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PHYSIOLOGICAL RELEVANCE OF A tRNA-DEPENDENT MECHANISM FOR
MEMBRANE MODIFICATION IN *ENTEROCOCCUS FAECIUM*

by

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A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Molecular Biology and Microbiology
in the College of Medicine
University of Central Florida
Orlando, Florida

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Thesis Chair: Dr. Hervé Roy

Abstract

Enterococci were once thought to be harmless, commensal organisms that colonize the gastrointestinal tract of humans and other mammals. In the last 30 years, however, concern has grown in the clinical setting over two particular species, *Enterococcus faecalis* and *Enterococcus faecium*, which are frequently found to be the etiologic agents of nosocomial infections. Aminoacyl-phosphatidylglycerol synthases (aaPGSs) are integral membrane proteins that add amino acids to phosphatidylglycerol (PG) in the cellular envelope of bacteria. Addition of amino acids to PG confers resistance to various therapeutic antimicrobial agents, and contributes to evasion of the host immune response in a number of clinically relevant microorganisms. *E. faecium* possesses two distinct aaPGSs: aaPGS1 and aaPGS2. In addition, another gene coding for a putative hydrolase (*pHyd*) is located in the same operon as *aaPGS2*, and has no known function. To investigate the physiological relevance of aa-PG formation, and the function of *aaPGS1*, *aaPGS2*, and *pHyd* in *E. faecium*, we generated individual knockouts of these genes using a markerless deletion strategy. Deletion of *aaPGS1* did not noticeably alter lipid aminoacylation, whereas deletion of *aaPGS2* led to a loss of aa-PG synthesis. Deletion of *pHyd* also led to a loss of lipid aminoacylation; however, additional experiments are needed to verify that expression of *aaPGS2* (which resides just downstream in the same operon) is unaffected in the *pHyd*-deletion strain. Development of the mutant strains described here will enable us to investigate additional phenotypes associated with these genes, and to determine whether

aa-PG formation contributes to antibiotic resistance in *E. faecium* as in several other pathogenic microorganisms.

This work is dedicated to my lovely fiancée, Valorie, our son Lain, and my parents, Sam and Barbara. This would not have been possible without your patience and perpetual encouragement.

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Table of Contents

I. Chapter One: Introduction	1
I.1 Background.....	1
I.2 Relevance.....	5
II. Chapter Two: Materials and Methods	8
II.1 Bacterial Strains and Growth Media.....	8
II.2 Construction of Integrative Plasmids	8
II.3 Transformation of <i>E. coli</i> XL1B Competent Cells.....	9
II.4 DNA Preparation.....	10
II.4.1 <i>E. coli</i> and Plasmid Preparation	10
II.4.2 Large-Scale Preparation of Plasmid Suitable for <i>E. faecium</i> Electroporation....	10
II.5 Generation of <i>E. faecium</i> knockout strains	11
II.5.1 Preparation of Competent <i>E. faecium</i> TX1330 Cells	11
II.5.2 Electrotransformation of <i>E. faecium</i> TX1330 Cells	12
II.5.3 Plasmid Integration	13
II.5.4 Plasmid Curing and Excision.....	13
II.5.5 Replica Plating.....	14
II.6 Lipid Analysis	15
II.6.1 Lipid Extraction.....	15
II.6.2 Thin-layer Chromatography (TLC).....	16

II.7	Growth Kinetics.....	16
III.	Chapter Three: Results.....	17
III.1	Generation of <i>E. faecium</i> Knockout Strains.....	17
III.2	Replica Plating/Negative Selection.....	21
III.3	Lipid Analysis of Δ <i>aaPGS1</i> , Δ <i>aaPGS2</i> , and Δ <i>pHyd</i> Strains.....	22
III.4	Bacterial Growth Kinetics of Δ <i>aaPGS1</i> , Δ <i>aaPGS2</i> , and Δ <i>pHyd</i> Strains.....	25
IV.	Chapter Four: Discussion.....	27
V.	References.....	31

List of Figures

Figure 1: tRNA-dependent mechanism for membrane lipid aminoacylation	2
Figure 2: Pathways for lipid biosynthesis and aminoacylation in bacteria.....	4
Figure 3: Allelic replacement strategies in <i>E. faecium</i>	18
Figure 4: Growth inhibition of <i>E. faecium</i> mediated by <i>pCl-Phe</i> and the marker <i>PheSA294G</i>	20
Figure 5: TLC analysis of membrane lipids from <i>E. faecium</i> cultured in various conditions.....	23
Figure 6: TLC analysis of lipids from <i>E. faecium</i> wild-type and mutant strains	24
Figure 7: <i>pHyd-aaPGS2</i> operon in <i>E. faecium</i> TX1330	24
Figure 8: Growth kinetics of <i>E. faecium</i> wild-type and mutant strains.....	25

List of Tables

Table 1: Parameters for the growth of <i>E. faecium</i> wild-type and mutant strains.....	26
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I. Chapter One: Introduction

I.1 Background

The cell wall of Gram-positive bacteria, such as enterococci, contains a thick layer of peptidoglycan, which presents a significant barrier to the environment. Underneath the peptidoglycan is the cytoplasmic membrane, a complex, composite structure containing a variety of phospholipids including the anionic component phosphatidylglycerol (PG) that contributes to the net negative charge of the membrane (Roy, 2009). There are several known mechanisms of modification of the inner cell envelope that confer protection to the cell against a variety of environmental stresses. One such mechanism involves addition of amino acids (aa) to PG by aminoacyl-phosphatidylglycerol synthases (aaPGSs). aaPGSs are integral membrane proteins that utilize aa-tRNAs as substrates to aminoacylate PG in the bacterial membrane (Figure 1). Addition of certain aa to PG lowers the net negative charge of the cell envelope, which in turn decreases the permeability of the membrane to cationic antimicrobial peptides (CAMPs) produced during the host immune response, and to various therapeutic antimicrobial agents such as vancomycin (for review see Roy, 2009).

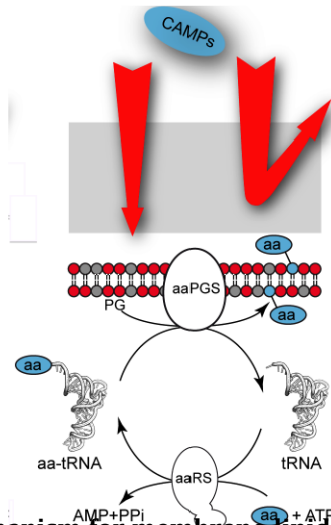


Figure 1: tRNA-dependent mechanism for membrane lipid aminoacylation. aaPGSs are integral membrane proteins that transfer amino acids (i.e., Lys, Ala, or Arg) from aa-tRNA to phosphatidylglycerol (PG) in the membrane, thereby reducing the net negative charge of the cellular envelope and providing resistance to various cationic antimicrobial peptides (CAMPs). (Adapted from Roy, 2009).

