LIPIDOMIC ANALYSIS OF THE STREPTOCOCCAL CELLULAR MEMBRANE

by

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This dissertation is dedicated to my parents, Ian and Fiona, and my brother, Andrew.

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Streptococci are Gram-positive bacteria that natively colonize niches throughout the human body. While carriage of streptococci is mostly asymptomatic, they can be major human pathogens causing a variety of diseases across all age groups. Research on streptococcal mechanisms of pathogenicity, colonization, and transmission have identified virulence factors such as proteins, extracellular polysaccharides, intercellular communication pathways, and gene regulation pathways. These virulence factors have been demonstrated to aid in adherence to and invasion of human tissues and evasion of the immune system. A major component of the streptococcal cell is poorly understood: the cellular membrane. The cellular membrane is a critical site in host-pathogen interactions, providing an anchor for extracellular polysaccharides, protecting the internal workings of the bacterial cell, aiding transport of nutrients, and promoting survival in harsh conditions. The overarching goal of my dissertation work was to elucidate the biosynthetic pathway of phosphatidylcholine (PC) in *Streptococcus mitis* and *S. oralis*, members of the Mitis group streptococci, and to characterize the streptococcal cellular membrane using a culture medium that mimics the human host. In my research, I utilized lipidomics coupled with isotope tracing to discover that the glycerophosphocholine (GPC) pathway is required for PC biosynthesis

in the Mitis group streptococcal species *S. mitis*, *S. oralis*, and *S. pneumoniae*. Briefly, the GPC pathway scavenges the major human metabolites GPC and lysophosphatidylcholine (lysoPC) and acylates them to form PC. Additionally, I determined that *S. pneumoniae*, *S. pyogenes*, and *S. agalactiae* synthesize PC when cultured in the presence of human serum. I demonstrate that lysoPC is the major substrate scavenged for an abbreviated GPC pathway in *S. pyogenes* and *S. agalactiae*. Furthermore, I characterized the structure of the novel aminoacylated glycolipid lysyl-glucosyl-diacylglcyerol (Lys-Glc-DAG) in *S. agalactiae*. I experimentally confirmed that the enzyme multiple peptide resistance factor (MprF) is the biosynthetic enzyme responsible for the lysine modification of Glc-DAG, establishing a novel glycolipid substrate for the *S. agalactiae* MprF. The MprF of *S. agalactiae* also catalyzes the addition of lysine to phosphatidylglycerol, forming lysyl-phosphatidylglycerol (Lys-PG), as expected based on prior knowledge of MprF in other bacteria. Using *in vitro* assays, I show that the lysine lipids, Lys-PG and Lys-Glc-DAG, impact the cellular membrane physiology such that *S. agalactiae* lacking the two lysine lipids are unable to survive in acidic conditions, have a more net negative outer surface charge, and exhibit significantly reduced human cell adherence and invasion. Taken together, my research provides critical insight into the cellular membrane of streptococci, evidence of cellular membrane remodeling through scavenging of major human metabolites, and the characterization of a novel aminoacylated glycolipid which impacts *S. agalactiae* colonization and invasion potential.

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LIST OF ABBREVIATIONS

aaPG	Aminoacylated phosphatidylglycerol
Ala-PG	Alanyl-phosphatidylglycerol
ANI	Average nucleotide identity
Arg-PG	Arginyl-phosphatidylglycerol
C_{55}-P	Undecaprenyl phosphate
CAMPs	Cationic antimicrobial peptides
CDP-choline	Cytidine diphosphate choline
CDP-DAG	Cytidine diphosphate diacylglycerol
CL	Cardiolipin
CPS	Counts per second
CPT	1,2-DAG cholinephosphotransferase
Da	Daltons
DAG	Diacylglycerol
DHDAG	Dihexosyldiacylglycerol
DM	Streptococcal defined media
ESI/MS	Electrospray ionization mass spectrometry
GAS	Group A *Streptococcus*; *Streptococcus pyogenes*
GBS	Group B *Streptococcus; Streptococcus agalactiae*
Glc_2-DAG	Diglucosyl-diacylglycerol
Glc-DAG	Glucosyl-diacylglycerol
GPC	Glycerophosphocholine
LTA	Lipoteichoic acid
Lys-Glc-DAG	Lysyl-glucosyl-diacylglycerol
LysoPC	Lysophosphatidylcholine
Lys-PG/L-PG	Lysyl-phosphatidylglycerol
MHDAG	Monohexosyldiacylglycerol
MIC	Minimum inhibitory concentration
MprF	Multiple peptide resistance factor
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
NPLC	Normal phase liquid chromatography
PA	Phosphatidic acid
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
Pcs	Phosphatidylcholine synthase
PE	Phosphatidylethanolamine

PG	Phosphatidylglycerol
Pmt	Phospholipid *N*-methyltransferase
pPC	Plasmanyl phosphatidylcholine
RPLC	Reverse phase liquid chromatography
THB	Todd-Hewitt Broth
TIC	Total ion chromatogram
TLC	Thin layer chromatography

CHAPTER 1

INTRODUCTION

1.1 Author contributions

The contents of this chapter were written and prepared by Luke Joyce. This chapter was edited by Luke Joyce and Kelli Palmer.

1.2 Streptococci and clinical significance

Streptococci are Gram-positive bacteria of the phylum Firmicutes. Streptococci were first described in 1874 by Theodor Billroth (1, 2). In 1879, Louis Pasteur demonstrated that *Streptococcus* was the primary agent in newborn and maternal death at the time (1, 2). In 1933, Rebecca Lancefield proposed the first pseudo-taxonomical classification of streptococci by grouping the hemolytic streptococci based on the serological reactivity of the extracellular carbohydrate structures, carbohydrate C, presented by each streptococcal species (3). While refined due to technological advances in the last 80 years, the Lancefield classifications are still used today to describe some streptococci. These well-studied organisms are major constituents of the human microbiome, colonizing niches from the oral cavity and nasopharynx to the gastrointestinal and genitourinary tracts (4–9). Streptococci in the oral cavity are some of the first colonizers after birth and promote the formation of a healthy oral microbiome (4, 5). While the typical carriage of streptococci in the human body is commensal and asymptomatic, many streptococcal species can cause disease ranging from mild superficial infections to severe invasive

disease. The switch from commensal to opportunistic pathogen is not well understood and is a topic of extensive research focusing on streptococcal virulence factors and host risk factors.

The streptococci are a significant burden to healthcare systems worldwide. Based on surveillance from the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), over 1.5 million deaths are attributed to streptococcal infections each year, with a higher prevalence in underdeveloped countries (10). There are three major human pathogenic streptococci, namely *Streptococcus pneumoniae*, *Streptococcus pyogenes* (Group A *Streptococcus*; GAS), and *Streptococcus agalactiae* (Group B *Streptococcus*; GBS) which are mainly responsible for streptococcal death around the world and have a combined estimate of over 9,500 deaths per year in the United States (11). Within the United States, these streptococci result in over 4 million minor and invasive infections each year and are classified as serious or concerning threat levels (depending on the species) for antibiotic resistance by the CDC 2019 Antimicrobial Resistance Threat report (11).

The Viridans group streptococci (VGS) are a large taxonomic group of streptococci that do not fall into a Lancefield classification group as they are non-reactive to the antisera used (3, 12). The VGS natively reside throughout the human body as commensal organisms (12). Within the VGS, the Mitis group streptococci encompass *S. pneumoniae* and its close relatives *Streptococcus mitis* and *Streptococcus oralis* (12, 13). *S. pneumoniae* is a major human pathogen causing community-acquired bacterial pneumonia, bacteremia, otitis media, and sepsis (9, 11). *S. mitis* and *S. oralis*

are causative agents of opportunistic bacteremia and infective endocarditis in immunocompromised individuals (4, 12, 14–16).

With the current threat of disease, mortality, and rising antibiotic resistance within the streptococci, research has focused primarily on mechanisms of virulence, pathogenicity, and antimicrobial resistance. Despite the extensive history of research on streptococci, there is very little knowledge available about streptococcal membrane physiology and biochemistry. The cellular membrane has been documented to play critical roles in virulence and pathogenesis in a variety of bacteria such as *Legionella, Brucella,* and *Staphylococcus*, yet the role of the cellular membrane in pathogenesis of streptococci is still a novel and underexplored area (17–19).

1.3 Streptococcal taxonomy

James Sherman, in 1937, devised a taxonomic scheme for streptococci that was based on a range of tests which included Lancefield groupings, hemolytic activity, and phenotypic tests such as fermentation, bile solubility, and temperature of growth, resulting in four groups: the VGS, pyogenic streptococci, lactic acid streptococci, and a group now known as the enterococci (20). This taxonomic scheme is still largely used today with modifications of phenotypic tests and incorporation of genomic sequences to help differentiate species.

1.3.1 Mitis group streptococci

The Mitis group streptococci are one of six taxonomic groups of streptococci classified as part of the VGS (12, 13, 16). Within the Mitis group streptococci, this dissertation focuses on the species *S. mitis* and *S. oralis,* along with their close relative and major human pathogen *S. pneumoniae* (21). These three species share >99% 16S rRNA sequence identity and frequently exchange genetic material through inter-species horizontal gene transfer (21–25). *S. mitis* and *S. oralis* are native colonizers of the oral cavity, often being the initial colonizers and promoting biofilm formation by the oral microbiota (4, 5). They are also opportunistic pathogens, responsible for infective endocarditis and bacteremia in immunocompromised individuals (14–16, 26). Previous work by Adams *et al* in 2017 identified a unique membrane physiology of *S. mitis* and *S. oralis* that allows them to survive loss-of-function mutations in the enzyme CdsA, which lead to loss of synthesis of the major anionic phospholipids phosphatidylglycerol and cardiolipin (27). CdsA is required for the generation of CDP-DAG, an essential substrate in the major phospholipid biosynthesis pathways in bacteria. During the study, the observation was made that *S. mitis* and *S. oralis* possess the zwitterionic phospholipid phosphatidylcholine (PC). Before this observation, PC had not been described as a component of the streptococcal cellular membrane. This is of interest because PC is a major component of eukaryotic cell membranes and has been linked to host-microbe interactions in pathogenic bacteria (17, 18). In published research (28) described in this dissertation (Chapter 2), I characterize, for the first time in Gram-positive bacteria, the rare glycerophosphocholine (GPC) pathway as being required for PC biosynthesis in streptococci, a pathway which requires scavenging of major human metabolites.

1.3.2 *Streptococcus pneumoniae*

Streptococcus pneumoniae, commonly referred to as pneumococcus, is taxonomically assigned within the Mitis group streptococci (13). It is a long-studied bacterium, first isolated independently by both Louis Pasteur and George Sternberg, in 1881 (1, 2). Fredrick Griffith used *S. pneumoniae* in 1928 to demonstrate transformation, and later in 1944 Avery *et al* used it to show that DNA was the molecule exchanged between bacteria during transformation (29, 30). *S. pneumoniae* natively resides in the nasopharynx and upper respiratory tract of healthy individuals (8, 9). However, it is a major human pathogen. It is considered the primary etiological agent of community-acquired bacterial pneumonia and meningitis in children and the elderly, along with many invasive diseases such as bacteremia, sepsis, and otitis media in babies (9, 11). The pneumococcus is responsible for over 2 million infections each year within the United States, costing approximately $1.3 billion in medical costs; this is after effective vaccine usage began in 2000, which targets the most prevalent antibiotic-resistant serotypes (11). Studies of the pneumococcus have focused on virulence factors such as the pneumolysin and capsule polysaccharides (which are the target of pneumococcal vaccines), and the genetic variability among strains (8). Comprehensive analyses of their cellular membrane are lacking, with only a few published studies utilizing thin-layer chromatography (TLC), which lacks molecular sensitivity and specificity as it requires known standards for identification of lipids (31–34). A more comprehensive study of the membrane of *S. pneumoniae* may identify novel lipids, mechanisms of virulence and survival, and potential strategies to combat antimicrobial resistance. I discovered PC in the membrane of *S. pneumoniae* and the presence of the rare GPC pathway which was published in the Journal of Bacteriology in 2019 and presented in Chapter 2 of this dissertation. I further go on to show that *S. pneumoniae* scavenges major human

metabolites from human serum in order to synthesize PC, which is presented in Chapter 3 of this dissertation.

