.02 and 1.00). Significance of changes was calculated using the beta negative binomial distribution previously described in Trapnell et al.\textsuperscript{115} using a significance level of p < 0.05. Expression levels of genes related to lipid biosynthesis are found
in Figure 2-2 and Supplemental Figure 2-2. Additionally, genes with greatest fold changes are found in the Appendix.

2.5.3 Zeta Potential Measurement Methods

We performed surface charge measurement of the susceptible and HLDR resistant strains using zeta potential\textsuperscript{116}. Frozen culture was streaked onto CAMHB plus blood plates and grown overnight for single colony selection and then and inoculated into 50 ml CAMHB broth and grown overnight. The following day, the cultures were diluted to 0.5 McFarland standard and one 4 ml culture was grown per condition. The diluted cultures were incubated at 37 °C with shaking for 1 hour. One (1) ml of the culture was placed in a Malvern zeta-sizing cuvette. Zeta potential (surface charge) was measured using a Zetasizer Nano ZS (ZEN3600) Dynamic Light Scattering System (Malvern Instruments) and compared between susceptible and HLDR paired strains.

2.5.4 Lipidomic Methods

We performed comparative lipidomics across all four mutation types. WT and HLDR matched isolates were grown overnight and diluted to an OD of 1 in 2.5 ml liquid culture in quintuplicate. Liquid cultures were spun down to the cell pellet and whole cell lipids were extracted using the Bligh-Dyer method\textsuperscript{117}. Samples were then stored at -20 until the lipids could be analyzed via liquid chromatography/mass spectrometry (LC/MS). The peak intensities were normalized to 100 for each lipid with WT or HLDR being the normalizing lipid. The WT lipid was chosen as the normalizing lipid for PG, and Cardiolipin because they were most abundant in WT compared to the HLDR isolate. The HLDR lipid was chosen as the normalizing lipid for CDP-DAG, Glua-DAG and PI because they were most abundant in HLDR compared to the WT
isolate. Statistical analysis was performed with 1-way anova and every column was means compared p ≤ 0.05 (*), p ≤0.01 (**), p ≤0.001 (***) , p≤0.0001 (****).

2.5.5 Carboxyfluorescein Liposome Stability Assay

We performed a liposome disruption assay in triplicate to assess the activity of daptomycin on varying compositions of liposomes. Equimolar ratios of PG:PC, CL:PC and PA:PC with a PC only liposome control were created using the reverse phase method containing carboxyfluorescein. Liposomes were then suspended in a buffer solution and subjected to varying concentrations of daptomycin from 3.125μg/ml-1000μg/ml. Fluorescence increase due to daptomycin as a result of carboxyfluorescein release was measured using a Varian Eclipse Spectrophotometer with an excitation wavelength of 492 and an emission wavelength of 512. Daptomycin activity was measured as a function of normalization to 100% release by triton x-100. Additionally, to assess PG% on daptomycin activity, PG:PC liposomes were created in triplicate with varying mole fraction ratios converted to PG% (50%, 40%, 20%, 15%, 10%, 5% and 0%) and subjected 35μg/ml daptomycin. Statistical analysis was performed with 1-way anova and every column was means compared p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) , p≤0.0001 (****).

2.5.6 Surface Plasmon Resonance

We performed surface plasmon resonance in triplicate to assess the binding of daptomycin on varying compositions of liposomes. In equimolar ratios (1:1) of PG:PC, CL:PC and PA:PC with a PC only liposome control were created using the reverse phase method. Liposomes were bound to carboxymethyl dextran hydrogel surface sensor chip that was treated with sphingosine and subjected to varying concentrations of daptomycin from 3.125μg/ml-
35 μg/ml. Baseline, stable liposome and peak daptomycin binding readings were collected. Daptomycin binding was normalized to liposome binding and presented as daptomycin/lipid unit. Statistical analysis was performed with 1-way anova and every column was means compared p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) , p ≤ 0.0001 (****).

2.5.7 Accession Number

All nucleotide sequences generated during this study have been uploaded to NCBI under BioProject Accession: PRJNA420593.

2.6 Supplemental Figures and Tables

Supplemental Table 2-1: Available C. striatum isolates Isolate naming convention: W = WT, R = Resistant, P = Isolated from a patient, E = Evolved from a patient isolate in culture under daptomycin selection, # = Different isolate source, (a,b,c) = Isolate from same patient collected at different time points. All isolates’ genomes were sequenced. WP1a was used as the reference genome. All other genomes were mapped to this genome and SNPs found in the resistant and not the susceptible isolate from each pair were analyzed further. Transcriptomics was performed on
WPIa and RP1b. Lipidomics was performed on the WT and resistant matched pairs of: WP1a:RP1b, WP1c:RE1c, WP2:RE2, WP5:RE5.

Supplemental Figure 2-1: Nonsynonymous mutations in biosynthetic pathways SNPs were clustered by functional category (using gene ontology). We found that pgsA2 was the only gene related to cellular biosynthetic function mutated with the exception of 8 additional mutations observed only in the in vivo evolved isolate. These mutations are consistent with the longer evolutionary time (17 days) experienced by the isolate between susceptible and resistant states, in contrast to the only 24 hours of in vitro evolution.
Supplemental Figure 2-2: Phosphatidylinositol lipid biosynthesis pathways The lipid synthesis pathways were constructed with KEGG. 8 WT and HLDR isolate paired genomes were compared and nonsynonymous single nucleotide polymorphisms identified. The metabolites names and structures are on the left, with key lipids colored in green. R1 represents the 16:0 carbon chain and R2 represents the 18:1 carbon chain. The enzyme nomenclature for each enzymatic step and their corresponding genes are located next to the appropriate synthesis arrow. SNP mutations for each enzyme/gene unit are to the far right with the number of mutations out of the 8 HLDR isolate, unless no mutations were present in any of the isolates. On the left side,
black bars indicate the functional completeness of the PI synthesis pathway. WT and HLDR proceed through the entire synthesis pathway producing PI. The green color of the lipid PI indicates a 0.65-4.25-fold buildup of that metabolite in the HLDR isolates compared to WT. Fold change was calculated using \((b-a)/a\), where ‘b’ is the largest value and ‘a’ is the smallest to maintain a positive number.