Alanylation and lysinylation of PG in the cytoplasmic membrane using aa-tRNAs as amino acid donors was initially discovered in the 1960's, but it wasn't until recently that a correlation was established between a gene called *mprF* (multiple protein resistance factor) and this process (Peschel et. al., 2001). The *mprF* pathway was first investigated in *Staphylococcus aureus*, where lysinylation of PG in the membrane was shown to decrease bacterial susceptibility to the host immune response, and confer resistance to different classes of cationic bactericidal agents such as daptomycin (a lipopeptide) and vancomycin (a glycopeptide) (for review see Roy, 2009). Recently, it was discovered that aminoacylation of membrane PG produces similar effects in a number of clinically relevant organisms such as *Bacillus anthracis* (Samant et. al., 2009), *Mycobacterium tuberculosis* (Maloner et. al., 2009), and *Listeria monocytogenes* (Thedieck et. al., 2006).

aaPGSs consist of a C-terminal hydrophilic domain and an N-terminal integral membrane domain. It was previously established that the hydrophilic C-terminal domain, which protrudes into the cytoplasm, is the location of the catalytic site of the enzyme (Roy, 2009). aaPGSs use aa-tRNAs located in the cytoplasm to covalently attach aa to the polar head of PG in the membrane, and it has been shown that these enzymes are able to compete equitably with the protein biosynthesis machinery for the cell's available pool of aa-tRNAs (Roy & Ibba, 2008). The main lipid constituents of the bacterial membrane are either anionic or neutrally charged, depending on the identity of the phospholipid polar head, which is determined by directing lipid biosynthesis through one of two biosynthesis pathways. The aaPGS pathway is known to branch from the biosynthesis pathway that produces anionic phospholipids (Figure 2)(Roy, 2009). Modification of pre-existing PG by aaPGSs is an efficient way for bacteria to adapt the electrostatic properties of their membrane without the need for *de novo* lipid synthesis (for review see Roy, 2009).

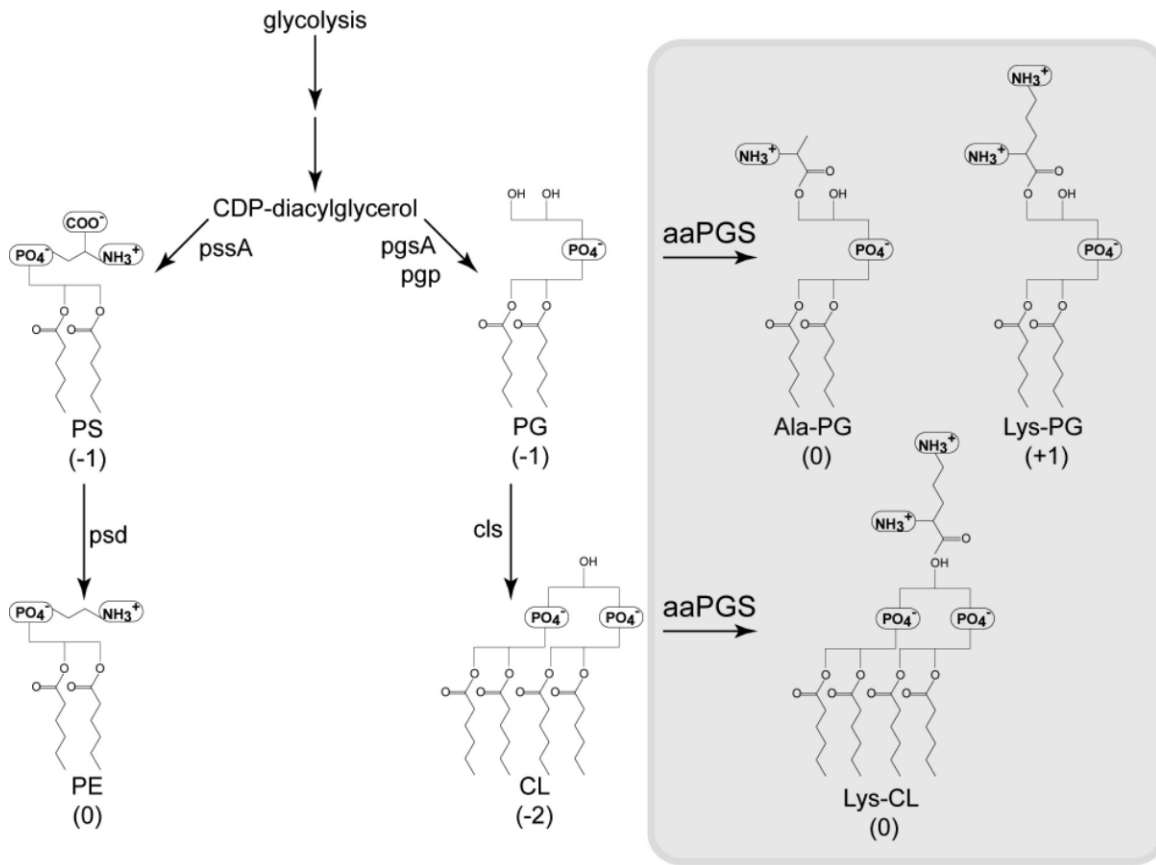


Figure 2: Pathways for lipid biosynthesis and aminoacylation in bacteria. The structure of the polar head groups of common membrane phospholipids in bacteria. Addition of amino acids to pre-existing anionic components (PG, CL) allows for modification of the electrostatic properties of the membrane without *de novo* lipid synthesis. Numbers in parentheses represent net charge at neutral pH. CDP, cytidine diphosphate; PG, phosphatidylglycerol; aaPGS, aminoacyl-phosphatidylglycerol synthase; pgs, phosphatidylglycerophosphate synthase; PS, phosphatidylserine; pss, PS synthase; psd, PS decarboxylase; pgp, phosphatidylglycerophosphatase; CL, cardiolipin; cls, CL synthase. (Adapted from Roy, 2009).

Currently, 348 *aaPGS* homologs have been identified that are distributed in over 200 species of bacteria. There are three substrate aa-tRNAs that have been described for aaPGSs: Ala-tRNA^{Ala}, Lys-tRNA^{Lys}, and Arg-tRNA^{Lys} (Roy & Ibba, 2009). Interestingly, many of the available genome sequences for aaPGS-containing organisms encode two or more aaPGS paralogs. Recent work has shown that two aaPGS paralogs found in *Clostridium perfringens* exhibit distinct substrate specificities: one enzyme uses Lys-tRNA^{Lys} to transfer Lys to PG, while the other uses Ala-tRNA^{Ala} to transfer Ala (Roy et. al., 2008). In contrast, investigations have shown that some aaPGSs have a relaxed specificity, which enables the transfer of a variety of aa by a single enzyme. Specifically, the aaPGS in *Bacillus subtilis* is able to catalyze the addition of either Lys or Ala to PG, and aaPGS2 in *Enterococcus faecium* is able to transfer Lys, Arg, or Ala (Roy et. al., 2009).

I.2 Relevance

Enterococci are commensal organisms that inhabit the gastrointestinal tracts of humans and other mammals. In the last 30 years concern has grown in the clinical setting over two particular species, *E. faecalis* and *E. faecium*, which are frequently identified as the etiologic agents of nosocomial infections, including urinary tract infections, intra-abdominal, pelvic and soft tissue infections, and bacteremia and endocarditis (Malani et. al., 2002). Until recently, nosocomial infections caused by *E. faecalis* far outnumbered other enterococcal infections; however, *E. faecium* is now responsible for over 40% of such infections (Zhang et. al., 2012). In U.S. hospitals, a primary concern is that a growing number of *E. faecium* isolates are resistant to vancomycin (referred to as vancomycin-

resistant enterococci, or VRE), which is often used as a last-resort antimicrobial agent. In addition, over 80% of clinical isolates worldwide are now found to be resistant to ampicillin (Zhang et. al., 2012). A sophisticated understanding of enterococcal physiology and genetics is needed to facilitate the development of new antimicrobials to combat multi-drug resistant strains (Kristich et. al., 2007).