1.3.3 *Streptococcus pyogenes* (GAS)

The GAS are β-hemolytic bacteria that asymptomatically colonize the human nasopharynx of up to 35% of children and adults (35). GAS usually causes minor infections such as streptococcal pharyngitis, commonly known as strep throat, and impetigo (6). However, GAS can also cause invasive infections such as necrotizing fasciitis, rheumatic fever, and sepsis, resulting in up to 2.5 million infections within the United States each year (6, 11). Streptococcal superantigens are a group of fourteen different virulence factors that have been identified along with other virulence factors such as streptokinase and streptolysin O that aid the GAS in invasive infections (36). From the initial step of colonization, progression through the mucosal layers, invasion of epithelial cells and continued infection in the human host, the GAS are required to transition between unfriendly nutritional and environmental landscapes while evading the human immune system (36, 37). The cellular membrane has been shown to aid bacteria in surviving unfavorable environmental conditions, yet there has only been one study characterizing the lipids in the cellular membrane of the GAS (38). I used normal phase liquid chromatography coupled with electrospray ionization mass spectrometry (NPLC-ESI/MS) to analyze the membrane lipids of GAS, presented in Chapter 3 of this dissertation.

1.3.4 *Streptococcus agalactiae* (GBS)

The GBS are β-hemolytic bacteria that natively colonize the genitourinary tract and are a major health threat to pregnant women and neonates (7). Pregnant women are periodically screened for GBS, and approximately 30% test positive for GBS carriage (7, 39). For positive carriers, intrapartum antibiotic prophylaxis is prescribed to help prevent GBS colonization of infants during natural birth (40, 41). Approximately 2% of colonized neonates present with invasive diseases such as pneumonia, sepsis, soft skin infections, and meningitis, which can result in long lasting neurological conditions (42–45). Up to 15% of invasive diseases in babies result in death, even with prophylaxis (46). Not only are newborns affected; the mother can also develop an ascending infection into the uterus during pregnancy. Ascending infections can progress to *in utero* infections of the fetus, resulting in premature or stillbirth but rarely sepsis in the mother, at least in developed countries (47).

Similar to the streptococci described above, investigation of colonization and pathogenesis of the GBS has focused on adhesins and virulence factors, and comprehensive knowledge of the cellular membrane is lacking (48). A more complete understanding of the GBS cellular membrane could reveal novel lipids, mechanisms of virulence and survival, targets for vaccine development and treatments, or rapid clinical diagnostic tests. I characterized a plasmalogen, plasmanyl-PC, present in the GBS cellular membrane only when GBS are cultured in the presence of human serum, which is described in Chapter 3 of this dissertation. I further discovered a novel cationic glycolipid in the membrane of the GBS, identified the biosynthetic pathway for this lipid, and analyzed phenotypic

impacts of lysine-modified lipids on the GBS in context of host colonization and infection, which are presented in Chapter 4 of this dissertation.

1.4 The cellular membrane of streptococci

The cellular membrane is composed of amphiphilic lipids and serves as a barrier for cells to separate the cytosol from the exogenous environment, thereby protecting the internal workings of the cell while allowing diffusion of certain ions in and out of the cell. The membrane is also responsible for anchoring proteins and extracellular polysaccharides that are involved in a range of processes from cellular maintenance to pathogenesis (49, 50). Additionally, many extracellular polysaccharides such as wall teichoic acids are synthesized while attached to the lipid membrane before being exported and anchored in the cell wall (51). The cellular membrane is also a highly dynamic surface and is remodeled in response to extracellular conditions such as pH, nutrient levels, temperature shifts, and membrane-active antimicrobials (52–54). Despite its critical functions for the cell, the lipid membrane is poorly characterized for many human pathogens.

Generally, lipids have two fatty acid acyl chains, which can be saturated or unsaturated, that make up the hydrophobic, internal portion of the membrane. Most lipids are glycerolipids, meaning the acyl chains are linked via an ester bond to a glycerol backbone, forming the molecule diacylglycerol (DAG) (53). The head groups of glycerolipids fall into two categories based on the linkage to DAG, either a phosphate linkage (known as phospholipids), or non-phosphate-containing head groups.

To date, the cellular membrane of the streptococci has mainly been investigated using TLC, which lacks the ability to perform comprehensive lipidomics due to the lack of molecular sensitivity and specificity (31). To overcome these shortfalls in studying the lipidome in general, NPLC-ESI/MS protocols were previously established (31). NPLC separates molecules based on polarity through association with a stationary phase chromatography column and gradient mobile phases. As the molecules leave the LC column, they are ionized into a mass spectrometer to gain molecular information. The resulting data output are a total ion chromatogram indicating the retention time and relative abundance of the molecules and the mass spectrum of mass to charge ratio (*m/z)* which is used to determine the structure of the molecule (Figure 1.1).

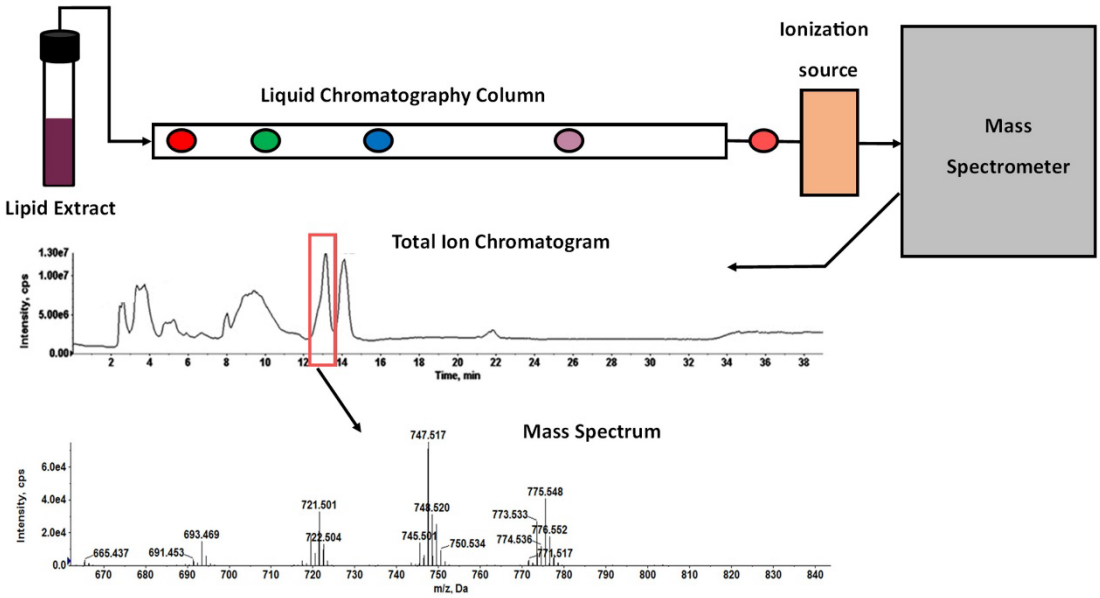

Figure 1.1. Schematic of lipidomic analysis via liquid chromatography coupled with mass spectrometry. Lipid extracts are injected onto a silica chromatography column and separated based on polarity. Lipid molecules are ionized as they leave the column, and mass spectrometry is performed. Resulting data is a total ion chromatogram (TIC) indicating the relative abundance of each molecule and the retention time on the chromatography column followed by the mass spectrum (MS) of each lipid molecule indicating the mass to charge ratio (*m/z*, Da). Daughter ion fragmentation spectra (not depicted) are further generated during mass spectrometry.

There are a handful of studies investigating the lipidomes of the major pathogenic streptococci (32–34, 38, 55, 56). These studies determined the streptococcal lipidome to be comprised of the anionic phospholipids, phosphatidylglycerol (PG) and cardiolipin (CL) (Figure 1.2), and glycolipids that contain a hexose moiety attached directly to DAG (see Figure 1.3 below). In this dissertation, the lipidomes of major pathogenic streptococci were investigated in depth using the sensitive NPLC-ESI/MS technique, leading to the discovery of several major membrane lipids that were previously unidentified in these bacteria.

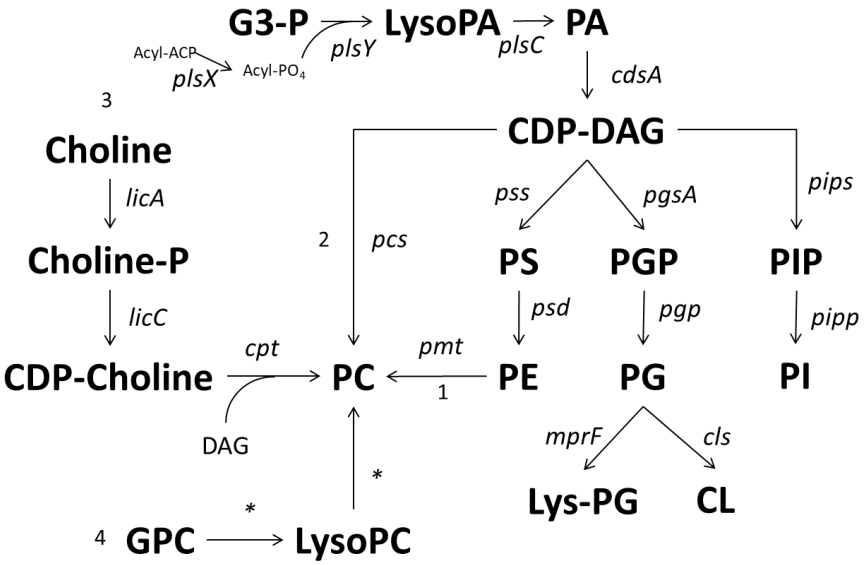

Figure 1.2. Major biosynthetic pathways of phospholipids in bacteria. Abbreviations: G3-P, glycerol-3-phosphate; LysoPA, lyso-phosphatidic acid; PA, phosphatidic acid; CDP-DAG, CDP-diacylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PGP, phosphatidylglycerol-3-P; PG, phosphatidylglycerol; CL, cardiolipin; Lys-PG, lysyl-phosphatidylglycerol; PIP, phosphatidylinositolphosphate; PI, phosphatidylinositol; PC, phosphatidylcholine; GPC, glycerophosphocholine; LysoPC, lyso-phosphatidylcholine;. Known phosphatidylcholine biosynthesis pathways: 1) Methylation pathway, 2) Phosphatidylcholine synthase pathway, 3) CDP-Choline pathway, and 4) Glycerophosphocholine pathway. * denotes unknown genes. Figure adapted from Joyce *et al* 2019 (28).

1.5 Phospholipids

The majority of Firmicutes synthesize the anionic phospholipids PG and CL (53). The phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are less widespread (53). The phospholipids phosphatidylinositol (PI) and PC, which are predominantly known as eukaryotic phospholipids, had previously not been reported within Firmicutes (53). The known biosynthetic pathways and genes for each lipid are depicted in Figure 1.2. Lysophospholipids are phospholipids with a single acyl chain attached to the glycerol backbone and are minor lipid species in the cellular membrane. The presence of these lipids can be the result of lipid turnover and/or cellular membrane remodeling, and they can be re-acylated to form the full lipid again (57). In the case of lysophosphatidic acid, it is the precursor to phosphatidic acid, a required substrate for phospholipids.

The head group of PG can be modified with an amino acid decoration to mask the negative charge of the phosphate linkage (19, 58). The amino acids typically used by bacteria are lysine, alanine, or arginine (59). Aminoacylated PG (aaPG) have been well documented within *Bacillus*, *Staphylococcus,* and *Enterococcus* species (58, 60, 61). The various aaPG lipids have been shown to aid bacterial survival in different environmental conditions such as temperature or pH changes and increase resistance to antimicrobial peptides such as human cationic antimicrobial peptides, bacteriocins, and antibiotics (19, 58, 62). The addition of the amino acid to the PG headgroup is mediated via the multiple peptide resistance factor (MprF) enzyme (19). MprF utilizes a charged tRNA as an amino acid donor and facilitates a nucleophilic attack to transfer the amino acid onto the PG head group at the C-terminus domain (52, 63). The amino acid-decorated PG is then flipped

to the outer leaflet of the membrane by the N-terminal flippase domain to mask the negative charges exposed to the exogenous environment and effect the desired phenotype (64).