Supplemental Figure 2-3: Structures of the key phospholipids These lipids are critical to bacterial membrane composition. \(R_1 = 16:0\) carbon chain and \(R_2 = 18:1\) carbon chain, black indicates the glycerol backbone, red indicates the phosphate group and green represents the functional group.
Supplemental Figure 2-4: Comparison of HLDR and WT lipid membranes and surface charge. a. WP1a and RP1b isolates were imaged with transmission electron microscopy after 1 hour with or without the addition of 10μg/ml daptomycin. WP1a without daptomycin and RP1b with and without daptomycin show no membrane irregularities, while WP1a with daptomycin shows membrane blebbing and disruption. b, all available WT and HLDR isolate pairs were checked for surface membrane charge changes. Charge was not indicative of HLDR and the range of surface charge between the most and least negative WT and HLDR isolates were not significant, while there was significant variability in charge for both WT and HLDR when compared within MIC group. Statistical analysis was performed with 1-way anova and paired means analysis, p ≥ 0.05 (ns), p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) , p ≤ 0.0001 (****).
2.7 Acknowledgments

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Chapter 3

Impact of Amoxicillin/Clavulanate followed by Autologous Fecal Microbiota Transplantation on Fecal Microbiome Structure and Metabolic Potential

Collaboration Statement

This work is the result of collaboration between Christopher Bulow, Amy Langdon, Tiffany Hink, Meghan Wallace, Kimberly A. Reske, Sanket Patel, Xiaoqing Sun, Sondra Seiler, Susan Jones, Jennie H. Kwon, Carey-Ann D. Burnham, Gautam Dantas, and Erik R. Dubberke for the CDC Prevention Epicenter Program. C.B. and A.L. designed and performed experiments, analyzed data and wrote the manuscript. T.H., M.W., K.A.R., S.P., X.S., S.S., and S.J. performed experiments and analyzed data. J.H.K., CA.B., G.D. and E.R.D. designed experiments, analyzed data, and wrote the manuscript.

3.1 Abstract

Strategies to prevent multidrug-resistant organism (MDRO) infections are scarce but autologous fecal microbiota transplantation (autoFMT) may limit gastrointestinal MDRO expansion. AutoFMT involves banking one’s feces during a healthy state for later use in restoring gut microbiota following perturbation. This pilot study evaluated the effect of autoFMT on gastrointestinal microbiome taxonomic composition, resistance gene content, and metabolic capacity after exposure to amoxicillin-clavulanic acid (amox/clav). Ten healthy participants were
enrolled. All received five days of amox/clav. Half were randomized to autoFMT, derived from stool collected pre-antimicrobials, by enema and half to saline enema. Participants submitted stool samples pre- and post-amox/clav and enema and during a 90 day follow-up period. Shotgun metagenomic sequencing revealed taxonomic composition, resistance gene content, and metabolic capacity. Amox/clav altered significantly altered gut taxonomic composition in all participants (n=10, p<0.01); however, only three participants exhibited major changes at the phylum level following exposure. In the cohort as a whole, beta-lactamase genes were enriched following amox/clav (p<0.05) and predicted metabolic capacity was significantly altered (p<0.01). Species composition, metabolic capacity and beta-lactamase abundance returned to pre-antimicrobial exposure state seven days after either autoFMT or saline enema (p>0.05, compared to enrollment). Alterations to microbial metabolic capacity occurred following antimicrobial exposure even in participants without substantial taxonomic disruption, potentially creating open niches for pathogen colonization. Our findings suggest that metabolic potential is an important consideration for complete assessment of antimicrobial impact on the microbiome. AutoFMT was well tolerated and may have contributed to phylogenetic recovery.

3.2 Introduction

The spread of multidrug resistance among pathogenic organisms has rendered many treatment options ineffective. The World Health Organization has described the situation as the dawn of a post-antimicrobial era. The human gut serves as a reservoir for many resistant organisms and their resistance genes, and this can lead to infection in the colonized host and transmission of resistance between commensals and pathogens. Once established in the gut, antimicrobial resistant bacteria can persist for extended durations even in the absence of additional antimicrobial exposure. Novel therapeutic approaches are essential to limit or even
reverse colonization with resistant organisms and the associated risk of infection and transmission between hosts.

Despite the growing antimicrobial resistance threat, there are no established methods to effectively reverse the effects of antimicrobial exposure on commensal or pathogenic bacteria. Several strategies have been proposed for mitigating the threat of resistance but each carries risk. For example, using non-absorbable broad-spectrum antimicrobials such as rifaximin has may confine exposure to the gut and prevent systemic resistance selection\(^57\). However, this practice still leads to resistance selection in the gut and allows opportunistic pathogens to gain access in this critical body habitat\(^58\).