The current project focused on two aaPGS paralogs found in *E. faecium*, aaPGS1 and aaPGS2, as well as a gene encoding a putative hydrolase (*pHyd*) that is located in an operon with *aaPGS2*. Recent work in *E. faecium* showed that aaPGS2 is triple-specific for Ala, Lys, and Arg *in vitro*; however, the activity of aaPGS1 could not be reconstituted and its function remains unclear (Roy et. al., 2009). aaPGS1 displays 32% identity with aaPGS2, indicating that both proteins may exhibit similar functions. Among the five species of enterococci for which the genomes are available, *aaPGS2* and *pHyd* are conserved and organized in a single operon. In contrast, *aaPGS1* is present in only two of the species (*E. faecium* and *E. faecalis*), and the genetic context of *aaPGS1* is not conserved between these two species.

Currently, the physiological relevance of *aaPGS1*, *aaPGS2*, and *pHyd* in *E. faecium* remains unknown, and the phenotypes associated with these genes have yet to be described. Despite the establishment that aaPGS2 modifies PG with Ala, Arg, and Lys *in vitro*, it is not known whether these modifications provide antibiotic resistance *in vivo*. Only within the last several years have effective tools for targeted mutagenesis in enterococci been developed. Our research has focused on the establishment of genetic techniques for the markerless deletion of *aaPGS1*, *aaPGS2*, and *pHyd* in *E. faecium*. A markerless deletion

strategy was selected in order to avoid detection of phenotypical changes resulting from polar effects, or antibiotic resistances associated with insertion of a selection marker into the genome. We characterized preliminary phenotypes associated with these genes using pre-established biochemical techniques and comparative analysis of growth in a variety of conditions.

II. Chapter Two: Materials and Methods

II.1 Bacterial Strains and Growth Media

E. faecium strains were stored at -80°C in Todd-Hewitt broth (THB; prepared according to the manufacturer's instructions) supplemented with 15% glycerol. Bacto agar was used as the solidifying agent in all semi-solid media. *p*-Cl-phenylalanine was obtained from Alfa Aesar. When required for selective growth, gentamycin (RPI) was used at a concentration of 25 mg/l for *E. coli* and 250 mg/l for *E. faecium*.

II.2 Construction of Integrative Plasmids

pTEX5500ts is an *E. coli* – *E. faecium* shuttle vector that contains two markers for antibiotic resistance (chloramphenicol and gentamicin), and a thermosensitive origin of replication for Gram-positive hosts (Nallapareddy et. al., 2006). In *E. faecium*, this plasmid exhibits optimal replication at the permissive temperature of 28°C, with dramatic loss of replication at 42°C. We added a mutated version of the gene *pheS*, which has been used successfully as a counterselectable marker in the context of *E. faecalis* (Kristish et. al., 2007). Specifically, the chloramphenicol resistance gene of the plasmid pTEX5500ts was substituted with *pheSA294G*, which encodes for the α -subunit of the *E. faecium* phenylalanyl-tRNA synthetase (PheRS) carrying a mutation in its active site. The mutated α -subunit reconstitutes with the genome-encoded β -subunit to form a functional heterotetrameric PheRS ($\alpha_2\beta_2$). This mutated version of PheRS allows for tRNA misacylation with the substrate analog para-chloro-phenylalanine (*p*Cl-Phe), which results

in growth inhibition. The plasmid containing the *pheSA294G* gene was named pTEX5500ts-*pheSA294G*.

The upstream and downstream regions of the targeted *aaPGS1*, *aaPGS2*, and *pHyd* loci were individually amplified and ligated using PCR. pTEX5500ts-*pheSA294G* contains two multiple cloning sites (MCS) that include digestion sites for the restriction enzymes *NheI* and *HindIII*. These restriction sites were also included at the terminal ends of each of the amplicons corresponding to the upstream and downstream regions of the targeted genes. The PCR products and pTEX5500ts-*pheSA294G* were digested with *NheI* and *HindIII*, and the digested plasmid was dephosphorylated with antarctic phosphatase. Following phenol-chloroform extraction and ethanol precipitation, the PCR products were ligated into pTEX5500ts-*pheSA294G* using T4 DNA ligase.

II.3 Transformation of *E. coli* XL1B Competent Cells

Transformation solutions were prepared by adding 60 μ l H₂O and 20 μ l KCM (0.5 M KCl, 0.15 M CaCl₂, 0.25 M MgCl₂) to 20 μ l of each ligation product. Competent cells (*E. coli* strain XL1B) were thawed on ice, and 100 μ l were added to each transformation solution. The cells were placed on ice for 20 minutes, followed by room temperature for 10 minutes. 800 μ l LB (Luria-Bertani) broth was added and the cells were allowed to recover at 37°C for 1 h with no agitation. Cells were plated on LB agar with 25mg/l gentamycin. 12 colonies exhibiting gentamycin resistance were selected and used for isolation of plasmid DNA.

II.4 DNA Preparation

II.4.1 *E. coli* and Plasmid Preparation

Cells harvested from 1.5 ml of an overnight culture, grown in LB broth supplemented with gentamycin, were resuspended in 150 μ l Solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0). Cells were lysed by addition of 300 μ l Solution II (0.2 M NaOH, 1% SDS; made fresh) and neutralized by addition of 200 μ l of Solution III (5 M potassium acetate, 2M glacial acetic acid). Cellular debris, genomic DNA, and proteins were precipitated for 5 min on ice and removed by centrifugation at 20,000 x g for 10 min at 4°C. Plasmid in 400 μ l of supernatant was retrieved by precipitation after addition of isopropanol (270 μ l) and centrifugation. The pellet was washed with 80% ethanol, dried, and resuspended in 50 μ l of water containing 25 mg/l of RNase A. Plasmids were screened by restriction digest and verified by sequencing (Genewiz, Inc.), and positive clones were selected for large-scale plasmid preparation.

II.4.2 Large-Scale Preparation of Plasmid Suitable for *E. faecium* Electroporation

Cells harvested from 500 ml of an overnight culture were resuspended in 20 ml of Solution I (see above). Cells were lysed in 40 ml of Solution II (made fresh; see above). Cellular debris, proteins, and genomic DNA were precipitated for 10 min on ice following addition of 20 ml of ice-cold Solution III (see above), and removed by centrifugation at 7000 x g for 15 min. The supernatant was filtered through gauze, and plasmid DNA in 80 ml of supernatant was retrieved by precipitation with 48 ml (0.6 vol) of isopropanol and

centrifugation. The pellet was washed with 70% ethanol, dried, and resuspended in 6 ml TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). RNA was precipitated by addition of 6 ml of 5 M LiCl and subsequent centrifugation at 4°C. Plasmid in 12 ml supernatant was retrieved by precipitation and centrifugation following addition of 12 ml (1 vol) of isopropanol. The pellet was again washed with 70% ethanol, dried, and resuspended in 1 ml TE containing 25 mg/l RNase A. Following incubation for 30 min at room temperature, 1 ml of Solution IV (1.6 M NaCl, 13% PEG 8000; made fresh) was added. After incubation for 30 min on ice, the plasmid was collected by centrifugation at 4°C, resuspended in 400 µl TE, and extracted with phenol-chloroform. Plasmid was precipitated with 0.25 volume of 10 M ammonium acetate, and 2 volumes 100% ethanol and washed in 70% ethanol. The dried pellet was dissolved in 300 µl ddH₂O, which yielded a final concentration of approximately 3 mg/ml for each construct. Samples were resolved on a 0.8% agarose gel, along with previously prepared plasmid containing insert, and plasmid with no insert as controls.