1.6 Glycolipids

Glycolipids are comprised of a hexose moiety, typically glucose, galactose, or mannose, that is directly attached to DAG via a glycotransferase enzyme (65, 66). Monohexosyl-DAG containing a single hexose can be used as a substrate for synthesis of dihexosyl-DAG (Figure 1.3). Glycolipids are important anchoring lipids for polymers such as lipoteichoic acid in Gram-positive bacteria (67, 68). The hexose moiety utilized is species-dependent. *S. pneumoniae* first adds glucose followed by galactose to form galactosyl-glucosyl-DAG (Gal-Glc-DAG) (32, 69) and both GAS and GBS use only glucose, producing a diglucosyl-DAG (Glc_2-DAG) (68, 70).

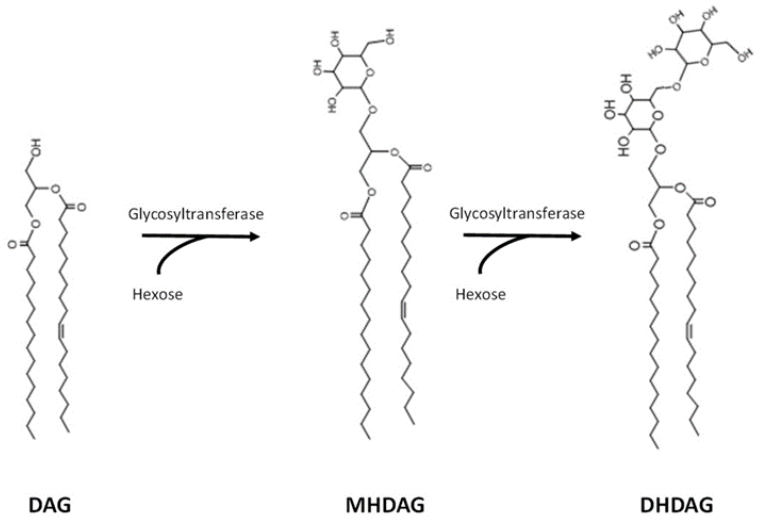

Figure 1.3. Glycolipid biosynthesis pathway. A hexose moiety is added to diacylglycerol (DAG) via a glycosyltransferase to form monohexosyl-DAG (MHDAG). A second hexose is added to form dihexosyl-DAG (DHDAG). The hexose moiety can be glucose, galactose, or mannose and is species-dependent. *S. agalactiae* synthesizes diglucosyl-DAG (Glc_2-DAG) whereas *S. pneumoniae* synthesizes galactosyl-glucosyl-DAG (Gal-Glc-DAG).

The functions of glycolipids in bacteria are poorly understood, other than to function as lipoteichoic acid anchors. Immunological studies have shown that microbial glycolipids are recognized by the host immune system (71). Particularly, the Glc-DAG of the *S. pneumoniae* and GBS is recognized by natural killer T cells in a CD1d-dependent manner (72). Similarly, the lipid anchor Glc-DAG of GAS is immunogenic and is recognized by the macrophage-inducible C-type lectin (70). One proposed function of glycolipids is to act as a lipid substitute in phosphate-limiting conditions (73), but the complete functions of glycolipids within Gram-positive bacteria are unknown. The fact that streptococcal glycolipids activate the immune system of humans is evolutionarily unfavorable and suggests that they may have a broader physiological role in the streptococcal membrane other than to act as lipid anchors for lipoteichoic acids.

1.7 Research summary

Previous work in the Palmer lab identified a unique membrane physiology within the Mitis group streptococci which allowed them to survive when exposed to daptomycin, a last line antibiotic. During that study, it was observed that the streptococci under investigation synthesized PC, which had not been reported before. I investigated the biosynthetic pathway for PC utilizing multiple techniques. Ultimately ruling out the three well known PC biosynthetic pathways, I determined that the rare GPC pathway, which relies on scavenging of major human metabolites, is used by the Mitis group streptococci (Chapter 2). This work led to a lipidomic survey of the three major pathogenic streptococci in context of the human host and the characterization of an abbreviated GPC pathway in GAS and GBS (Chapter 3). Furthermore, we observed the plasmalogen

plasmanyl-PC in the membrane of the GBS, which had not been previously identified in a facultative anaerobic microbe. Finally, a relatively large proportion of the lipid extract of the GBS was comprised of an unknown lipid molecule. Investigations with labeled isotopes revealed a novel aminoacylated glycolipid, lysyl-glucosyl-diacylglycerol (Chapter 4). The addition of the amino acid lysine onto Glc-DAG is reminiscent of lysine modification of PG via the enzyme MprF. I hypothesized that the GBS MprF was lysinylating both PG and Glc-DAG. Through genetic knockouts and heterologous host expression, I demonstrated that MprF is responsible for the observed lysinylation of both glycolipid and phospholipid substrates. The identification of an expanded substrate repertoire for the GBS MprF, and a novel amino acid-modified glycolipid structure, presents exciting avenues of research into the membrane physiology of the GBS. The work presented in this dissertation has broadened the knowledge of the biosynthetic pathways of PC and characterized the cellular membrane components of the major pathogenic streptococci in depth. Ultimately, this work has revealed new and exciting genetic, proteomic, and membrane physiology research avenues to be pursued.

1.8 References

1. Watson DA, Musher DM, Jacobson JW, Verhoef J. 1993. A brief history of the pneumococcus in biomedical research: a panoply of scientific discovery. Clin Infect Dis 17:913–24.
2. Ferretti JJ, Stevens DL, Fischetti VA. 2016. *Streptococcus pyogenes:* Basic Biology to Clinical Manifestations [Internet]. Oklahoma City (OK): Univeristy of Oklahoma Health Sciences Center.
3. Lancefield RC. 1933.A serological differentiation of human and other groups of hemolytic streptococci. J Exp Med 57:571–95.

4. Mitchell J. 2011. *Streptococcus mitis*: Walking the line between commensalism and pathogenesis. Mol Oral Microbiol 26:89–98.

5. Kolenbrander PE, London J. 1993. Adhere today, here tomorrow: Oral bacterial adherence. J Bacteriol 175:3247–3252.

6. Cunningham MW. 2000. Pathogenesis of Group A Streptococcal Infections. Clin Microbiol Rev 13:470–511.

7. Wilkinson HW. 1978. Group B Streptococcal Infection in Humans. Annu Rev Microbiol 32:41–57.

8. Mitchell AM, Mitchell TJ. 2010. *Streptococcus pneumoniae*: Virulence factors and variation. Clin Microbiol Infect 16:411–418.

9. Weiser JN, Ferreira DM, Paton JC. 2018. *Streptococcus pneumoniae*: transmission, colonization and invasion. Nat Rev Microbiol 16:355–367.

10. Carapetis JR, Steer AC, Mulholland EK, Weber M. 2005. The global burden of group A streptococcal diseases. Lancet Infect Dis 5:685–94.

11. CDC. 2019. Antibiotic resistance threats in the United States, 2019. Atlanta, GA: US Department of Health and Human Services, CDC.

12. Doern CD, Burnham CAD. 2010. It's not easy being green: The Viridans Group Streptococci, with a focus on pediatric clinical manifestations. J Clin Microbiol 48:3829–3835.

13. Facklam R. 2002. What happened to the streptococci: Overview of taxonomic and nomenclature changes. Clin Microbiol Rev 15:613–630.

14. Murdoch DR, Corey GR, Hoen B, Miró JM, Fowler VG, Bayer AS, Karchmer AW, Olaison L, Pappas PA, Moreillon P, Chambers ST, Chu VH, Falcó V, Holland DJ, Jones P, Klein JL, Raymond NJ, Read KM, Tripodi MF, Utili R, Wang A, Woods CW, Cabell CH, Investigators TI. 2009. Clinical presentation, Etiology and Outcome of Infective Endocarditis in the 21st Century: The International Collaboration on Endocarditis-Prospective Cohort Study. Arch Intern Med 169:463–473.

15. Marín M, Gudiol C, Garcia-Vidal C, Ardanuy C, Carratalà J. 2014. Bloodstream infections in patients with solid tumors: Epidemiology, antibiotic therapy, and outcomes in 528 episodes in a single cancer center. Medicine (Baltimore) 93:143–149.

16. Shelburne SA, Sahasrabhojane P, Saldana M, Yao H, Su X, Horstmann N, Thompson E, Flores AR. 2014. *Streptococcus mitis* strains causing severe clinical disease in cancer patients. Emerg Infect Dis 20:762–771.

17. Conover GM, Martinez-Morales F, Heidtman MI, Luo Z-Q, Tang M, Chen C, Geiger O, Isberg RR. 2008. Phosphatidylcholine synthesis is required for optimal function of *Legionella pneumophila* virulence determinants. Cell Microbiol 10:514–528.

18. Comerci DJ, Altabe S, De Mendoza D, Ugalde RA. 2006. *Brucella abortus* synthesizes phosphatidylcholine from choline provided by the host. J Bacteriol 188:1929–1934.

19. Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, Kalbacher H, Nieuwenhuizen WF, Jung G, Tarkowski A, Van Kessel KPM, Van Strijp JAG. 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. J Exp Med 193:1067–1076.

20. Sherman JM. 1937. The Streptococci. Bacteriol Rev 1:3–97.

21. Kilian M, Riley DR, Jensen A, Brüggemann H, Tettelin H. 2014. Parallel Evolution of *Streptococcus pneumoniae* and *Streptococcus mitis* to Pathogenic and Mutualistic Lifestyles. MBio 5:e01490-14.

22. Kawamura Y, Hou X-G, Sultana F, Miura H, Ezaki T. 1995. Determination of 16S rRNA Sequences of *Streptococcus mitis* and *Streptococcus gordonii* and Phylogenetic Relationships among Members of the Genus *Streptococcus*. Int J Syst Bacteriol 45:406–408.

23. Scholz CFP, Poulsen K, Kilian M. 2012. Novel Molecular Method for Identification of *Streptococcus pneumoniae* Applicable to Clinical Microbiology and 16S rRNA Sequence-Based Microbiome Studies. J Clin Microbiol 50:1968–1973.

24. Skov Sørensen UB, Yao K, Yang Y, Tettelin H, Kilian M. 2016. Capsular Polysaccharide Expression in Commensal *Streptococcus* Species: Genetic and Antigenic Similarities to *Streptococcus pneumoniae*. MBio 7:e01844-16.

25. Jensen A, Valdórsson O, Frimodt-Møller N, Hollingshead S, Kilian M. 2015. Commensal Streptococci Serve as a Reservoir for β-Lactam Resistance Genes in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 59:3529–3540.

26. Gudiol C, Bodro M, Simonetti A, Tubau F, González-Barca E, Cisnal M, Domingo-Domenech E, Jiménez L, Carratalà J. 2013. Changing aetiology, clinical features, antimicrobial resistance, and outcomes of bloodstream infection in neutropenic cancer patients. Clin Microbiol Infect 19:474–479.

27. Adams HM, Joyce LR, Guan Z, Akins RL, Palmer KL. 2017. Streptococcus mitis and S. oralis Lack a Requirement for CdsA, the Enzyme Required for Synthesis of Major Membrane Phospholipids in Bacteria. Antimicrob Agents Chemother 61:e02552-16.

28. Joyce LR, Guan Z, Palmer KL. 2019. Phosphatidylcholine Biosynthesis in Mitis Group Streptococci via Host Metabolite Scavenging. J Bacteriol 201:e00495-19.