A healthy fecal microbiome defends against pathogen and MDRO invasion through colonization resistance\(^66\). Fecal microbiota transplantation (FMT) has been proposed as a method of restoring the microbiome to a healthy state after treatment with antimicrobials\(^67\). By displacing infectious or resistant microbes, the new community can restore species diversity, antimicrobial susceptibility, and colonization resistance\(^67\). FMT from healthy allogeneic donors (alloFMT) has been remarkably successful in treating *Clostridium difficile* infection (CDI)\(^67,68\). Some studies indicate patients who receive alloFMT for CDI may have a reduction in MDROs in feces as well as infections\(^69-75\). However, this approach has been found to inadvertently allow transmission of resistance genes from donor to recipient\(^76\). Donor feces may also transmit pathogens or pathobionts which are being asymptomatically harbored by the allogeneic donor. Additionally, microbiota structure varies significantly between individuals and a poor donor-recipient match may lead to dysbiosis or FMT failure\(^77-80\). Studies to date of the effects of FMT on the microbiome have primarily relied on 16S ribotype-based analyses of bacterial taxonomic composition and diversity\(^81-84\). While such studies have been transformative in demonstrating
how well taxonomic compositions match between FMT donors and recipients over time, they are generally not designed to illuminate functional changes in the microbiome. Accordingly, complementary approaches are required to enable a higher resolution understanding of the impact of FMTs on the composition, dynamics, and transmission of resistance genes and metabolic capacity encoded by the microbiome.

Autologous fecal microbiota transplantation (autoFMT) is a method that could potentially restore the gut to a healthy state while avoiding the risks of donor resistance genes and donor-recipient mismatch. AutoFMT involves storage of a healthy person’s fecal material for later use to restore the gut microbiota after perturbation, such as antimicrobial use. Bolstering the commensal microbiome by autoFMT following exposure to antimicrobials may be effective at combating colonization with MDROs. AutoFMT is predicted to have a more desirable safety profile compared to alloFMT because the feces originated from the participant and was collected during a healthy state. The purpose of this study was to evaluate the effects of five days of amoxicillin/clavulanate (amox/clav) on microbiome taxonomic composition, resistance gene content, and predicted metabolic capacity and the effectiveness of autoFMT versus placebo in microbiome restoration.

3.3 Results

3.3.1 Enrollment

Ten healthy participants were enrolled in the study; participant characteristics are given in Table 1. Two participants experienced adverse events >30 days post enema: one patient was treated with antimicrobials for an ear infection, and the second was diagnosed with *H. pylori* infection and treated with antimicrobials. Neither infection was determined to be related to
amox/clav, autoFMT, or saline enema. There was no difference between study groups in the number of bowel movements per day post-enema or in bowel movement consistency post-enema as measured by Bristol stool type (Mann-Whitney U, p>0.05 for all).

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<tr>
<th>Variable</th>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>3 (30)</td>
</tr>
<tr>
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<td>3 (30)</td>
</tr>
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<tr>
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</tr>
<tr>
<td>Constipation in the past year</td>
<td>2 (20)</td>
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</tbody>
</table>

Table 3-1: Demographics of study population (N=10) $^a$Hysterectomy (2), migraine headaches, mild hearing loss, history of sports injuries, arthritis, history of back surgery, history of ulcer, history of knee surgery, thyroid partial $^b$Vegan plus fish

3.3.2 Taxonomic Compositional Analysis

Among healthy volunteers (n=10), five days of amox/clav resulted in a significant taxonomic shift from the enrollment composition as measured by Bray-Curtis distance ($p<0.01$). The taxonomic composition across all subjects and time points is visualized in Figure 3-1. Both
the autoFMT and saline groups returned to baseline taxonomic composition by seven days post-treatment (Bray-Curtis distance to enrollment, $p>0.05$).
Figure 3-1: Taxonomic composition Taxonomic composition over time was determined by (a) metagenomic sequencing and (b) qualitative culturomics. (c) Shannon index of diversity was calculated using species data from metagenomic sequencing. Species composition was significantly different after amox/clav by type II Adonis test of Bray-Curtis distance ($p<0.01$). The most obvious change post amox/clav was a Proteobacteria bloom in subject 8. Numbers preceded by ‘S’ at the top of columns indicate participant identification number.

Major taxonomic diversity reduction (Shannon index change of at least -1) occurred in three of 10 participants. Two of these shifts (in participants 5 and 8) occurred immediately following amox/clav. A bloom in Bacteroidetes and reduction in Actinobacteria and Firmicutes contributed to the diversity loss in Participant 5. A Proteobacterial bloom and loss of Bacteroidetes and Actinobacteria appeared to drive diversity reduction in Participant 8. Both participants were randomized to the autoFMT treatment group. Additionally, participant 4 experienced a reduction in diversity due to loss of Proteobacteria and Actinobacteria seven days after autoFMT treatment. In each of these three cases, diversity was restored by the next timepoint (Shannon index within 0.5 of pre-shift value and restoration of reduced phyla).

3.3.3 Resistance Gene Analysis

The resistance genes detected by metagenomic sequencing included a wide range of common resistance genes. The resistance genes found across the most participants were tetracycline destructases$^{121,122}$ and efflux pumps, but the total number of copies of resistance genes was dominated by beta-lactamases. The beta-lactamases were also the mechanistic category that showed most reaction to treatment by amox/clav. As expected, the number of beta-lactamases significantly increased in the study cohort after exposure to amox/clav ($p=0.0017$), while the count of non-beta-lactamase antimicrobial inactivation resistance genes was not
enriched (Figures 3-2 and 3-3). Beta-lactamase gene levels returned to baseline (not significantly different from enrollment level) in both the saline (p>0.05) and autoFMT (p>0.05) groups.

**Figure 3-2: Beta-lactamase gene abundance** Beta-lactamase genes were significantly enriched after amox/clav (two-tailed t-test, \( p=0.0017 \)). Participants randomized to autoFMT (red) and saline (grey) both returned to baseline by day 90.
Figure 3-3: Resistance gene enrichment after amox/clav  Enrichment of resistance genes after amox/clav was determined by comparing normalized counts (RPKM) post amox/clav to enrollment. Genes are grouped by resistance mechanism. Beta-lactamases (black) were most enriched and formed a majority of the antibiotic inactivation enzymes enriched. Efflux pumps were also enriched and functional metagenomic selections suggest co-occurrence of beta-lactamases and efflux pumps on a mobile element.