II.5 Generation of *E. faecium* knockout strains

II.5.1 Preparation of Competent *E. faecium* TX1330 Cells

Enterococci are known to exhibit poor transformability. However, *E. faecium* TX1330, a community derived fecal isolate, is one of a few strains that are efficiently transformable by electroporation (Nallapareddy et. al., 2006). Previous studies have reported a relatively high transformation yield, with transformants ranging from 1,200 to 4,000 cfu/µg of plasmid. Methods that have been previously described were used to prepare and transform *E. faecium* TX1330 (Nallapareddy et. al., 2006).

Electrocompetent cells were prepared in THB liquid media supplemented with 0.25 M sucrose, by incubation with slow agitation for 24 h at 37°C. These culture conditions promote weakening of the bacterial cell wall, which occurs naturally during the late stationary phase of cell growth. Viability of the weakened cells was maintained in a hypertonic medium (containing sucrose) with low mechanical stress (low agitation). After growth, 1 L of cells was harvested by centrifugation at 7000 x g for 10 minutes at 4°C. The cells were sequentially washed with 400 ml, 200 ml, 100 ml, 50 ml, and 30 ml of ice cold 10% glycerol, and centrifuged at 5000 x g for 10 min at 4°C. The pellets were resuspended in 50 ml 10% glycerol and transferred to 50 ml conical tubes, which were centrifuged at 3220 x g for 15 minutes at 4°C. The pellets were then washed with 10% glycerol and 0.25 M sucrose, and frozen at -80°C. Freezing the cells is an essential step in the preparation of the *E. faecium* competent cells, as it further weakens the cell wall (Friesenegger et. al., 1991).

II.5.2 Electrotransformation of *E. faecium* TX1330 Cells

E. faecium TX1330 cells were electroporated according to a protocol adapted from Friesenegger et. al., 1991. Competent cells were thawed on ice, and 45 µl of cells were transferred to a 0.1 cm cuvette. 1.2 ng of plasmid DNA (pTEX5500ts-*pheSA294G*) was added to the cuvette and placed on ice. Electrotransformation was performed at 1.8 kV potential, 200 Ω resistance, and 25 µF capacitance. No arcing was observed, and 0.8 ml of ice-cold THB, supplemented with 0.25 M sucrose, was immediately added to the cuvette and placed on ice. As a control, THB with 0.25 M sucrose was added to 45 µl *E. faecium* cells

that had not been electroporated. The cells were allowed to recover for 2 h at room temperature, and then plated on THB agar supplemented with 0.25 M sucrose and gentamycin (250 mg/l). Plates were incubated at 28°C for 72 h.

II.5.3 Plasmid Integration

Isolated colonies were used to inoculate 5 ml cultures of THB (supplemented with gentamycin) and grown overnight at the non-permissive temperature (i.e., 42°C, a temperature that inhibits plasmid replication) to allow for plasmid integration into the genome by a single homologous recombination event. Genomic DNA was extracted using the same protocol described above for purification of plasmid DNA from *E. coli*, with the modification of agitating the cells by vortexing after addition of solutions I, II, and III. Diagnostic PCR was performed using appropriate oligonucleotides, and the products were resolved on a 0.8% agarose gel to determine where the plasmid had integrated. Controls of wild-type *E. faecium* genomic DNA, as well as free plasmid DNA, were included for comparison. Once it was determined that plasmid integration had taken place, either in the upstream or downstream region of the targeted loci, stock solutions of the corresponding cultures were placed at -80°C for storage after addition of 15% (v/v) glycerol.

II.5.4 Plasmid Curing and Excision

Isolated colonies from clones in which plasmid integration had been verified were used to inoculate 5 ml cultures of THB media supplemented with gentamycin and grown at 42°C. These cultures were subjected to five serial passages in THB (supplemented with gentamycin) to completely cure the cells of remaining free plasmid. Following this step,

plasmid excision by homologous recombination was allowed during three additional passages in 5 ml THB with no gentamycin. Various types of minimal media, containing low-level amounts of peptone and yeast extract as a source of amino acids (ranging from 60 to 250 mg/l), were screened to test the efficacy of using *pCl-Phe* (provided at 15 mM) as a counterselectable marker. Our investigations revealed that 125 mg/l of peptone and yeast extract was sufficient to allow for efficient growth of wild-type *E. faecium*, but inhibit growth of one of the deletion strains. The cells were washed several times with 100 mM NaCl, and 500 cfu were plated on minimal media supplemented with 15mM *pCl-Phe*.

II.5.5 Replica Plating

After it was determined that the *pheS* counterselection strategy was not an efficient tool for markerless mutation in *E. faecium* (see Chapter Three: Results, *Generation of Knockout Strains of E. faecium*), replica plating and negative screening were employed to identify clones in which the integrated plasmid had been excised. Once the cells had been subjected to three serial passages in the absence of selective pressure (i.e., gentamycin), 500 cfu were plated on a master plate of THB agar (no gentamycin) and incubated overnight at 42°C. Clones were replica plated on THB agar (no gentamycin), and THB agar supplemented with gentamycin (250mg/l), and incubated overnight at 42°C. Colonies present on the THB (no gentamycin) plate, but absent on the THB + gentamycin plate were assumed to have lost the plasmid (as well as the targeted loci) in a second recombination event during the non-selective serial passages. Several colonies were selected and screened using PCR analysis.

II.6 Lipid Analysis

II.6.1 Lipid Extraction

To monitor the biosynthesis of aa-PG in *E. faecium*, we first tested the wild-type strain in several different growth conditions. *E. faecium* strain TX1330 was grown in Brain Heart Infusion (BHI) broth, THB, and a minimal media adjusted to different pH values (6.4, 6.8, and 6.7 respectively). The minimal media contained 70 mM of a $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 7.3 or 5.8), 15 mM NH_4Cl , 0.4% glucose, 1 g/l of yeast extract, 1 g/l of peptone. *E. faecium* deletion strains (ΔaaPGS1 , ΔaaPGS2 , or ΔpHyd) were grown in minimal media adjusted to pH 6.7, since these growth conditions yielded a satisfactory level of lipid aminoacylation with the wild-type strain. Total lipids were extracted and analyzed using 1-dimensional thin-layer chromatography (1D TLC). The total lipids were extracted using the Bligh-Dyer method (Bligh, E.G., Dyer, W.J., 1959) with the following modifications (Roy, H., Ibba, M., 2007). The harvested cell pellets were resuspended in 0.2 ml of 120 mM sodium acetate, pH 4.5. 0.75 ml of chloroform:methanol (1:2, v:v) was added to the resuspended cells, and mixed by vortexing for 10 min at room temperature. Subsequently, 0.25 ml of chloroform and 0.25 ml of 120 mM sodium acetate, pH 4.5, were added and mixed thoroughly by vortexing. The organic phase (containing the lipids) and the aqueous phase were separated by centrifugation, and the organic phase was collected and dried. Finally, the dried lipids were resuspended in 50 μl of chloroform:methanol (2:1, v:v).