29. Griffith F. 1966. The significance of pneumococcal types. J Hyg (Lond) 64:129-i4.

30. Avery OT, Macleod CM, McCarty M. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type iii. J Exp Med 79:137–158.

31. Goldfine H, Guan Z. 2015. Lipidomic Analysis of Bacteria by Thin-Layer Chromatography and Liquid Chromatography/Mass Spectrometry, p. 125–139. *In* Hydrocarbon and Lipid Microbiology Protocols.

32. Meiers M, Volz C, Eisel J, Maurer P, Henrich B, Hakenbeck R. 2014. Altered lipid composition in *Streptococcus pneumoniae cpoA* mutants. BMC Microbiol 14:1–12.

33. Trombe M-C, Lanéelle M-A, Lanéelle G. 1979. Lipid composition of aminopterin-resistant and sensitive strains of *Streptococcus pneumoniae*. Biochim Biophys Acta - Lipids Lipid Metab 574:290–300.

34. Brundish DE, Shaw N, Baddiley J. 1967. The phospholipids of Pneumococcus I-192R, A.T.C.C. 12213. Biochem J 104:205–11.

35. Kaplan EL, Top FH, Dudding BA, Wannamaker LW. 1971. Diagnosis of Streptococcal Pharyngitis: Differentiation of Active Infection from the Carrier State in the Symptomatic Child. J Infect Dis • 123:490–501.

36. Wilkening R V, Federle MJ. 2017. Evolutionary Constraints Shaping *Streptococcus pyogenes*-Host Interactions. Trends Microbiol 25:562–572.

37. Pezzulo AA, Gutiérrez J, Duschner KS, McConnell KS, Taft PJ, Ernst SE, Yahr TL, Rahmouni K, Klesney-Tait J, Stoltz DA, Zabner J. 2011. Glucose depletion in the airway surface liquid is essential for sterility of the airways. PLoS One 6:e16166.

38. Cohen M, Panos C. 1966. Membrane Lipid Composition of *Streptococcus pyogenes* and Derived L Form. Biochemistry 5:2385–2392.

39. Doran KS, Nizet V. 2004. Molecular pathogenesis of neonatal Group B Streptococcal infection: No longer in its infancy. Mol Microbiol 54:23–31.

40. Schuchat A. 1998. Epidemiology of Group B Streptococcal disease in the United States: shifting paradigms. Clin Microbiol Rev 11:497–513.

41. Ohlsson A, Shah VS. 2014. Intrapartum antibiotics for known maternal Group B Streptococcal colonization. Cochrane database Syst Rev CD007467.

42. Schuchat A. 1999. Group B *Streptococcus*. Lancet 353:51–56.

43. Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ. 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol 96:498–503.

44. Nandyal RR. 2008. Update on group B streptococcal infections: perinatal and neonatal periods. J Perinat Neonatal Nurs 22:230–7.

45. Edwards MS, Rench MA, Haffar AA, Murphy MA, Desmond MM, Baker CJ. 1985. Long-term sequelae of group B streptococcal meningitis in infants. J Pediatr 106:717–22.

46. Gaschignard J, Levy C, Romain O, Cohen R, Bingen E, Aujard Y, Boileau P. 2011. Neonatal Bacterial Meningitis: 444 Cases in 7 Years. Pediatr Infect Dis J 30:212–7.

47. Hall J, Adams NH, Bartlett L, Seale AC, Lamagni T, Bianchi-Jassir F, Lawn JE, Baker CJ, Cutland C, Heath PT, Ip M, Le Doare K, Madhi SA, Rubens CE, Saha SK, Schrag S, Sobanjo-Ter Meulen A, Vekemans J, Gravett MG. 2017. Maternal Disease With Group B *Streptococcus* and Serotype Distribution Worldwide: Systematic Review and Meta-analyses. Clin Infect Dis 65:S112–S124.

48. Armistead B, Oler E, Adams Waldorf K, Rajagopal L. 2019. The Double Life of Group B *Streptococcus*: Asymptomatic Colonizer and Potent Pathogen. J Mol Biol 431:2914–2931.

49. Rosch JW, Hsu FF, Caparon MG. 2007. Anionic lipids enriched at the ExPortal of *Streptococcus pyogenes*. J Bacteriol 189:801–806.

50. Rajendram M, Zhang L, Reynolds BJ, Auer GK, Tuson HH, Ngo K V., Cox MM, Yethiraj A, Cui Q, Weibel DB. 2015. Anionic Phospholipids Stabilize RecA Filament Bundles in *Escherichia coli*. Mol Cell 60:374–84.

51. Brown S, Santa Maria JP, Walker S. 2013. Wall teichoic acids of gram-positive bacteria. Annu Rev Microbiol 67:313–36.

52. Roy H. 2009. Tuning the properties of the bacterial membrane with aminoacylated phosphatidylglycerol. IUBMB Life 61:940–953.

53. Sohlenkamp C, Geiger O. 2016. Bacterial membrane lipids: Diversity in structures and pathways. FEMS Microbiol Rev 40:133–159.

54. Marr AG, Ingraham JL. 1962. Effect of temperature on the composition of fatty acids in *Escherichia coli*. J Bacteriol 84:1260–7.

55. Fischer W. 1977. The polar lipids of Group B Streptococci. II. Composition and positional distribution of fatty acids. Biochim Biophys Acta 487:89–104.

56. Curtis J, Kim G, Wehr NB, Levine RL. 2003. Group B Streptococcal phospholipid causes pulmonary hypertension. Proc Natl Acad Sci U S A 100:5087–90.

57. López-Lara IM, Geiger O. 2017. Bacterial lipid diversity. Biochim Biophys acta Mol cell Biol lipids 1862:1287–1299.

58. Roy H, Ibba M. 2008. RNA-dependent lipid remodeling by bacterial multiple peptide resistance factors. Proc Natl Acad Sci U S A 105:4667–4672.

59. Roy H, Ibba M. 2009. Broad range amino acid specificity of RNA-dependent lipid remodeling by multiple peptide resistance factors. J Biol Chem 284:29677–29683.

60. den Kamp JA, Redai I, van Deenen LL. 1969. Phospholipid composition of *Bacillus subtilis*. J Bacteriol 99:298–303.

61. Nahaie MR, Goodfellow M, Minnikin DE, Hájek V. 1984. Polar lipid and isoprenoid quinone composition in the classification of Staphylococcus. J Gen Microbiol 130:2427–37.

62. Dare K, Shepherd J, Roy H, Seveau S, Ibba M. 2014. LysPGS formation in Listeria monocytogenes has broad roles in maintaining membrane integrity beyond antimicrobial peptide resistance. Virulence 5:534–46.

63. Hebecker S, Krausze J, Hasenkampf T, Schneider J, Groenewold M, Reichelt J, Jahn D, Heinz DW, Moser J. 2015. Structures of two bacterial resistance factors mediating tRNA-dependent aminoacylation of phosphatidylglycerol with lysine or alanine. Proc Natl Acad Sci U S A 112:10691–10696.

64. Ernst CM, Kuhn S, Slavetinsky CJ, Krismer BB, Heilbronner S, Gekeler C, Kraus D, Wagner S, Peschel A. 2015. The Lipid-Modifying Multiple Peptide Resistance Factor Is an Oligomer Consisting of Distinct Interacting Synthase and Flippase subunits. MBio 6:1–9.

65. Jorasch P, Wolter FP, Zähringer U, Heinz E. 1998. A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose residues to 1,2-diacylglycerol: expression of ypfP in Escherichia coli and structural analysis of its reaction products. Mol Microbiol 29:419–30.

66. Jorasch P, Warnecke DC, Lindner B, Zähringer U, Heinz E. 2000. Novel processive and nonprocessive glycosyltransferases from *Staphylococcus aureus* and *Arabidopsis thaliana* synthesize glycoglycerolipids, glycophospholipids, glycosphingolipids and glycosylsterols. Eur J Biochem 267:3770–83.

67. Kiriukhin MY, Debabov D V., Shinabarger DL, Neuhaus FC. 2001. Biosynthesis of the glycolipid anchor in lipoteichoic acid of *Staphylococcus aureus RN4220*: Role of YpfP, the diglucosyldiacylglycerol synthase. J Bacteriol 183:3506–3514.

68. Doran KS, Engelson EJ, Khosravi A, Maisey HC, Fedtke I, Equils O, Michelsen KS, Arditi M, Peschel A, Nizet V. 2005. Blood-brain barrier invasion by Group B *Streptococcus* depends upon proper cell-surface anchoring of lipoteichoic acid. J Clin Invest 115:2499–2507.

69. Edman M, Berg S, Storm P, Wikström M, Vikström S, Ohman A, Wieslander A. 2003. Structural features of glycosyltransferases synthesizing major bilayer and nonbilayer-prone membrane lipids in *Acholeplasma laidlawii* and *Streptococcus pneumoniae*. J Biol Chem 278:8420–8.

70. Imai T, Matsumura T, Mayer-Lambertz S, Wells CA, Ishikawa E, Butcher SK, Barnett TC, Walker MJ, Imamura A, Ishida H, Ikebe T, Miyamoto T, Ato M, Ohga S, Lepenies B, van Sorge NM, Yamasaki S. 2018. Lipoteichoic acid anchor triggers Mincle to drive protective immunity against invasive group A *Streptococcus* infection. Proc Natl Acad Sci U S A 115:E10662–E10671.

71. Zajonc DM, Girardi E. 2015. Recognition of microbial glycolipids by Natural Killer T cells. Front Immunol 6:1–11.

72. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li X, Li Y, Imamura M, Kaneko Y, Okawara A, Miyazaki Y, Gómez-Velasco A, Rogers P, Dahesh S, Uchiyama S, Khurana A, Kawahara K, Yesilkaya H, Andrew PW, Wong C-H, Kawakami K, Nizet V, Besra GS, Tsuji M, Zajonc DM, Kronenberg M. 2011. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. Nat Immunol 12:966–74.

73. Diercks H, Semeniuk A, Gisch N, Moll H, Duda KA, Hölzl G. 2015. Accumulation of novel glycolipids and ornithine lipids in *Mesorhizobium loti* under phosphate deprivation. J Bacteriol 197:497–509.

CHAPTER 2

PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN MITIS GROUP STREPTOCOCCI VIA HOST METABOLITE SCAVENGING

2.1 Author contributions

This chapter was published as an original manuscript in collaboration with Ziqiang Guan (ZG) and Kelli Palmer (KP) in *Journal of Bacteriology* in 2019. The experiments for this work were designed by Luke Joyce (LJ), ZG, and KP. LJ performed all experiments and lipidomic analyses were performed by ZG. The manuscript was written by LJ and KP. *Journal of Bacteriology*, 201, 2019, DOI: 10.1128/JB.00495-19

2.2 Abstract

The Mitis group streptococci include the major human pathogen *Streptococcus pneumoniae* and the opportunistic pathogens *Streptococcus mitis* and *Streptococcus oralis*, which are human oral cavity colonizers and agents of bacteremia and infective endocarditis in immunocompromised patients. Bacterial membrane lipids play crucial roles in microbe-host interactions; for many pathogens, however, the composition of the membrane is poorly understood. In this study, we characterized the lipidomes of selected species of Mitis group streptococci and investigated the mechanistic basis for biosynthesis of the phospholipid phosphatidylcholine (PC). PC is a major

lipid in eukaryotic cellular membranes, but it is considered to be comparatively rare in bacterial taxa. Using liquid chromatography-mass spectrometry in conjunction with stable isotope tracing, we determined that Mitis group streptococci synthesize PC via a rare host-metabolite-scavenging pathway, the glycerophosphocholine (GPC) pathway, which is largely uncharacterized in bacteria. Our work demonstrates that Mitis group streptococci, including *S. pneumoniae*, remodel their membranes in response to the major human metabolites GPC and lysophosphatidylcholine.

2.3 Importance

We lack fundamental information about the composition of the cellular membrane even for the best-studied pathogens of critical significance for human health. The Mitis group streptococci are closely linked to humans in health and disease, but their membrane biology is poorly understood. Here, we demonstrate that these streptococci scavenge major human metabolites and use them to synthesize the membrane phospholipid PC. Our work is significant because it identifies a mechanism by which the major human pathogen *S. pneumoniae* and the primary human oral colonizers *S. mitis* and *S. oralis* remodel their membranes in response to host metabolites.