Additionally, functional metagenomic selections also provided information about gene co-occurrence. Genes encoding beta-lactamase CblA and an efflux pump from the AcrB family co-occurred on 22 different functional metagenomic contigs in eight different selections. Twelve such co-occurrences had exactly 114 bp between the genes, suggesting a conserved multi-gene cassette.
3.3.4 Metabolic Capacity Analysis

Metabolic capacity of the microbiome shifted significantly following amox/clav, even when subjects with broad phylogenetic differences post amox/clav (participants 5 and 8) were excluded. In order to assess metabolic perturbations, we structured an index of metabolic capacity (IMC) from the metagenomic sequencing data. Genes with metabolic functions were grouped into 37 functional categories. Principal component analysis of IMC (Figure 3-4) demonstrated an effect of amox/clav on metabolic capacity. IMCs of post-antimicrobial samples cluster apart from baseline IMCs. The Bray-Curtis distance between baseline and post-antimicrobial IMCs was significantly different by type II Adonis ($p<0.01$). This remained true when we excluded participants with obvious taxonomic shifts at these time points (5 and 8) to control for intrinsic metabolic differences between phyla ($p<0.01$). Additionally, both saline and autoFMT groups returned to baseline composition (not significantly different from enrollment) by day seven post treatment ($p=0.99$ and $p=0.84$ respectively).
Figure 3-4: **Index of metabolic capacity** Principal component analysis (PCA) of metabolic pathway data (IMC) from participants without obvious taxonomic disturbances (all participants excluding 5 and 8). IMC was derived from metagenomic sequencing data. Normally distributed confidence ellipses are shown. Post amox/clav samples have significantly different IMCs than enrollment samples by type II Adonis test of Bray-Curtis distance ($p<0.01$). This is true with or without participants 5 and 8. IMC returned to baseline state by 90 days with saline or autoFMT at similar rates.

Each metabolic category comprising the IMC was tested for contribution to the difference between enrollment and post amox/clav IMC using a Random Forest model. Thirteen of the 37 metabolic categories comprising the IMC drove the change in metabolic capacity from enrollment to post amox/clav. Each of these metabolic pathway categories was enriched post-amox/clav. Factors and their relative contribution are found in Supplemental Figure 3-1 and are ranked according to their contribution to this difference in Supplemental Table 1. AutoFMT did
not affect IMC differently than the saline control. IMC returned to baseline state by 30 days with saline or autoFMT. Saline or autoFMT did not result in different rates of return to baseline IMC. We found significant differences between individual participants’ IMCs when all time points were compared by type II Adonis ($p<0.01$).

### 3.4 Conclusions

This pilot study evaluated the impact of amox/clav on the taxonomic composition, resistome, and metabolic potential of the microbiome, and whether autoFMT could reverse any changes. Analysis of antimicrobial perturbation of the gut microbiome is complicated by the wide variation between baseline taxonomic composition of healthy guts\textsuperscript{123,124}. Additionally, while antimicrobial use has been previously demonstrated to reduce species diversity and cause diarrhea, the specific taxonomic changes observed vary greatly by individual\textsuperscript{123}.

Despite the dramatic and rapid perturbations observed acutely following antimicrobial exposure, longer-term robustness and resilience has been observed in prior reports\textsuperscript{123-126}. Most pilot studies and case reports after amoxicillin or ciprofloxacin indicate return to baseline by two months after exposure\textsuperscript{123-126}. However, while overall diversity and structure may be restored, some species remain missing. These analyses have been limited by resolution of 16S rRNA sequencing data and have been unable to detect resistance and metabolic genes. These factors may play critical roles in determining the robustness of the restructured microbiome to antimicrobial perturbation and pathogen invasion. Using shotgun sequencing we tracked resistance and metabolic genes not detectable in previous 16S rRNA analyses of taxonomy.

Among healthy volunteers, five days of amox/clav exposure (875 mg BID) led to significant taxonomic shifts ($p<0.01$), beta-lactamase gene enrichment ($p=0.0017$), and predicted
metabolic capacity alteration ($p<0.01$). Interestingly, only two participants demonstrated obvious phylum-level taxonomic perturbation of the type readily detected by traditional analysis. However, amox/clav led to enrichment of 13 metabolic gene categories and significant alteration from enrollment. This significant shift in metabolic capacity was observed even in the absence of phylum level differences. Previous work has demonstrated that metabolic pathways are more conserved within individuals than phylogenetic structure\textsuperscript{127}. The disruption of these pathways observed here may be more important than taxonomic alterations when predicting acquisition and/or proliferation of an MDRO.

This study’s limitations included small sample size and limited taxonomic perturbation following amox/clav. In addition, the most obvious taxonomic shifts occurred in participants randomized to autoFMT. These factors limited assessment of autoFMT’s effects on taxonomic restoration. However, this provided an opportunity to assess metabolic perturbation in the absence of broad taxonomic shifts. Small sample size limited our ability to assess the implications of the observed shift in metabolic capacity. Future work is necessary to explore perturbations caused by more disruptive antimicrobial regimens and the ability of autoFMT to restore gut phylogeny.

Despite these limitations, this study contributed to our understanding of the gut microbiome in several important ways. This study supports the concept of the healthy microbiome’s resilience following perturbation. AutoFMT was found to be safe and well tolerated, with no adverse events. By seven days following amox/clav phylogeny, beta-lactamase content, and predicted metabolic capacity returned to baseline.
Most strikingly, this study demonstrates the importance of assessing gut metabolic potential which was significantly altered even in the absence of taxonomic shifts. This shift in metabolic capacity can only be detected using shotgun sequencing or metabolite profiling. Further work is necessary to determine the implications of this shift in metabolic capacity on risk of MDRO colonization and host metabolism.