II.6.2 Thin-layer Chromatography (TLC)

5 μ l of lipid extract preparations were spotted on 10 cm, 250 micron HLF silicagel TLC plates, which were developed with chloroform:methanol:water (14:6:1, v:v:v) for 20 min in 1-dimension. For detection of phospholipids the TLC plates were first stained with primuline, followed by ninhydrin staining to detect aminoacylated phospholipids.

II.7 Growth Kinetics

Growth curves of wild-type *E. faecium* and the deletion mutants were compared in 96-well plates containing 100 μ l M9 minimal media, pH 6.7. 5 ml starter cultures were grown in THB at 37°C, the OD₆₀₀ was determined, and the cultures were washed with 100 mM NaCl to remove residual media. The cultures were then diluted in M9 minimal media (pH 6.7) and 10,000 cfu were used to inoculate each well (1 OD = 185,000 cfu/ μ l). Cell plates were incubated in a 96-well plate reader (Synergy H1 Hybrid Microplate Reader, BioTek) at 37°C for 20 h with high agitation, and the OD₆₀₀ was measured every 6 mins.

III. Chapter Three: Results

III.1 Generation of *E. faecium* Knockout Strains

Phenotypical microarray (PM) is a powerful method used to investigate gene function. This technique allows comparison of the respiration rates of wild-type and knock-out strains in an array of nearly 2000 different growth conditions to detect phenotypical changes induced by specific genetic modifications. To generate knockouts of *aaPGS1*, *aaPGS2*, and *pHyd*, we employed a markerless deletion strategy to circumvent the use of antibiotic markers that could interfere with PM analysis. We started with an established protocol for allelic exchange in the genome of *E. faecium* that uses a shuttle plasmid with a thermosensitive origin of replication (pTEX5500ts) harboring a mutated copy of the target gene (in this case a deletion). This procedure (developed by Nallapareddy et. al., 2006) involves a two-step process to first integrate the mutated allele into the genome by homologous recombination, followed by a second recombination event between duplicated regions in the chromosome to yield a recombinant strain carrying either the wild-type allele or the mutated allele, depending on the site of recombination (Figure 2A). Since the frequency of plasmid excision is low (<1/1200, Nallapareddy et. al., 2006), and selection of recombinants is more convenient than laborious screening, we first investigated the efficacy of using a counterselectable marker, *pheSA294G*, to select for recombinant strains in which plasmid had been successfully excised (Figure 2B).

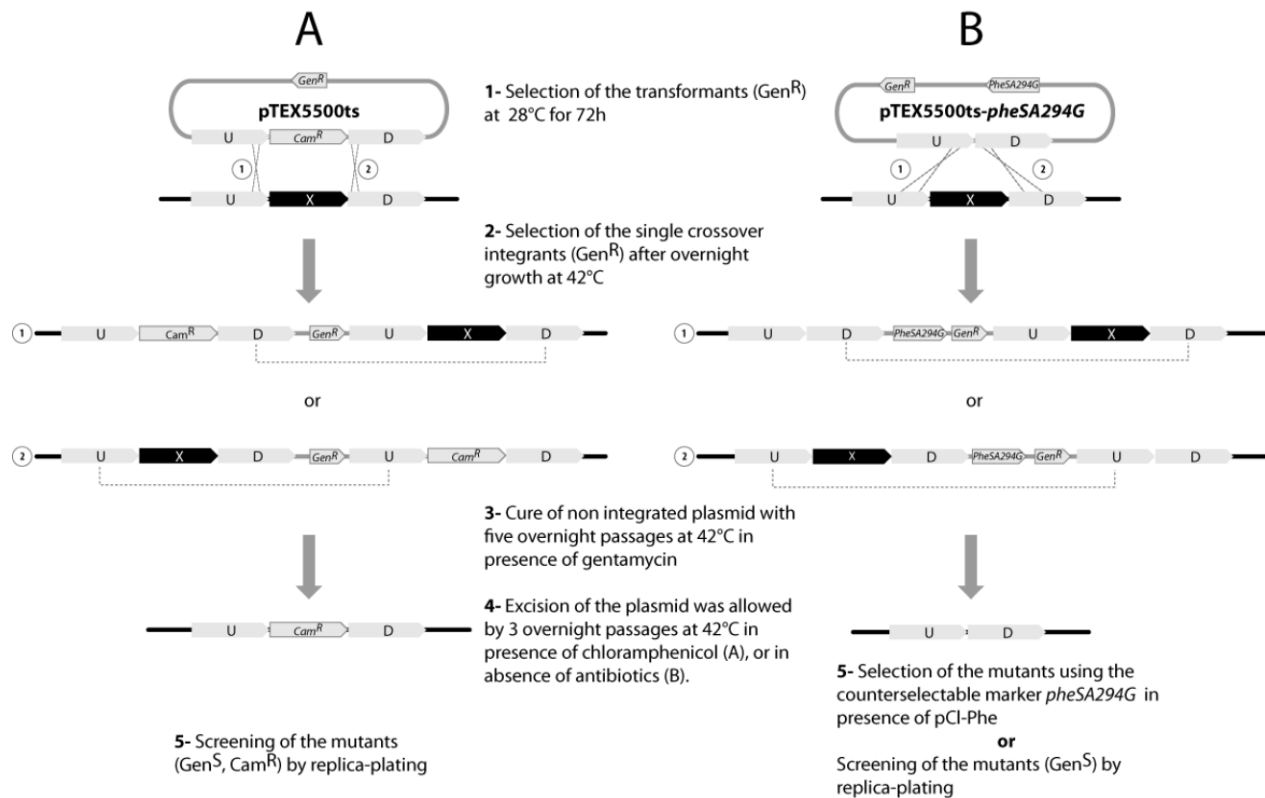


Figure 3: Allelic replacement strategies in *E. faecium*. A. Allelic replacement method as developed by Nallapareddy et. al. B. Modification of the method described in panel A yielding a markerless allelic replacement strategy. Both strategies involve the two-step integration-excision of the thermosensitive replicating plasmid pTEX5500ts (Nallapareddy et. al., 2006). Plasmid replication is maintained at permissive temperature (28°C), and is inhibited at restrictive temperature (42°C). U and D indicate the upstream and downstream regions of the target gene (X). Chromosomal and plasmid DNA is represented by black and gray lines, respectively, and recombination events are indicated by dashed lines. Gen^R , gene for gentamycin resistance; Cam^R , gene for chloramphenicol resistance. The successive steps of both methods are indicated in the figure (1-5). Gen^R , Gen^S , and Cam^R indicate the resistance (R) or sensitivity (S) phenotypes used for selection of the recombinants at the various steps of the methods.