2.4 Introduction

The Mitis group streptococci are Gram-positive bacteria that natively inhabit the human oral cavity, nasopharynx, and gastrointestinal tract (1). They include the species *Streptococcus mitis* and *Streptococcus oralis*, which are among the first colonizers of the human oral cavity from birth and facilitate host-microbe-microbe interactions by creating anchors for biofilm formation with

other oral microbiota (2, 3). *S. mitis* and *S. oralis* are also opportunistic pathogens that cause bacteremia and infective endocarditis (4–7). The Mitis group streptococci also include the major human pathogen *Streptococcus pneumoniae*. *S. pneumoniae* has >99% 16S rRNA sequence identity (8–10), exchanges capsule biosynthesis and antibiotic resistance genes (11, 12), and shows antibody cross-reactivity (13) with *S. mitis* and *S. oralis*.

We, and others, recently reported that certain Mitis group streptococci have unusual membrane physiology, in that they can proliferate while lacking the major anionic phospholipids phosphatidylglycerol (PG) and cardiolipin (CL) (14, 15). More specifically, *S. mitis* and *S. oralis* can tolerate deletion or inactivation of cdsA, the gene encoding phosphatidate cytidylyltransferase (CdsA) (14, 15). CdsA catalyzes synthesis of CDP-diacylglycerol (CDP-DAG), a key intermediate in the synthesis of major phospholipids (Figure 2.1). Deletion or inactivation of *cdsA* in Mitis group streptococci and the corresponding loss of PG and CL confer high-level resistance (MICs of >256 μg/mL) to daptomycin, a last-line lipopeptide antibiotic (14–16).

Unexpectedly, lipidomic analysis of *S. mitis* and *S. oralis* by normal-phase liquid chromatography (NPLC)-electrospray ionization (ESI)-mass spectrometry (MS) revealed the presence of phosphatidylcholine (PC) in wild-type strains and in *cdsA*-null mutants (14). To our knowledge, PC in streptococci had not been described previously. Overall, the lipid compositions of streptococci are understudied and poorly characterized. Previous studies analyzing lipids of

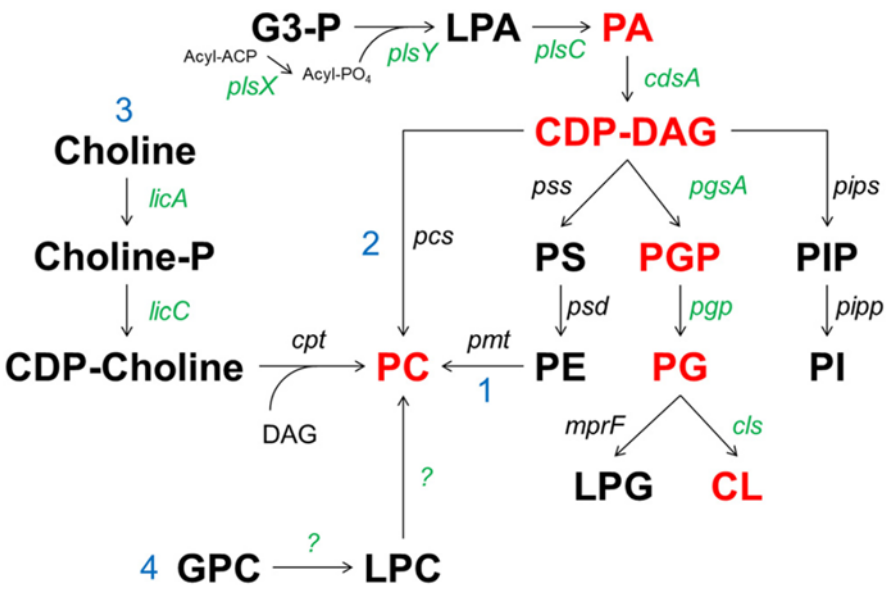

Figure 2.1. Phospholipid pathways in bacteria, including pathways for PC biosynthesis. Shown are the methylation pathway (pathway 1), the Pcs pathway (pathway 2), the CDP-choline pathway (pathway 3), and the GPC pathway (pathway 4). Lipids detected in THB-cultured SM43 cells are shown in red. Genes present in SM43 are shown in green. PA, phosphatidic acid; PS, phosphatidylserine; G3-P, glycerol-3-phosphate; PGP, PG-3-phosphate; LPG, lysyl-phosphatidylglycerol; PIP, phosphatidylinositol phosphate; PI, phosphatidylinositol; LPC, lysoPC; LPA, lysophosphatidic acid; acyl-ACP, acyl-acyl carrier protein. Other abbreviations are defined in the text.

streptococci primarily used thin-layer chromatography, whose limitations in analytical sensitivity and molecular specificity prohibit comprehensive lipidomic identification; those studies did not detect PC (17–22). PC is a biologically significant lipid. As a zwitterionic phospholipid, PC promotes bilayer formation (23), reduces the rate of protein folding to allow correct protein configurations (24, 25), aids in resistance to antimicrobials targeting prokaryotic membranes (26), aids in survival of environmental fluctuations such as temperature shifts (27), and is a major component of eukaryotic membranes. There is evidence that PC plays important roles in host-microbe interactions. *Legionella* strains lacking functional PC biosynthesis exhibit decreased

virulence because of poor recognition by host macrophages and reduced motility (28). *Brucella abortus* and *Agrobacterium tumefaciens* also exhibit diminished virulence when PC biosynthesis is inactivated (29–31). In contrast, *Pseudomonas aeruginosa* strains lacking PC show no detectable alterations in virulence (32).

Because PC may affect how Mitis group streptococci interact with the human host, in this study we investigated the mechanism for PC biosynthesis in these organisms. There are four experimentally confirmed PC biosynthesis pathways in bacteria (Figure 2.1), two of which are widespread and well characterized, namely, the phosphatidylethanolamine (PE) methylation pathway (23, 33) and the PC synthase (Pcs) pathway (34, 35). The PE methylation pathway uses PE as a starting substrate; it is methylated via phospholipid N-methyltransferase (Pmt) in three subsequent steps to form monomethylphosphatidylethanolamine, dimethylphosphatidylethanolamine, and finally PC, using S-adenosylmethionine as the methyl group donor (33). The PE methylation pathway is utilized by mammalian liver cells (23, 36) and bacteria, including *Rhodobacter sphaeroides* and *Sinorhizobium meliloti* (23). The Pcs pathway is exclusive to prokaryotes and is a one-step reaction in which a Pcs enzyme condenses choline with CDP-DAG to form PC (34). The presence of either *pmt* or *pcs* genes has been used to identify bacterial taxa likely to produce PC (23).

A third pathway, the CDP-choline pathway (referred to as the Kennedy pathway in eukaryotes), was recently identified in the Gram-negative human oral colonizer *Treponema denticola* (37). In this pathway, choline is scavenged from the environment and activated to CDP-choline via the

LicAC enzymes. Many host-associated bacteria possess LicAC and utilize host-derived choline to decorate a wide range of extracellular structures (23), including the type IV lipoteichoic acid (LTA) of *S. pneumoniae*, *S. mitis*, and *S. oralis* (38–42). In the CDP-choline pathway, CDP-choline is condensed with DAG by a 1,2-DAG cholinephosphotransferase (CPT) to form PC.

A fourth pathway, the glycerophosphocholine (GPC) pathway, has been reported for only two organisms, namely, the Gram-negative plant pathogen *Xanthomonas campestris* (43) and *Saccharomyces cerevisiae* (44). In eukaryotic cells, GPC is a breakdown product of choline-containing membrane phospholipids. Yeast can utilize GPC as the source for glycerol-3-phosphate, choline, or phosphate, depending on the environmental conditions (45). GPC is a major human metabolite present in saliva and blood (46, 47). In the GPC pathway, GPC is scavenged from the environment and acylated twice to form the intermediate lysophosphatidylcholine (lysoPC) and then PC. The genetics underlying the GPC pathway in *X. campestris* have not been fully elucidated. Moser *et al*. 2014 identified two *X. campestris* acyltransferases that performed the second acylation from lysoPC to PC (43). Yeast possess a fully elucidated GPC pathway (44, 48).

Here, we use NPLC-ESI/MS and other biochemical and genetic approaches to investigate PC biosynthesis in Mitis group streptococci, using type strains and an infective endocarditis isolate (Table 2.1). We determined that these organisms synthesize PC by the rare GPC pathway via scavenging of the host metabolites GPC and lysoPC.

2.5 Results

PC biosynthesis by model *S. mitis* and *S. oralis* strains used in this study.

We previously reported lipidomic analysis by NPLC-ESI/MS of three Mitis group infective endocarditis isolates cultured in the rich, undefined, laboratory growth medium Todd- Hewitt broth (THB). We determined that these organisms possessed PC in their membranes (14) (Table 2.1). We confirmed these results using the *S. mitis* and *S. oralis* type strains ATCC 49456 and ATCC 35037, respectively (Table 2.1).

Table 2.1. Summary of major glycolipids and phospholipids detected in Mitis group streptococci in routine laboratory media.

				Detection of[b]:								
Streptococcal species	Strain	Source	Growth medium[a]	DAG	MHDAG	DHDAG	PA	PG	CL	C_{55}-P	PC	L-PG
S. mitis	ATCC 49456	Type strain	THB	+	+	+	+	+	+	+	+	−
S. oralis	ATCC 35037	Type strain	THB	+	+	+	+	+	+	+	+	−
	1647[c]	Endocarditis	THB	+	+	+	+	+	+	+	+	−
	1648[c]	Endocarditis	THB	+	+	+	+	+	+	+	+	−
S. pneumoniae	D39	Historical strain	THB+Y	+	+	+	+	+	+	+	+	−
	TIGR4	Bacteremia	THB+Y	+	+	+	+	+	+	+	+	−
Mitis group	1643 (SM43)[c]	Endocarditis	THB	+	+	+	+	+	+	+	+	−
	SM43Δ*cdsA*	This study	THB	+	+	+	+	−	−	+	+	−
	SM43Δ*pgsA*	This study	THB	+	+	+	+	−	−	+	+	−

[a]THB+Y, THB supplemented with 0.5% yeast extract.
[b]MHDAG, monohexosyldiacylglycerol; DHDAG, dihexosyldiacylglycerol; PA, phosphatidic acid; C_{55}-P, undecaprenyl phosphate; L-PG, lysylphosphatidylglycerol; +, detected; -, not detected.
[c]Lipid profiles were previously reported by Adams *et al*. 2017 (14).

In this study, we used one of the infective endocarditis isolates, referred to as SM43, for most of our mechanistic studies of PC biosynthesis. Phylogenetic assignments within the Mitis group are difficult, due to variable phenotypes and highly conserved 16S rRNA sequences (8, 9). SM43 was initially assigned to the *S. mitis* species using standard biochemical techniques (49). We analyzed

a complete SM43 genome sequence using an average nucleotide identity (ANI) calculator (50, 51). ANI values are used for molecular species definitions (52). Two bacterial strains with ANI of >95% in their shared genes are considered to be the same species, and those with ANI of >70% are considered to be the same genus (53, 54). SM43 possesses 94.3% ANI with *S. oralis* ATCC 35037 and 86.9% ANI with *S. mitis* ATCC 49456. Based on these data showing a close phylogenetic relationship between SM43 and *S. oralis*, we refer to SM43 as a Mitis group *Streptococcus* strain in this study (Table 2.1).

PC biosynthesis in SM43 is not via the PE methylation or Pcs pathway.

The well-characterized PE methylation pathway for PC biosynthesis is catalyzed by the enzyme Pmt (Figure 2.1). This pathway is excluded because SM43 does not synthesize PE (14) and does not possess *pmt*.