3.5 Methods

This was a prospective, randomized controlled pilot study (NCT 02046525) to determine the effect of autoFMT on the intestinal microbiome versus placebo (saline enema) after five days of amox/clav, administered at 875 mg twice per day. The study participants and investigators were blinded to the treatment assignment until after all analyses were completed. This study was approved by the Washington University School of Medicine Human Research Protection Office. Written informed consent was obtained from all study participants.

3.5.1 Study Participants

Ten healthy volunteers were recruited to participate. Written informed consent was obtained from all study participants. Inclusion criteria included being generally healthy and between 21-70 years of age. Exclusion criteria included a history of allergic reaction to beta-lactam antimicrobials or contraindications to amox/clav; any non-topical antimicrobial exposure or tube feeds as a primary source of nutrition in the six months prior to enrollment; pregnant or risk of becoming pregnant during the study period; gastroenteritis in the last three months; any non-elective hospitalization in the previous 12 months; incontinent of feces; prior resection or alteration of the stomach, small bowel, or colon; unwillingness to receive an enema or FMT;
known colonization with an MDRO; anticipated change in diet, medications, or elective surgery during the study period; or a history of an intestinal disorder.

3.5.2 Study Procedures

Participants submitted stool samples to investigators at enrollment, immediately post-amox/clav, and at days 1, 7, 30, and 90 post-enema (saline or autoFMT). Once the pre-antimicrobial specimen was obtained, the participant was instructed to take five days of amox/clav at 875 mg twice daily (BID). Participants were requested to return the bottles of amox/clav to confirm all doses had been taken. The participant was then randomized in a 1:1 fashion to 100 mL of placebo (non-bacteriostatic saline) or autoFMT product. The autoFMT product was thawed overnight at room temperature. The study enema was administered 24 to 48 hours after the last dose of amox/clav. In order to maintain blinding of the study participant, the study enema was delivered to the study clinic in an opaque bag, and the participant was instructed to not turn around when the enema was being prepared and administered. A vial of the participant’s autoFMT product was opened in the room, regardless if randomized to placebo or autoFMT, prior to preparing the enema for administration in order to prevent the participant from attempting to guess which study group she or he had been assigned to based on the odor during the procedure. The investigators doing the sequencing work remained blinded to the study group until after the sequencing was completed.

3.5.3 Fecal Processing

Participants were provided with sealable feces collection devices. After the participant collected a bowel movement, samples were delivered within 2 hours of collection. Upon receipt, the feces was immediately processed and 1.5g was reserved for feces culture, 1 g for Clostridium
difficile culture, approximately 23g for feces pulverization and sequence-based analyses, and the remainder of the subject’s first sample was used to prepare the FMT product.

### 3.5.4 FMT Product Preparation

To prepare the FMT product, the remaining feces was weighed and transferred to a sterile container. Non-bacteriostatic saline was added to the feces in a volume of twice the weight of the feces. A sterile spatula was used to emulsify the mixture for 3-5 minutes. Then the mixture was allowed to rest for five minutes. The feces/saline mixture was then poured through a stainless steel strainer to remove large particulate matter. Four 2 mL aliquots were frozen for genomic analysis. The remaining filtrate was drawn into 60 mL syringes (50 mL filtrate each, up to four syringes total). If the patient was randomized to saline, two 60 mL syringes were filled with non-bacteriostatic saline. The filled syringes and aliquots were stored at -80 °C.

### 3.5.5 Quantitative Cultiromics

Fresh feces (1.5g) was added to an equal amount of 1X PBS and mixed thoroughly. Immediately, six ten-fold serial dilutions were made from the homogenized specimen. 10ul and 100ul of the 1/10 and 1/100 dilutions were plated to two each of the following media: TSA II with 5% sheep blood (BAP, BBL BD, Franklin Lakes, NJ), Columbia Colistin Nalidix Agar with 5% sheep blood (CNA, BBL BD), MacConkey (MAC, BBL BD) and Chocolate agar (CHOC, BBL BD). The BAP and CHOC were incubated at 35⁰C in CO₂. The CNA and MAC were incubated at 35⁰C in air. The plates were read at 24h and 72h. 10 µL and 100 µL of the 1/10³ and 1/10⁶ dilutions were plated to two each of the following media: Brucella Blood agar (BBA, Anaerobe Systems, Morgan Hill, CA), Bacteroides Bile Esculin agar (BBE, Anaerobe Systems), Laked blood with kanamycin and vancomycin (LKV, Anaerobe Systems), cycloserine-cefoxitin
fructose agar with horse blood and taurocholate (CCFA_HT, Anaerobe Systems), and phenylethyl alcohol blood agar (PEA, Anaerobe Systems). These plates were incubated at 35°C anaerobically for seven days. The plates were read at 48 h, 4-5 d, and 7 d. 1 g of fresh feces was processed for culture of *C. difficile*, as previously described\textsuperscript{128}.

All growth was observed and recorded semi-quantitatively. All distinct colonies were identified using VITEK MS IVD v2.0 MALDI-TOF MS (bioMerieux). For any isolate that was not identified, a Gram stain was performed. All isolates were stored in TSB with glycerol at -80°C.

3.5.6 Metagenomic DNA Extraction and Sequencing

Metagenomic DNA was extracted from 0.5g of feces via phenol-chloroform for each sample as previously described.\textsuperscript{72} DNA from each sample was sheared to 500–600 bp using the Covaris E220 sonicator (intensity, 4; duty cycle, 10%; cycles per burst, 200; treatment time, 75 s; temperature, 4 °C; sample volume, 130 µL). The sonication product was purified with the Qiagen PCR Purification Kit and eluted in 63 µL nuclease-free water (pre-warmed at 50°C).

End repair and barcode ligation reactions were performed in triplicate for each sample. End repair was performed in a Bio-Rad thermocycler using the reagents: 2.5 µL T4 DNA ligase buffer with 10mM ATP (10X) (NEB B0202S), 1 µL dNTP (1mM), 0.5 µL T4 Polymerase (NEB, M0203S), 0.5 µL T4 PNK (NEB M0201S), and 0.5 µL Taq Polymerase (NEB, M0267S).