The counterselectable marker *pheSA294G* has been successfully utilized in the context of *E. faecalis* (Kristich et. al., 2007). We introduced *pheSA294G* into the plasmid pTEX5500ts to yield the plasmid pTEX5500ts-*pheSA294G*. Specifically, the open reading frame of the chloramphenicol resistance gene of pTEX5500ts, which is under the control of the synthetic promoter *aad9* and constitutively expressed in *E. faecium* (Podbielski et. al., 1996), was replaced with that of *pheSA294G*. The *pheSA294G* gene encodes for a mutated version of the α -subunit of the *E. faecium* phenylalanyl-tRNA synthetase (PheRS), which, when reconstituted with the genome-encoded β -subunit to form a functional heterotetrameric PheRS, allows for tRNA^{Phe} misacylation with the substrate analog para-chloro-phenylalanine (*pCl*-Phe). Misacylation results in mis-incorporation of *pCl*-Phe instead of Phe during protein synthesis, which, in turn, inhibits bacterial growth. *pCl*-Phe toxicity is mainly determined by the relative abundance of PheRS and PheRS_{A294G}, and the relative availability of Phe and *pCl*-Phe in the cell. Therefore, it was critical to use a medium containing a low concentration of Phe in order to observe toxicity conferred by *pCl*-Phe incorporation. To this end we screened media supplemented with 15 mM *pCl*-Phe and containing variable amounts (from 60 to 250 mg/l; see Chapter Two: Materials and Methods) of yeast extract and peptone as sources of amino acids. Our initial investigations revealed that a medium containing 15 mM *pCl*-Phe and 125 mg/l peptone and yeast extract allowed for efficient growth of wild-type *E. faecium*, but inhibited growth of a strain electrotransformed with the plasmid pTEX5500ts-*pheSA294G*- Δ *aaPGS1* (designed to delete *aaPGS1*; Clone 1, Figure 4). Further investigations demonstrated that *pCl*-Phe toxicity

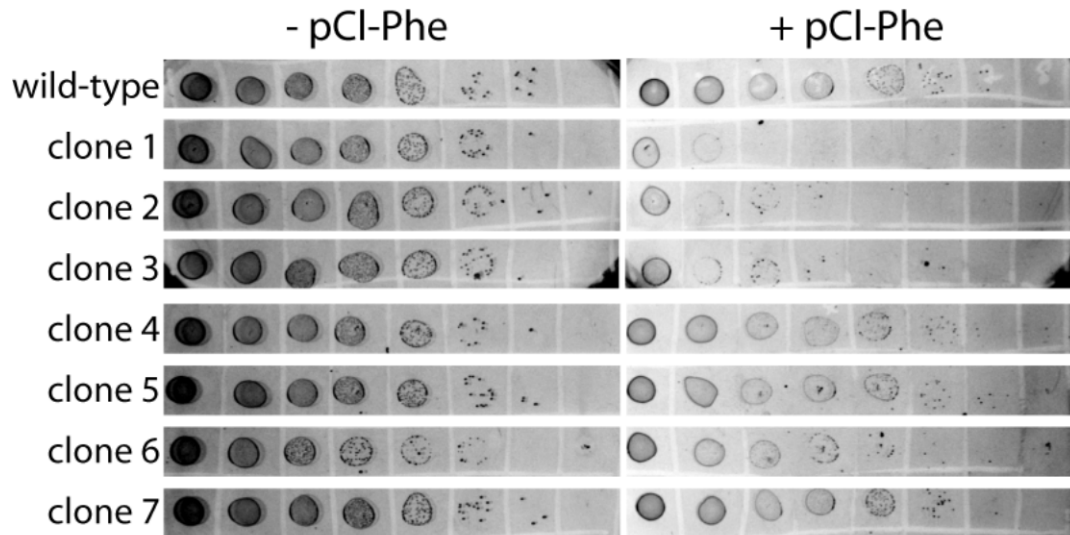


Figure 4: Growth inhibition of *E. faecium* mediated by *pCl-Phe* and the marker *PheSA294G*. *E. faecium* was transformed with the plasmid pTEX5500ts-*pheSA294G-ΔaaPGS1*. Wild-type *E. faecium* and several transformants (clones 1 – 7) were cultured in Todd-Hewitt broth during 8 h at 37°C. Cells were washed and adjusted to density $OD_{600nm} = 3$. 4 μ l of ten-fold serial dilutions were sequentially spotted (left to right) on minimal media in the absence or presence of 15 mM *pCl-Phe*. Analysis of plasmid integration by PCR showed that clone 1 has a double insertion of the plasmid in the upstream and downstream region of the *aaPGS1* locus; clones 2 and 3 exhibit integration of the plasmid in the *pheS* locus; clones 4-7 exhibit single integration of the plasmid in either the upstream or downstream region of *aaPGS1*.

varied depending on the integration state of the plasmid, making the use of *pheSA294G* impractical as a counterselectable marker in *E. faecium*. Chromosomal analysis of several integrants revealed that integration of the plasmid pTEX5500ts-*pheSA294G-ΔaaPGS1* at the *pheS* locus induced sensitivity to *pCl-Phe*. In contrast, integration of the plasmid at the *aaPGS1* locus resulted in *pCl-Phe* resistance instead of sensitivity. Upon determining that *pheSA294G* could not be used for the counterselection of recombinants that have lost the integrated plasmid, a screening strategy using replica plating was used as an alternative approach.

III.2 Replica Plating/Negative Selection

The pTEX5500ts-*pheSA294G* plasmids harboring the targeted loci with the desired mutations (*ΔaaPGS1*, *ΔaaPGS2*, or *ΔpHyd*) were electrotransformed in *E. faecium*. Transformants (~100 cfu/μg of plasmid) were selected on semi-solid media containing gentamycin and incubated at permissive temperature (i.e., 28°C, a temperature that allows for plasmid replication) for 72 h. Integration of each plasmid into the chromosome was accomplished by inoculation of a single transformant in liquid media (THB) in the presence of gentamycin, and by incubation overnight at restrictive temperature (i.e., 42°C, a temperature that inhibits plasmid replication). Individual clones were isolated, and successful integration of each plasmid at the desired loci in the chromosome was verified using PCR. Free plasmid was cured from the integrants by five serial passages of the strains in liquid THB, in the presence of gentamycin at restrictive temperature. Excision of plasmid by a second round of homologous recombination between duplicated regions in the chromosome was accomplished by three additional serial passages in THB without gentamycin. 500 cfu were plated on THB without antibiotics, and then replica plated on THB containing gentamycin. Inspection of both plates allowed for identification of clones sensitive to gentamycin, from which the plasmid had been excised from the targeted loci. The rates for the second recombination event ranged from approximately 1/100 to 1/1000. Depending on the site of recombination, gentamycin sensitive recombinants carried either the wild-type allele, or the mutated alleles (Figure 2B). Identification of recombinants carrying the mutated alleles was performed using PCR.

III.3 Lipid Analysis of Δ aaPGS1, Δ aaPGS2, and Δ pHyd Strains

The main lipid constituents of the cytoplasmic membrane of *E. faecium* are PG, phosphatidylethanolamine (PE), cardiolipin (CL), glycolipids, and aa-PGs (dos Santos Mota et. al., 1970). Previous work revealed that aaPGS2 modifies PG with Ala, Lys, and Arg (Roy, 2009). Similar attempts to reconstitute the activity of aaPGS1 using several different aa-tRNAs were unsuccessful (unpublished results). These previous studies were limited by the fact that aaPGS function was investigated *in vitro* using *E. faecium* membrane extracts expressed in *E. coli*. It was not clear whether the lack of aaPGS1 activity was due to lack of expression in *E. coli*, or whether the wrong aa-tRNAs were provided as substrates *in vitro*. Hence, there was a need to investigate the function of these genes directly in *E. faecium*.