The Pcs pathway requires CDP-DAG and choline as substrates (Figure 2.1). Loss-of-function *cdsA* mutants arising spontaneously among Mitis group streptococci under daptomycin selection do not synthesize CDP-DAG but still synthesize PC (14). These results indicate that PC biosynthesis in Mitis group streptococci is via a CDP-DAG-independent pathway. To confirm these results, we generated a *cdsA* deletion in SM43. PC was detected in both wild-type and Δ*cdsA* SM43 strains cultured in THB. Figure 2.2A shows the relative abundance of PC in the SM43 membrane in mid-exponential phase. The ESI/MS spectra of the major PC species, including PC (30:1) at *m/z* 704, PC (32:1) at *m/z* 732, PC (34:1) at *m/z* 760, and PC (36:2) at *m/z* 786, are shown for SM43 (Figure 2.2B) and *S. mitis* type strain ATCC 49456 (Figure A.1A in Appendix A). The chemical structure

of PC (34:1) shown in Figure 2.2C was supported by tandem mass spectrometry (MS/MS) (Figure A.1B in Appendix A). The ESI/MS spectrum of PC in SM43Δ*cdsA* is shown in Figure 2.2D. Based on these results and the absence of a pcs ortholog in the SM43 and ATCC 49456 genomes (Figure 2.1), the Pcs pathway is excluded.

SM43 expresses a partial CDP-choline pathway.

The CDP-choline pathway requires activation of choline to CDP-choline by the LicA and LicC enzymes. This is followed by condensation of CDP-choline with DAG by a 1,2-DAG CPT enzyme to form PC (Figure 2.1). *S. mitis* and *S. oralis* express *licABC* for the activation of exogenous choline to CDP-choline, which is required for choline decoration of type IV LTA in these organisms and *S. pneumoniae* (38–42). Given that SM43 expresses *licABC*, we assessed the possibility that the CDP-choline pathway is used for PC biosynthesis in this organism.

The CPT of *T. denticola* possesses a CDP-alcohol phosphatidyltransferase domain (NCBI superfamily accession no. cl00453) (37). Only one SM43 predicted protein, phosphatidylglycerophosphate synthase (PgsA), possesses this domain. PgsA catalyzes the addition of glycerol phosphate to CDP-DAG to form phosphatidylglycerophosphate (55), which is required for subsequent PG and CL synthesis (Figure 2.1).

To investigate whether PgsA has CPT activity in SM43, a 284-bp region of *pgsA* encoding the catalytic domain was replaced with an erythromycin resistance cassette. The *pgsA* mutant has a significant growth defect, with a doubling time almost twice that of the wild-type strain (Figure A.2A in Appendix A). The defect is likely due to PgsA interaction with RodZ in membrane

homeostasis, as has been reported for *Bacillus subtilis* (56). Similar to SM43Δ*cdsA*, PC was present in SM43Δ*pgsA* cultured in THB (Figure 2.2E). As expected, the SM43Δ*pgsA* mutant lacked PG and CL (Table 2.1) and had high-level daptomycin resistance (MIC of >256 µg/mL), in agreement with experiments by Tran *et al*. 2019 utilizing *S. mitis/S. oralis* strains (57). We conclude that the sole candidate for CPT activity in SM43, PgsA, does not catalyze PC biosynthesis.

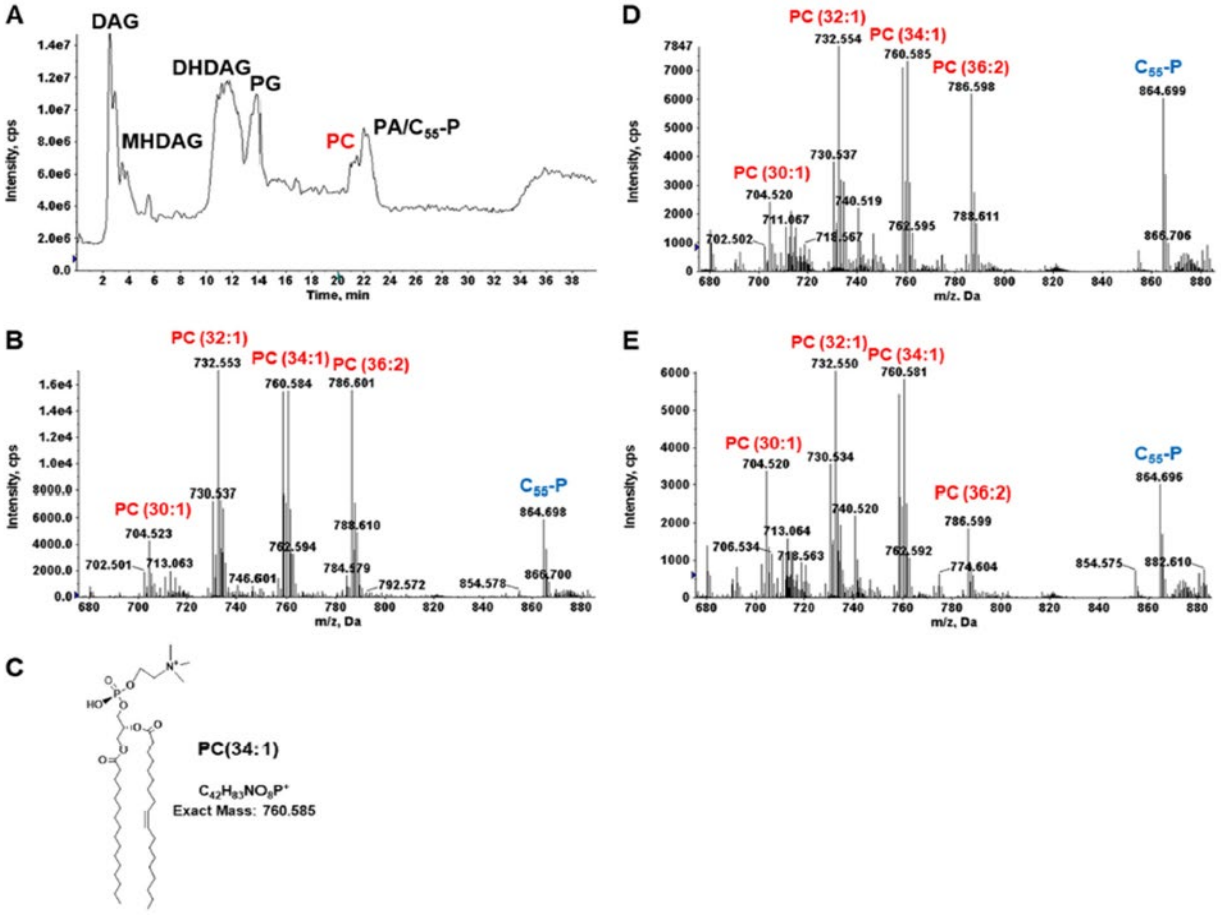

Figure 2.2. PC species detected in SM43, SM43Δ*cdsA*, and SM43Δ*pgsA*. (A) Positive-mode TIC of SM43 lipids. (B) ESI/MS of PC species in SM43. (C) Chemical structure of PC (34:1). (D) ESI/MS of PC species in SM43Δ*cdsA*. (E) ESI/MS of PC species in SM43Δ*pgsA*. The mass spectra shown were averaged from spectra acquired by NPLC-ESI/MS during the window of 20 to 21 min. PC species were detected by positive-ion ESI/MS as the M^+ ions. MHDAG, monohexosyldiacylglycerol; DHDAG, dihexosyldiacylglycerol; C_{55}-P, undecaprenyl phosphate.

To conclusively exclude the CDP-choline pathway, we performed stable isotope labeling experiments. SM43 was cultured in THB supplemented with 2 mM deuterated choline (choline-*d9*), in which all nine hydrogen atoms (1 Da) on the three methyl groups are replaced with deuterium atoms (2 Da), thereby increasing the mass of choline by 9 Da. If SM43 utilizes the CDP-choline pathway for PC biosynthesis, then a *m/z* shift of 9 Da would be observed for CDP-choline and PC species in choline-*d9* supplemented SM43 cultures. We observed choline-*d9* incorporation into CDP-choline via a shift of its M^+ ion from *m/z* 489 (Figure 2.3A, no choline-*d9* supplementation) to *m/z* 498 (Figure 2.3B, with choline-*d9* supplementation). The identification of CDP-choline and CDP-choline-*d9* was confirmed by MS/MS (Figure A.3 in Appendix A). In contrast, no *m/z* shift was observed for PC species between control (Figure 2.3C) and choline-*d9*-supplemented (Figure 2.3D) cultures. These data definitively eliminate the CDP-choline pathway as the SM43 PC biosynthesis pathway.

SM43 utilizes the GPC-scavenging pathway for PC biosynthesis.

GPC is a major human metabolite that is present in blood and saliva at concentrations of up to 40 μM and 10 μM, respectively (46). Using reverse-phase liquid chromatography (RPLC)-MS, we detected GPC (*m/z* 258.1) in THB (Figure A.4 in Appendix A), likely originating from the heart infusion component of the medium. Therefore, GPC is available for scavenging in the medium used for routine SM43 cultures.

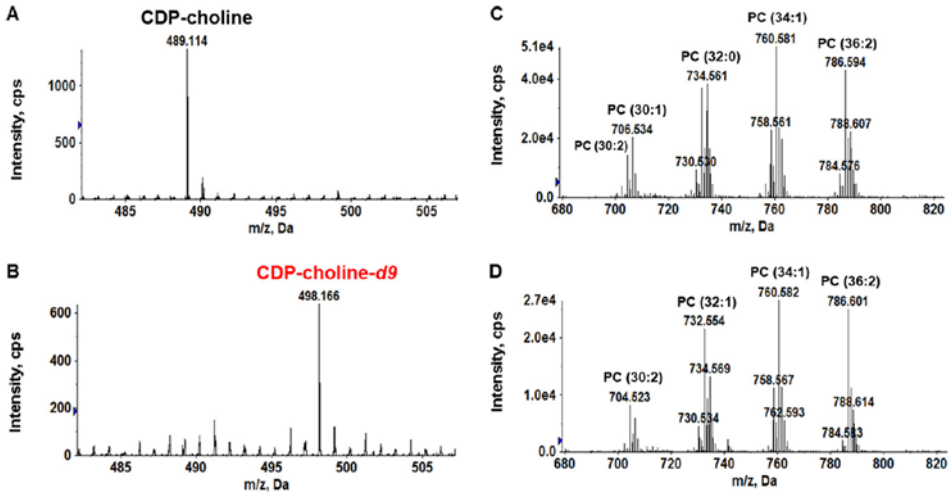

Figure 2.3. Exogenous deuterated choline (choline-*d*9) is used by SM43 to synthesize CDP-choline but not PC. CDP-choline and PC were detected in the soluble-metabolite extract and the lipid extract, respectively. Soluble metabolites were analyzed by RPLC-ESI/MS in positive-ion mode. Lipids were analyzed by NPLC-ESI/MS in positive-ion mode. (A) CDP-choline (M^+ ion at *m/z* 489.1) present in SM43 cultured in THB. (B) CDP-choline-*d*9 (M^+ ion at *m/z* 498.1) present in SM43 cultured in THB supplemented with 2 mM choline-*d*9. Note the expected mass shift (9 Da) between CDP-choline and CDP-choline-*d*9. (C) PC species detected in SM43 cultured in THB. (D) PC species detected in SM43 cultured in THB supplemented with choline-*d*9. No corresponding mass shift (9 Da) was detected in the PC species, excluding the possibility of SM43 using the CDP-choline pathway for PC synthesis.

To determine whether SM43 utilizes the GPC pathway for PC biosynthesis, we used stable-isotope-labeled GPC to trace the conversion of GPC into PC. SM43 was cultured in THB with and without 0.13 mM GPC-*d9* supplementation. A *m/z* shift of 9 Da was observed for all PC species (Figure 2.4A), demonstrating that SM43 uses the GPC pathway for PC biosynthesis. To confirm this result, SM43 and *S. mitis* ATCC 49456 were cultured in a chemically defined medium containing 0.5 mM choline (58, 59), with or without 0.13 mM GPC supplementation. SM43 and ATCC 49456 synthesized PC only when GPC was present in the defined medium (DM) (Figure 2.5 and Table 2.2). In summary, GPC-*d9* isotope tracking and DM experiments independently

confirmed that SM43 utilizes the GPC pathway for PC biosynthesis. Moreover, these results are not strain specific, as the *S. mitis* type strain also synthesized PC only when GPC was present in the growth environment.