The barcode ligation reaction was performed by adding 2.5 µL of unique sequencing barcode at 1uM to 500ng of end-repaired DNA. 0.8ul of T4 DNA ligase (NEB, M0202M) was incubated with the barcode and sample DNA mixture in a Bio-Rad thermocycler. Following
barcode ligation, samples were pooled into groups containing 6 barcodes. Pools were purified using the Qiagen PCR Purification Kit and MinELute columns and eluted in 15µl of EB.

Fragment size selection was performed using a Use 0.5 TBE 1.5% agarose gel and visualized with SYBR Safe DNA stain. Barcoded DNA fragments sized 400-900bp were cut from the gel and purified with the Qiagen Gel Extraction Kit and MinElute columns and eluted in 12 µL Buffer EB. 2 µL of the eluted mixture was used for PCR enrichment of size selected products. The enrichment reaction was prepared as follows: 12.5µl 2X Phusion HF Master Mix, 9.5 µL Nuclease-free water, 1ul Illumina PCR Primer Mix (F+R) (10uM), and 2 µL Gel-purified DNA. The reaction was performed in a Bio-Rad thermocycler using the program: 17 x [ 98C 30sec (0:30), 65C 30sec (0:30), 72C 30sec (0:30)] 72C 5min, 4C forever.

Size selection of the enriched products was performed using a Use 0.5 TBE 1.5% agarose gel and visualized with SYBR Safe DNA stain. Fragments sized 400-900bp were cut from the gel and purified with the Qiagen Gel Extraction Kit and MinElute columns and eluted in 15 µL of EB. DNA concentration was quantified using a Qbit fluorometer and all samples pools were combined at equal concentrations for sequencing. Prior to sequencing, pooled fragment size was assessed with a BioAnalyzer trace and barcode read distribution was assessed using a spike-in run on the Illumina sequencing platform.

To generate metagenomic sequencing reads, the Illumina NextSeq platform was used with the high output kit and settings to generate a minimum of 400 million paired-end reads per run. In total 70 samples were sequenced at least 1 million reads per sample to allow microbiome and resistome analyses. The depth of 1 million reads was established using previous studies of the gut microbiome\textsuperscript{129-131}. 

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Multiplexed Illumina paired-end shotgun metagenomic sequence reads were demultiplexed by barcode. Reads without exact match to barcode were discarded. The remaining reads were quality filtered using Trimmomatic v0.35 and parameters optimized by the Dantas lab (seed mismatches: 2, palindrome clip threshold: 30, simple clip threshold: 10, min Adapter length: 1, keep both Reads: TRUE, window size: 4, required quality: 20, leading: 10, trailing: 10, min length: 60).

3.5.7 Microbiome Taxonomic Composition Prediction

Taxonomic composition was determined by comparing unique indicator sequences from sequencing reads to clade-specific marker genes from approximately 17,000 reference genomes using MetaPhlAn 2.6.0\textsuperscript{72}. Analysis was performed using metaphlan2/2.6.0 with the following parameters: --blastdb ./metaphlan2/blastdb --input_type multifasta. The difference in species diversity was calculated using the Shannon index function from the R vegan package. The difference in species composition was calculated using Bray Curtis distance between samples and significance was tested with type II adonis.

3.5.8 Resistance Gene Prediction

The metagenomic DNA sequence was analyzed for both known and sequence-novel antimicrobial resistance genes using functional metagenomic selections and curated resistance gene databases for resistance gene identification, and ShortBRED for resistance gene abundance estimation\textsuperscript{132}. To supplement known resistance gene markers available via the well-curated CARD database, markers from novel, cryptic resistance gene unique to the studied samples were obtained by performing functional metagenomic selections on bacterial metagenomic DNA pooled by participant\textsuperscript{133}. Functional metagenomic identification of genes that conferred
resistance to amox/clav was performed as previously described\textsuperscript{65,72,134} by randomly shearing metagenomic DNA from each pool of feces into fragment libraries. These libraries were cloned into the natively pan-susceptible host \textit{E. coli} DH10B using vector PZE21. For each sample, the host cells containing library fragments were selected against amoxicillin and ampicillin at concentrations lethal to the untransformed host. Surviving colonies were pooled and the inserted fragments sequenced via the Illumina MiSeq platform (2x150bp). Sequencing reads were assembled into contigs with the PARFUMS pipeline\textsuperscript{135}. The contigs were searched for open reading frames with MetaGeneMark\textsuperscript{136} and annotated by hmmScan function of HMMER3\textsuperscript{137} against the Resfams core database\textsuperscript{138}, Pfams\textsuperscript{133}, and TIGRFAMS\textsuperscript{133}. Resulting annotations were then hand-curated\textsuperscript{133} in the following manner: Selections were excluded if >100 contigs were assembled because this suggests a failure in the assembly or selection since <100 unique resistance contigs are expected per selection. Within each contig, annotations were ranked by specificity to the selective agents and lowest E-value with preference given to Resfams annotations over Pfams or TIGRFAMS. In absence of a clear specific causative gene annotation (i.e. a beta-lactamase), the two best annotations with <90\% overlap were accepted. The sequences corresponding to accepted annotations were then pooled with known antimicrobial resistance genes sequences from CARD 2017\textsuperscript{133}. The genes were then quantified in unassembled metagenomic sequence using the ShortBRED pipeline with a clustering identity of 1.

3.5.9 Metabolic Pathway Prediction

The metabolic potential of the fecal microbial communities was inferred through functional potential profiling. The presence and abundance of metabolic pathways in the microbial communities was assessed using HUMAnN2 which maps unassembled shotgun sequencing reads to functionally annotated species pangenomes in order to predict function\textsuperscript{62}. 
Default parameters were used and details can be found at: http://huttenhower.sph.harvard.edu/humann2.