In the current study, we first screened media such as THB, BHI, and minimal media (at a variable pH values), for use in determining the level of aa-PG synthesis in the wild-type strain (see Materials and Methods). It was previously shown that aa-PG synthesis is enhanced in response to acidic pH in multiple organisms, e.g., *Staphylococcus aureus*, (Gould & Lennarz, 1970); *Rhizobium tropici* (Vinuesa et. al., 2003); *B. subtilis* (Houtsmuller & van Deenen, 1965). Figure 5 indicates that lipid aminoacylation is lower when *E. faecium* is grown in rich media such as BHI or THB, and higher when cultured in minimal media. The pH values of these cultures were similar, ranging from 6.4 for BHI to 6.7 for the minimal media, indicating that differences in media composition outside of pH can also affect aa-PG synthesis. However, lipid aminoacylation was further enhanced when the pH of the minimal media was decreased from 6.7 to 4.1. One ninhydrin-reactive spot that was

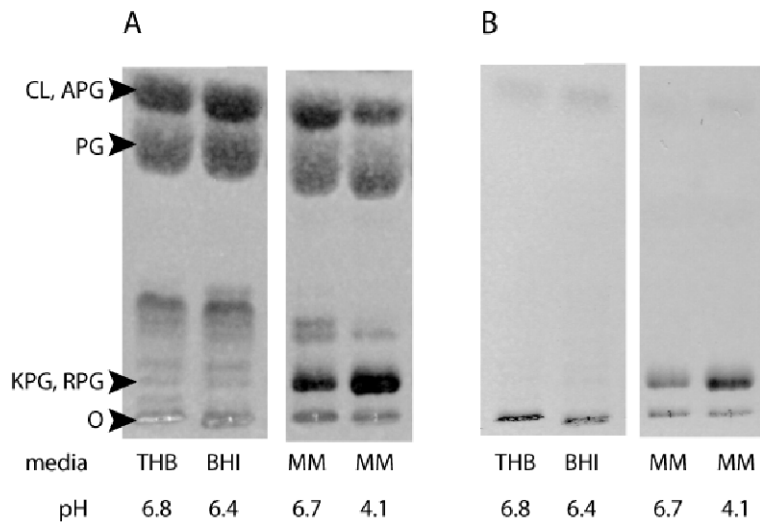


Figure 5: TLC analysis of membrane lipids from *E. faecium* cultured in various conditions. *E. faecium* was cultured overnight at 37°C in THB, BHI or minimal media (prepared at pH 6.8, pH 6.4, and pH 6.7, respectively) as described in materials and methods. The pH of each culture (indicated in figure) was measured just prior to harvesting the cells. Membrane lipids were prepared and analyzed on TLC silica gel as described in materials and methods. Total lipids were visualized by spraying the TLC plates with primuline, and amino containing lipids were visualized using ninhydrine. CL, cardiolipin; PG, phosphatidylglycerol; APG, Ala-PG; KPG, Lys-PG; RPG, Arg-PG; O, origin.

observed is consistent with that of Lys-PG and Arg-PG (Roy et. al., 2009); however, it could not be determined if the spot was due to the presence of one of these aminoacylated PGs, or both. Further analysis by 2D TLC will be required to identify the precise aa acylating the PG. It is worth mentioning that a very weak spot corresponding to that of Ala-PG was detected in samples extracted from *E. faecium* grown in either rich or minimal media (Figure 5).

Following identification of a suitable medium for enhancing lipid aminoacylation, wild-type and mutant strains were compared to determine if the mutations affected synthesis of aa-PG *in vivo*. As can be seen in figure 6, aa-PG synthesis was abolished in the Δ *aaPGS2* and Δ *pHyd* mutants, whereas no apparent change in lipid composition was

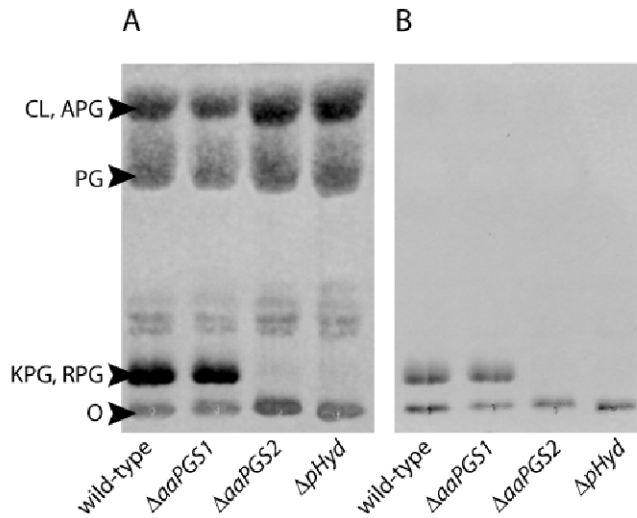


Figure 6: TLC analysis of lipids from *E. faecium* wild-type and mutant strains. Lipid extracts from *E. faecium* strains cultured in minimal media (prepared at pH 6.7) were analyzed by TLC (see Figure 5). Total lipids were visualized by spraying with primuline (A), and amino lipids were revealed with ninhydrin (B). CL, cardiolipin; PG, phosphatidylglycerol; APG, Ala-PG; KPG, Lys-PG; RPG, Arg-PG; O, origin.

observed in the $\Delta aaPGS1$ mutant. In light of the fact that aaPGS2 activity can be efficiently reconstituted in *E. coli* without expression of *pHyd*, we hypothesized that deletion of *pHyd* is not likely to directly affect aaPGS2 activity. Therefore, we considered the possibility that expression of *aaPGS2* may have been affected by the genetic manipulation we performed on *pHyd*. Re-examination of the operon revealed that there are alternative putative start codons for translation of *aaPGS2* and *pHyd*, and that deletion of *pHyd* may have removed the actual start site for *aaPGS2* expression (Figure 7).

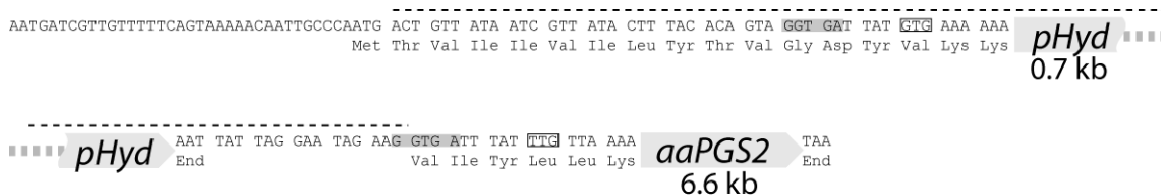


Figure 7: *pHyd-aaPGS2* operon in *E. faecium* TX1330. Amino acid sequences are those predicted by NCBI. Alternative initiator codons are boxed, and putative Shine-Dalgarno sequences are shaded. A dashed line indicates the sequence that has been deleted in the $\Delta pHyd$ construct.

Therefore, the *pHyd* mutant may, in fact, be a double knockout of both *pHyd* and *aaPGS2*. Further work is needed to verify that *aaPGS2* expression is unaffected in the $\Delta pHyd$ strain, and/or to generate a *pHyd* deletion strain in which the *aaPGS2* start site remains intact. Nevertheless, our findings, with respect to $\Delta aaPGS2$ and $\Delta aaPGS1$ are consistent with previous results (Roy, 2009) and with data recently obtained in *E. faecalis* (Bao et. al., 2012). Also, our work towards generating *E. faecium* knockout strains will facilitate construction of additional mutants that can be used to study the aaPGS pathway.