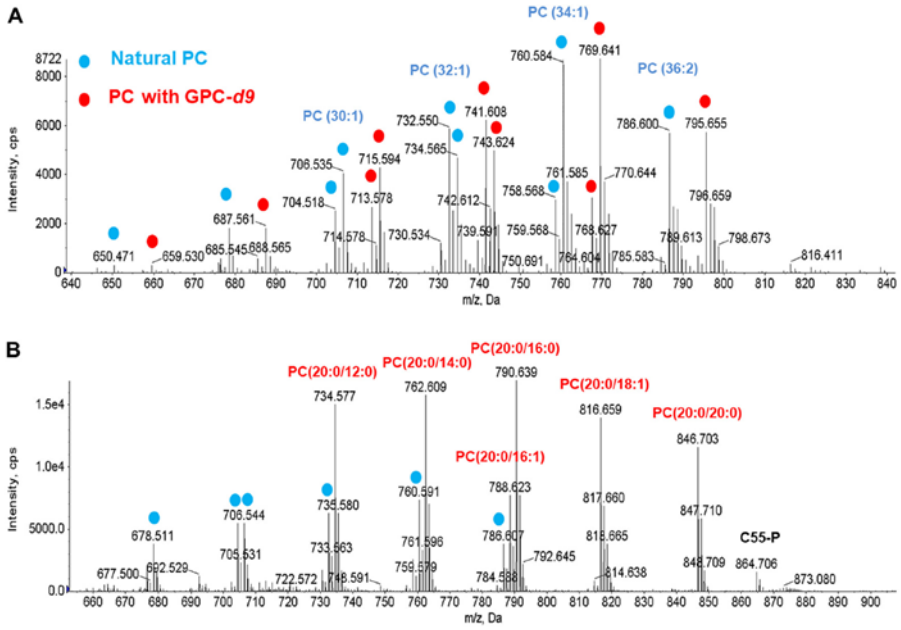

Figure 2.4. PC species in SM43 cultured in THB supplemented with GPC-*d*9 and lysoPC (20:0). (A) ESI/MS detection of PC species in SM43 cultured with GPC-*d*9. Blue dots indicate PC species normally detected in SM43 grown in THB, and red dots indicate GPC-*d*9-originating PC species. (B) ESI/MS detection of PC species in SM43 cultured in the presence of lysoPC (20:0). Blue dots indicate PC species normally detected in SM43 grown in THB, and red indicates lysoPC (20:0)-originating species. Incorporation of GPC-*d*9 and lysoPC (20:0) into PC indicates that the GPC pathway is utilized by SM43 for PC biosynthesis. C_{55}-P, undecaprenyl phosphate.

PC is not an essential component in the lipid membrane for SM43 and ATCC 49456, as evidenced by their abilities to grow in DM lacking GPC. To assess the impact of PC on growth dynamics, SM43 and ATCC 49456 were cultured in THB and in DM with or without GPC supplementation. GPC presence or absence had no impact on growth (Figure A.2B and A.2C in Appendix A).

Since lysoPC is an intermediate in the GPC pathway, we hypothesized that SM43 could also scavenge lysoPC from its environment. LysoPC is present in human blood at up to 200 µM (60). We supplemented THB with lysoPC (20:0), which has an acyl chain that is not usually observed in bacterial membranes. SM43 readily scavenged exogenous lysoPC (20:0) and acylated it to form PC (Figure 2.4B). The PG species in the SM43 membrane remained unchanged in the presence of lysoPC (20:0), indicating low transacylation activity (Figure A.5 in Appendix A). We conclude that SM43 scavenges both GPC and lysoPC from the environment to synthesize PC (Figure 2.6).

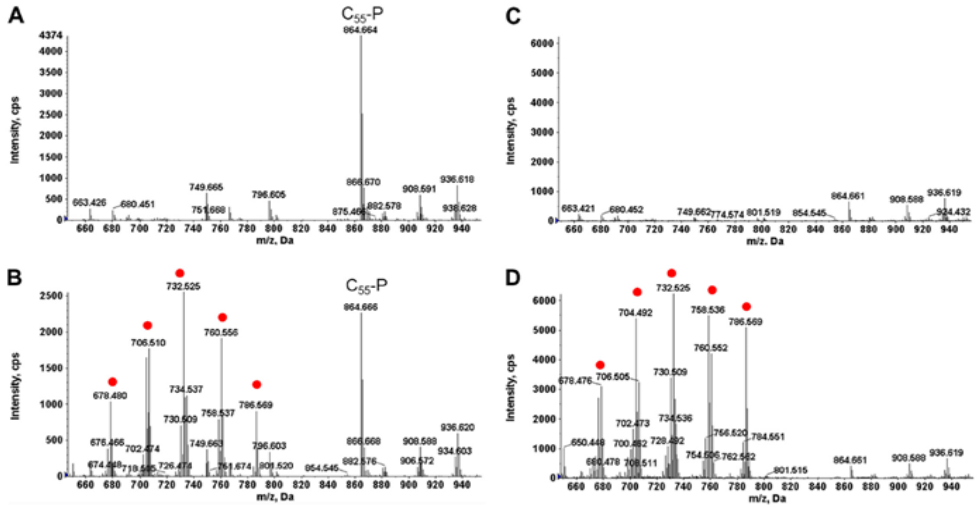

Figure 2.5. PC species in SM43 and *S. mitis* ATCC 49456 cultured in DM with or without GPC. PC species (red dots) detected in SM43 (A) and ATCC 49456 (C) cultured in DM and in SM43 (B) and ATCC 49456 (D) cultured in DM supplemented with GPC are shown. PC was detected only when GPC was present in the culture medium. C_{55}-P, undecaprenyl phosphate.

GPC-dependent PC synthesis by *S. pneumoniae* and *S. oralis*.

S. pneumoniae is a major human pathogen and a close relative of *S. mitis*. Surprisingly, there are only a few reports on lipid analysis of *S. pneumoniae* (17–19), for which thin-layer chromatography was used as the analytical technique. We applied liquid chromatography (LC)-ESI/MS, which has much higher sensitivity and specificity, to investigate the lipidome of *S.*

pneumoniae. PC was present in the membranes of *S. pneumoniae* D39 (Figure 2.7A and 2.7B) and TIGR4 (Figure 2.7C and 2.7D) cultured in THB supplemented with yeast extract (Table 2.1). Figure 2.7A and 2.7C show the positive-ion total ion chromatogram (TIC) and the relative abundance of PC in D39 and TIGR4, respectively, in early stationary phase. The ESI/MS spectra of the major PC species, including PC (30:1) at *m/z* 704, PC (32:1) at *m/z* 732, PC (34:2) at *m/z* 758, and PC (36:2) at *m/z* 786, are shown for D39 (Figure 2.7B) and TIGR4 (Figure 2.7D). We identified the presence of PC in infective endocarditis *S. oralis* isolates 1647 and 1648 in a previous study (14).

Table 2.2. Summary of major glycolipids and phospholipids detected in Mitis group streptococci cultured in DM with or without GPC.

				Detection of[a]:								
Streptococcal species	Strain	Source	Growth medium	DAG	MHDAG	DHDAG	PA	PG	CL	C_{55}-P	PC	L-PG
S. mitis	ATCC 49456	Type strain	DM	+	+	+	+	+	+	+	−	−
			DM + GPC	+	+	+	+	+	+	+	+	−
S. oralis	ATCC 35037	Type strain	DM	+	+	+	+	+	+	+	−	−
			DM + GPC	+	+	+	+	+	+	+	+	−
S. pneumoniae	D39	Historical strain	DM	+	+	+	+	+	+	+	−	−
			DM + GPC	+	+	+	+	+	+	+	+	−
Mitis group	1643 (SM43)	Endocarditis	DM	+	+	+	+	+	+	+	−	−
			DM + GPC	+	+	+	+	+	+	+	+	−

[a]MHDAG, monohexosyldiacylglycerol; DHDAG, dihexosyldiacylglycerol; PA, phosphatidic acid; C_{55}-P, undecaprenyl phosphate; L-PG, lysyl-phosphatidylglycerol; +, detected; -, not detected.

To determine whether *S. pneumoniae* and *S. oralis* utilize the GPC pathway for PC biosynthesis, *S. pneumoniae* D39 and *S. oralis* ATCC 35037 were cultured in DM with or without GPC supplementation. When GPC was not available, *S. pneumoniae* and *S. oralis* did not synthesize PC (Figure 2.8A and 2.8C); PC was present only when GPC was available in the medium (Figure 2.8B and 2.8D). We conclude that *S. pneumoniae* and *S. oralis* also scavenge GPC to synthesize PC.

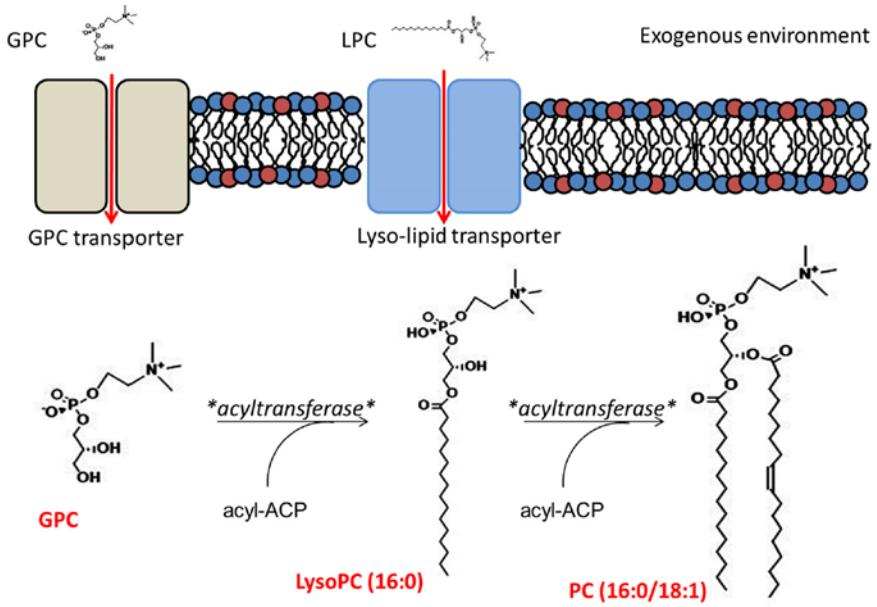

Figure 2.6. Proposed model for the GPC pathway in Mitis group streptococci. Exogenous GPC and lysoPC (LPC) are transported into the cell via unidentified transporters. GPC is sequentially acylated to form lysoPC and PC. Acyl chain lengths vary in the lipid membrane; representative chain lengths are shown.

2.6 Discussion

The lipid membrane is a dynamic site of interaction between microbial pathogens and their hosts. For many pathogens, however, the composition of the membrane is poorly understood. In this study, we characterized the lipidomes of selected species of Mitis group streptococci and investigated the mechanistic basis for biosynthesis of the phospholipid PC. We found that Mitis group streptococci remodel their membrane lipid compositions in response to the host metabolites GPC and lysoPC. To our knowledge, this is the first description of PC in *S. pneumoniae*, a major human pathogen that has been studied for over a century but whose membrane lipid composition remains poorly understood. There have been very few lipidomic studies performed in *S. pneumoniae* (17–19), and little is known about how *S. pneumoniae* remodels its membrane in

response to changing environments inside and outside the host. Here, we reported the first identification of PC in *S. pneumoniae* and demonstrated that *S. pneumoniae* synthesizes PC only when GPC, a major human metabolite, is available for scavenging.