In order to assess metabolic perturbations, we implemented an index of metabolic capacity (IMC) from the metagenomic sequencing data. To form this index we grouped genes with metabolic functions into 37 metabolic pathway categories using Gene Ontology (GO) terms. GO term grouping was performed using ASaiM\textsuperscript{139} and custom scripts. The ability of the predicted metabolic capacity to discriminate between sample groups was visualized using principal component analysis (PCA) and significance was tested using type II adonis of Bray-Curtis distance between samples. This test of significance was conducted with and without participants 5 and 8 to determine whether any difference observed was driven by the large taxonomic perturbations seen in those participants. Each metabolic category comprising the IMC was tested for contribution to the difference between enrollment and post amox/clav IMC using a Random Forest model. This was implemented using the Boruta package in R\textsuperscript{140}. Factors were ranked according to their contribution to this difference.

### 3.5.10 Statistical Analysis

Significance of differences between taxonomic composition at baseline and post amox/clav was calculated by determining Bray-Curtis distance between the communities and using a type II adonis with a significance level of $p<0.05$ with n=10. Significance of differences between IMC at enrollment and post amox/clav was calculated similarly. Bray-Curtis distance between IMCs was calculated and a type II adonis with a significance level of $p<0.05$ with n=10 was used. Because these are permutation tests, iteration cut-offs were set to detect significance up to $p<0.01$. Saline and autoFMT treatment groups taxonomy and IMC were compared to enrollment for return to baseline comparison. Enrichment of beta-lactamase genes in post-
antimicrobial samples relative to enrollment was calculated using a two-tailed t-test and a significance level of $p<0.05$. This test was also applied to the comparison between saline and autoFMT to enrollment baseline.

### 3.5.11 Accession Number

All nucleotide sequences generated during this study have been uploaded to NCBI under BioProject Accession: PRJNA446061.

### 3.6 Supplemental Figures and Tables

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**Supplemental Table 3-1: Ranked features contributing to index of metabolic capacity shift**

Each metabolic category comprising the IMC was tested for contribution to the difference between enrollment and post-amox/clav IMC using a Random Forest model. Features are ranked by their predicted contribution to difference between enrollment and post-amox/clav IMC. Features predicted to contribute to this difference are marked “Confirmed” while features predicted to have no effect are marked “Rejected.”
**Supplemental Figure 3-1: Features driving index of metabolic capacity shift** Metabolic features driving differences between enrolment and post amox/clav samples were identified by a Random Forest model. Participants 5 and 8 were excluded to eliminate factors related to broad taxonomic differences. Green indicates features predicted to drive differences while red indicates features predicted to have no effect. Blue boxplots indicate artificial shadow attributes used for Z score calibration\(^{140}\).
3.7 Acknowledgements

We are thankful to the Burnham, Dantas, and Dubberke labs for collaborative discussions. We thank Audrey Dang and Alaric D’Souza for insight and assistance in designing graphical representations of this data. This work was supported by awards to the authors from the Center for Disease Control and Prevention Epicenter Prevention Program Grant (1U1CI000033 301), to C.B. through an NHGRI training grant (T32 HG000045) administered by Michael Brent and Barak Cohen, to A.L. the NIH (TL1 TR000449), and to J.K. from the National Center for Advancing Translational Sciences of the National Institutes of Health (KL2 TR002346). We are thankful to Jessica Hoisington-Lopez, Eric Martin, Brian Koebbe, Keith Page, and Bonnie Dee in the Center for Genome Sciences and Systems Biology at Washington University in St. Louis School of Medicine. The content of this chapter does not necessarily represent the official views of the funding agencies.
Chapter 4

Conclusion and Future Directions

4.1 Mechanisms of Daptomycin Action and Resistance

Daptomycin is a broad spectrum antibiotic used to treat severe Gram-positive infections. Recent reports of high-level daptomycin resistance (HLDR) have raised concern for the continued clinical utility of this safeguarded drug. While alarming levels of resistance have emerged in *Streptococci* and *Enterococcus faecium* it is the ability of *Corynebacterium striatum* to rapidly transition to HLDR that is most dire. *C. striatum* poses such a serious threat due to its ability to rapidly and almost completely remove PG (from ~45% to <1%) from its outer membrane, demonstrated here. Furthermore, this work has shown that this elimination of PG occurs without additional compensatory genetic, transcriptomic changes. The HLDR mutants also exhibit no changes in cell wall thickness, cell surface charge, conversion of PG to cardiolipin, or membrane shape, changes suggested in reports of lower-level daptomycin resistance. PG absence from the membrane is reconciled by increased levels of PI. Liposome binding assays presented here have demonstrated that PG is the target of daptomycin and the lipids that remain in the HLDR stains (PA and CL) do not sufficiently interact with the drug. This work demonstrates *C. striatum* has the ability to completely remove PG from its membrane and further establishes that PG is the *in vivo* and *in vitro* target of daptomycin.
The remarkable ability of C. striatum to remove a previously presumed necessary membrane phospholipid highlights the importance of testing for resistance evolution in various species. This is particularly true in the case of lipopeptide antibiotics that target the mutable bacterial membrane. Further work is necessary to understand the ability of other commensals and pathogens to remove or reduce PG membrane content.

4.2 Autologous Fecal Microbiota Transplantation

The pilot autoFMT trial presented here assessed the effects of antimicrobials (amox/clav) on microbiome structure. Unlike previous work which has relied on phylogeny to infer metabolic and resistance capacity, the shotgun sequencing performed here allowed identification of particular genes of interest. In particular, the index of metabolic capacity (IMC) structured here was significantly perturbed following amox/clav treatment. This shift was driven by enrichment of 13 metabolic gene categories in response to the antimicrobial. This significant shift in metabolic capacity was observed even in the absence of phylum level differences. This underscores the importance of directly assessing metabolically linked genes. Previous work has demonstrated that metabolic pathways are more conserved within individuals than phylogenetic structure\textsuperscript{127}. The IMC structured here provides a framework for future studies to measure differences between individuals and following various perturbations. Further work is necessary to determine the implications of this shift in metabolic capacity on risk of MDRO colonization and host metabolism.