III.4 Bacterial Growth Kinetics of $\Delta aaPGS1$, $\Delta aaPGS2$, and $\Delta pHyd$ Strains

None of mutant strains ($\Delta aaPGS1$, $\Delta aaPGS2$, or $\Delta pHyd$) exhibited an altered growth rate compared to wild-type. However, only the $\Delta pHyd$ mutant, which may in fact be a double knock-out ($\Delta pHyd/\Delta aaPGS2$), exhibited a 20% increase of the maximal growth in comparison to the wild-type strain ($P < 0.05$)(Figure 8 and Table 1).

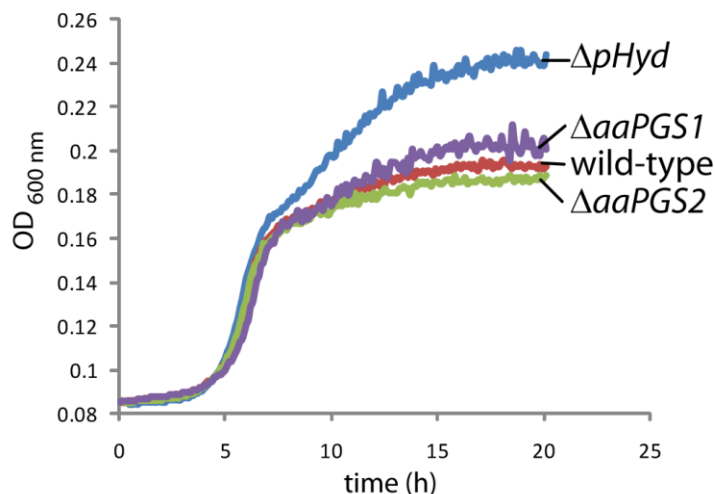


Figure 8: Growth kinetics of *E. faecium* wild-type and mutant strains. Bacterial growth was measured in a 96-well microplate as described in Materials and Methods.

Table 1: Parameters for the growth of *E. faecium* wild-type and mutant strains.

<i>E. faecium</i> Strain	Growth rate (OD/h)	Maximal growth (OD)
Wild-type	0.0431 ± 0.0033	0.1995 ± 0.0039
<i>ΔaaPGS1</i>	0.0395 ± 0.0038	0.2106 ± 0.008
<i>ΔaaPGS2</i>	0.043 ± 0.0029	0.1949 ± 0.0037
<i>ΔpHyd</i>	0.0382 ± 0.0011	0.2389 ± 0.0056*

Parameters were derived from bacterial growth curves (see Materials and Methods and Figure 8) with Grofit Package using the spline-fitting approach. Parameters are expressed as the mean ± SD (n=5). * indicates a value significantly different than that obtained with the wild-type strain (P<0.05).

IV. Chapter Four: Discussion

We modified the method previously described by Nallapareddy et. al. to construct the markerless deletions of the genes *aaPGS1*, *aaPGS2*, and *pHyd* in *E. faecium*. This strategy uses a two-step integration-excision of the shuttle plasmid pTEX5500ts, which harbors a cloned copy of the targeted gene with the desired mutation. In this strategy we evaluated the efficacy of the counterselectable marker *pheSA294G*, which exerts toxicity when bacteria are cultured in the presence of pCl-Phe. We found that *pheSA294G* cannot be used as a counterselectable marker when placed under the control of the synthetic promoter *aad9* found in pTEX5500ts. *pheSA294G* exhibited different levels of toxicity in the presence of pCl-Phe, which depended on how the plasmid was integrated in the genome of *E. faecium*. Only multi-copy insertions, or insertions of the plasmid at the wild-type *pheS* locus displayed the expected toxicity. Our results suggest that *pheSA294G* was not expressed in sufficient quantities to outcompete wild-type *pheS* expression, and that the synthetic promoter controlling expression of *pheSA294G* on the plasmid (*aad9*) may be weaker than the natural promoter of *pheS*. This problem may be fixed by placing *pheSA294G* under the control of the *E. faecium pheS* promoter.

As an alternative to the counterselection strategy using *pheSA294G*, we used negative-selection by replica plating to accomplish markerless deletion of *aaPGS1*, *aaPGS2*,

and *pHyd* from *E. faecium*. Phenotypical changes associated with these mutations were determined by investigating bacterial growth and lipid synthesis.

We first examined the influence of growing conditions on lipid aminoacylation levels in *E. faecium*. Aminoacylated lipids were present when *E. faecium* was cultured in minimal media, and were absent when cells were cultured in richer media such as THB or BHI. More work is needed to determine whether the aaPGS pathway may be triggered under starvation conditions, or if a specific compound in the minimal media, not present in BHI or THB, is responsible for triggering lipid aminoacylation. In addition, we showed that lipid aminoacylation is enhanced when the pH of the culture is lower, which is consistent with previous observations made in several other organisms such as *S. aureus* (Gould et. al., 1970) and *B. subtilis* (Houtsmuller et. al., 1965).

Mutant and wild-type strains were grown in minimal media and total lipids were extracted. Analysis of the extracts by TLC revealed that aaPGS2 aminoacylates PG *in vivo*, while the function of aaPGS1 could not be determined. Since the ninhydrin reaction spot observed from the wild-type extract is consistent with that seen when PG is acylated with either Lys or Arg, further analysis by 2D TLC will be necessary to positively identify the aa-PG.

Deletion of *pHyd* from *E. faecium* resulted in a similar loss of aa-PG as compared to wild-type; however, as mentioned above, this may have been due to the absence of *aaPGS2* expression in the $\Delta pHyd$ mutant. A clean $\Delta pHyd$ mutant will need to be obtained in order to determine the physiological relevance of the putative hydrolase.

We found that deletion of the targeted loci in *E. faecium* did not affect the growth rates of the mutant strains during exponential growth. However, $\Delta pHyd$, which may in fact be a double mutant ($\Delta pHyd-\Delta aaPGS2$), exhibited a 20% increase in maximal growth in comparison to the wild-type strain and the single deletion strain $\Delta aaPGS2$. Further analysis of a clean $\Delta pHyd$ mutant is needed to determine if the observed increase was due to the double knockout of $pHyd$ and $aaPGS2$, or if deletion of $pHyd$ alone conferred this effect.

Of particular interest is the fact that the function and phenotype associated with $aaPGS1$ remains unknown. For the next step in characterization of $aaPGS1$, $aaPGS1$ activity will be assayed using [^{14}C]aa-tRNAs and *E. faecium* $\Delta aaPGS2$ crude membrane extracts. The $\Delta aaPGS2$ strain will be useful for this assay as it will allow detection, with greater sensitivity, of $aaPGS1$ activity without it being obfuscated by activity associated with $aaPGS2$.

In conclusion, the goal of this project was to achieve markerless deletion of $aaPGS1$, $aaPGS2$, and $pHyd$ in *E. faecium*. Although substantial progress was made towards the generation of the mutant strains (with the exception of perhaps $\Delta pHyd$), there is more work to do in the future. A $\Delta pHyd$ mutant that does not interfere with the translation start site of $aaPGS2$ needs to be obtained and/or verified. In addition, all of the strains will be analyzed using phenotypic microarrays. This method will allow us to identify phenotypes (including antibiotic resistances) outside of those already investigated, and will allow us to establish relationships between $pHyd$, $aaPGS1$, $aaPGS2$. Further, these investigations may

provide intimations of the functions of *aaPGS1* and *pHyd*, two genes for which the physiological relevance is currently not known.

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