AutoFMT was found to be safe and well tolerated, with no adverse events. By seven days following amox/clav perturbations in phylogeny, beta-lactamase content, and IMC were reversed. It is important to note that both the autoFMT and the saline control group returned to
baseline resistance gene abundance and IMC. As only two perturbations in phylogenetic composition after amox/clav were observed and both were randomized to autoFMT, further work is necessary to determine whether autoFMT reverses such changes or if healthy microbiomes are capable of re-balancing phylogenetic composition without outside intervention.

This study’s limitations included small sample size and limited taxonomic perturbation following amox/clav. Approval for future work involving a greater number of healthy participants has been obtained. The work reported here has demonstrated the importance of determining metabolic capacity explicitly rather than drawing inferences from phylogeny. Future work should compare metabolite profiles (e.g. by mass spectrometry) to the metabolic capacity predicted by metagenomic sequencing. Furthermore, this data should be coupled with host response factors. The IMC detailed here is an important first step in more acutely predicting the metabolic capacity of the human gut.

4.3 General Conclusion

To prevent the arrival of a post-antibiotic era we must better understand the mechanisms of action and resistance and collateral effects of antimicrobials. The work described here makes progress toward understanding how daptomycin, a critical antimicrobial targets bacterial membranes and how high-level resistance can emerge rapidly in particular species. Furthermore, this work identifies important post-antimicrobial shifts in gut microbiome metabolic capacity that would have been overlooked by traditional 16S rRNA analyses. This continuing effort to understand antimicrobials and their effects is necessary to maintain medicine’s ability to control bacteria.
References


OptumRx. Cubicin (daptomycin) - First-Time Generic, 2016).


Scarpignato, C. *Rifaximin, a poorly absorbed antibiotic: pharmacology and clinical use.* (Karger, 2005).


Khoruts, A., Dicksveld, J., Jansson, J. K. & Sadowsky, M. J. Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent Clostridium difficile-


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Appendix: MRSA Epidemiology

Methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for community-acquired and healthcare associated infections\(^{141-143}\). When compared to their methicillin-susceptible counterparts, MRSA infections carry increased mortality risk and treatment costs\(^{144,145}\).

**Banked MRSA Genomic DNA**

A set of 1880 MRSA strains has been collected by Barnes-Jewish Hospital from 2005-2012\(^{146}\). These isolates were obtained during epidemiological surveillance of the SICU\(^{146}\). The isolates were obtained by nasal swab and genomic DNA from 504 randomly selected isolates was available\(^{146}\). 200 samples representing a span of collection years and SCC\(mec\) type were selected for whole genome sequencing.

**Sequencing and Genome Assembly**

Isolates were sequenced using the Illumina Hi Seq 2500 platform, generating 101bp paired-end reads. We assembled each genome de novo using SPADES\(^{108}\). 76 genome assemblies passed the quality control. These genomes were annotated using the Prokka v1.12 and the Pfam database\(^{109}\).
Phylogenetic Tree Construction

A pan genome and was constructed and core genome alignment was performed (from the .gff files generated by Prokka) using Roary and the following parameters: `roary -e --mafft -p 8 *.gff`. A phylogenetic tree of resulting from this core genome alignment is shown in Figure A-1. The 76 isolates clustered into three distinct clades.

**Figure A-1: Core genome alignment** The 76 core genomes obtained over time in the SICU cluster into three distinct branches one of which is noticeably distinct in this representation.
Resistance Gene Annotation

The three distinct clades observed by alignment and phylogenetic prediction did not correspond to year collected (Figure A-2). This could be the result of three distinct sources of MRSA entering the SICU (e.g. community, ER, outpatient care facility). To explore these clades further we mapped SCCmec type and mobile resistance gene abundance to the groups (Figure A-2). The top clade was the most phylogenetically distinct from the others and contained an even mix of SCCmec type IV and II. The middle clade was predominantly type IV (community-associated strains) and the bottom clade was predominantly type II (hospital-associated strains).

There does not appear to be a difference in resistance transferrable gene carriage between the groups. Work in progress assesses resistance mutations that are less not transferrable as well as multilocus sequence type (MLST). Additionally, the genomes will be compared to other global MRSA genomic datasets to help infer their origin. The five most similar published genomes to each of the strains will be determined and metadata from these nearest neighbors will be used to infer origin of each clade. Furthermore, a classifier will be constructed to determine which genes and metadata features effectively predict clade.
Figure A-2: Phylogeny with SCC*mec type, year, and resistance genes The phylogenetic tree is annotated by SCCmec type (Roman numerals) and year (Color of Roman numerals: Green is earliest and purple is latest). Additionally, resistance gene presence (red) or absence (green) is depicted. The three distinct clades observed in the 76 MRSA isolates did not correlate with year collected. The top clade was the most distinct and an even mix of SCC*mec type IV and II. The middle clade was predominantly type IV (community associated) and the bottom clade was predominantly type II (hospital associated).
Appendix: Extended Data

Chapter 2 Extended Genomic Data

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Table A-1: All SNPs observed Only SNP in all 8 HLDR isolates was in pgsA2
## Chapter 2 Extended Transcriptomic Data

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**Table A-2: Increased expression** Changes greater in magnitude than 25%
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<td>2447.44</td>
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<td>Uncharacterized protein</td>
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<td>3731.82</td>
<td>2780.39</td>
<td>-25</td>
</tr>
</tbody>
</table>

*Table A-3: Decreased expression* Changes greater in magnitude than 25%