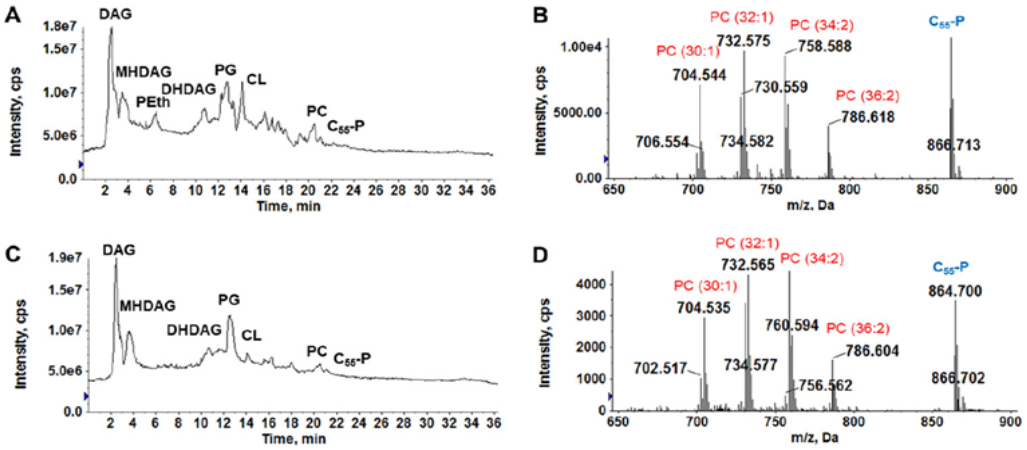

Figure 2.7. Positive-ion TIC and PC mass spectra for *S. pneumoniae* D39 and TIGR4 strains in early stationary phase. (A) TIC of *S. pneumoniae* D39 lipids. (B) ESI/MS of major PC species in *S. pneumoniae* D39. (C) TIC of *S. pneumoniae* TIGR4 lipids. (D) ESI/MS of major PC species in *S. pneumoniae* TIGR4. The mass spectra shown were averaged from spectra acquired by NPLC-ESI/MS during the window of 19.5 to 21 min. PC species were detected by positive-ion ESI/MS as the M^+ ions, while the coeluting undecaprenyl phosphate (C_{55}-P) was detected as the $[M+NH4]^+$ ion (*m/z* 864.7). MHDAG, monohexosyldiacylglycerol; DHDAG, dihexosyldiacylglycerol; PEth, phosphatidylethanol.

Very little is known about the GPC pathway in bacteria; however, a complete GPC pathway has been characterized in yeast (44, 48). The pathway includes a dual substrate transporter, Git1, for the uptake of glycerophosphoinositol and GPC, a GPC acyltransferase referred to as Gpc1, and the acyltransferase Ale1, performing lysoPC acylation. To fully elucidate the GPC pathway in streptococci, identification of transporters for GPC and lysoPC, as well as the acyltransferases, is required. However, no orthologs of the yeast GPC pathway components were identified in the SM43 genome. There are two acyltransferases encoded in the *S. mitis* genome, PlsY and PlsC,

which are responsible for phosphatidic acid biosynthesis in other organisms and may play a role in the acylation of either GPC or lysoPC. In *Mycobacterium tuberculosis*, a sugar-binding ABC transporter, UgpABCE, transports GPC (61). The UgpB substrate-binding domain is flexible in substrate affinity, binding to phosphate-containing substrates such as *sn*-glycerol 3-phopshate, glycerol 2-phosphate, and GPC (61–63). Given the reduced size of streptococcal genomes, it is possible that GPC uptake is also performed by a transport system with flexible substrate specificity.

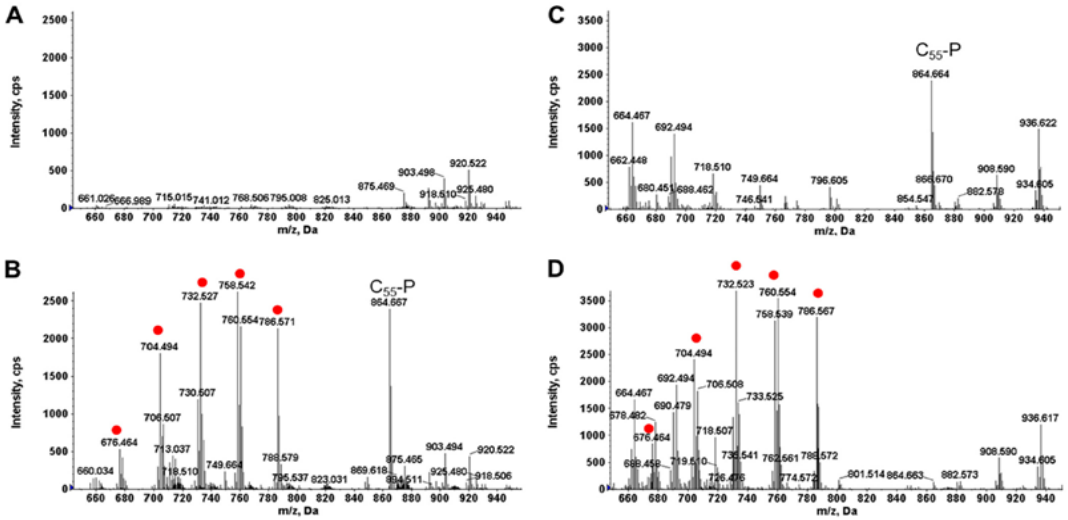

Figure 2.8. GPC pathway confirmation in *S. pneumoniae* D39 and *S. oralis* ATCC 35037. PC species (red dots) detected in D39 (A) and ATCC 35037 (C) cultured in DM and in D39 (B) and ATCC 35037 (D) cultured in DM supplemented with GPC are shown. PC was detected only when GPC was present in the culture medium. C_{55}-P, undecaprenyl phosphate

Is PC biosynthesis important for Mitis group streptococcal virulence? We expect that PC levels in the membrane would affect membrane charge, for example, which could in turn affect biofilm formation and interactions with the host immune system. More- over, GPC and lysoPC levels vary at different sites within the human body and in health and disease (<500 µM and <200 µM, respectively [46, 60]), which could affect the relative ratios of the zwitterionic PC to the anionic

phospholipids PG and CL in Mitis group streptococci colonizing these sites. Due to our limited understanding of the genes underlying the GPC pathway, at present our only method to control this pathway is by altering the in vitro growth medium. For this reason, *in vivo* studies are not currently feasible. However, by culturing Mitis group streptococci in DM with or without GPC, in future studies we can assess the biophysical impact of PC on streptococcal membranes in terms of lipid/microdomain organization, charge, rigidity, and protein composition, which would be informative from a basic science perspective.

Overall, our work highlights the importance of utilizing laboratory culture media that mimic the in vivo nutritional environments in which pathogens are found. Our identification of PC in the membranes of Mitis group streptococci, including the major human pathogen *S. pneumoniae*, and their utilization of the rare GPC pathway justify further investigation into streptococcal membrane biology, about which little is known.

2.7 Materials and Methods

Bacterial strains, media, and growth conditions.

The strains and plasmids used in this study are shown in Table A.1 of Appendix A. Streptococcal strains were grown in THB at 37°C in 5% CO_2 unless otherwise stated. *S. pneumoniae* THB cultures were supplemented with 0.5% yeast extract. Streptococcal chemically defined medium (58) was diluted from stock as described (59) and supplemented with 0.5 mM choline (referred to as DM), slightly modified from reference (64), unless otherwise stated. Media were supplemented with 130 µM GPC (Sigma-Aldrich) where stated. Erythromycin was used at 20 µg/mL for SM43.

Daptomycin susceptibilities were assessed using daptomycin Etest strips (bioMérieux) on Mueller-Hinton agar plates, according to CLSI standards (65).

Genome sequencing and assembly.

Genomic DNA was isolated using the Qiagen DNeasy blood and tissue kit according to the manufacturer's protocol, with the exception that cells were pretreated with 180 µL of 50 mg/mL lysozyme, 25 µL of 2,500 U/mL mutanolysin, and 15 µL of 20 mg/mL preboiled RNase A and incubated for 2 h at 37°C. Pacific Biosciences single-molecule real-time (SMRT) sequencing was performed by the Johns Hopkins Genome Core. The SM43 whole genome was assembled using the Unicyler assembly pipeline (66), combining SMRT long reads generated in this study and Illumina reads we had previously generated for SM43 (GenBank accession no. PRJNA354070) (14). Sequencing of SM43Δ*pgsA* was performed by Molecular Research DNA (Shallowater, TX), using Illumina HiSeq paired- end reads (2 by 150 bp).

Gene deletions in SM43.

Primers used in this study are shown in Table A.2 of Appendix A. The SM43 cdsA deletion construct was designed essentially as described previously (67, 68) (see Text A.1 in the appendix). For *pgsA* deletion, the protocol was the same as for *cdsA* deletion except that an erythromycin resistance marker with its native promoter was amplified from pG^{+}host4 (69) and inserted between the homologous flanking arms, to allow for selection-based screening of putative *pgsA* mutants. In the resulting construct, 284 bp encoding the catalytically active site of PgsA were deleted and

replaced with the erythromycin resistance marker. The replacement of *pgsA* was confirmed by whole-genome sequencing.

Natural transformation.

The natural transformation protocol was as described previously (68), with minor modifications. Briefly, 100 μL of exponential-phase preculture (optical density at 600 nm [OD600] of ~0.5) in THB was frozen with an equal volume of 10% glycerol. Precultures were thawed at room temperature, diluted in 900 μL of THB, further diluted 1:50 in prewarmed 5 mL THB, and incubated for 45 min at 37°C; 500 μL of culture was aliquoted with 1 μL of 1 mg/mL competence-stimulating peptide (DWRISETIRNLIFPRRK) and 1 μg/mL linear DNA construct. Transformation reaction mixtures were cultured for 2 h at 37°C in microcentrifuge tubes before being plated on THB agar, with selection as appropriate.

Lipidomics.

Unless otherwise noted, lipidomics analyses were performed on overnight cultures in stationary phase. Centrifugation was performed using a Sorvall RC6+ centrifuge. Cultures were pelleted at 4,280 X *g* for 5 min at room temperature. The supernatants were removed and stored at – 80°C until acidic Bligh-Dyer lipid extractions were performed as described previously (70), with minor modifications (see Text A.1 in Appendix A).

NPLC was performed on an Agilent 1200 quaternary LC system equipped with an Ascentis silica high-performance liquid chromatography (HPLC) column (5 μm, 25 cm by 2.1 mm; Sigma-

Aldrich), as described previously (70, 71). Data analysis was performed using Analyst TF1.5 software (Sciex, Framing- ham, MA) (see Text A.1 in Appendix A).

Metabolite extractions. Cultures were pelleted at 4,280 X *g* in a Sorvall RC6+ floor centrifuge at room temperature, washed once with 1X phosphate-buffered saline, and transferred to 1.5-mL micro- centrifuge tubes. Cells were pelleted and frozen at – 80°C until use. Metabolite extraction was performed as described previously (72), with minor modifications (see Text A.1 in Appendix A).

RPLC-ESI/MS analysis.

RPLC-ESI/MS analysis of water-soluble metabolites was performed using a Shimadzu LC system (including a solvent degasser, two LC-10A pumps, and a SCL-10A system controller) coupled to a TripleTOF 5600 mass spectrometer (Sciex) (see Text A.1 for detailed information on LC flow rate and mass spectrometer settings). Data acquisition and analysis were performed using Analyst TF1.5 software (Sciex).

Deuterated isotope and lysoPC tracking.

Deuterated isotope tracking was performed by addition of 2 mM choline-*d9* (Sigma-Aldrich) in 50 mL of THB; 3.7 mM GPC-*d9* (Toronto Research Chemicals) was added to 15 mL of THB to yield a final concentration of 130 µM. Cultures were grown in 5% CO2 at 37°C for 18 h before 10 mL of choline-*d9* culture or 5 mL of GPC-*d9* culture was removed for metabolite extraction and the remaining culture was pelleted for lipid extraction. LysoPC (20:0) was obtained from Avanti

Polar Lipids. Cultures were supplemented with 2 mg of lysoPC per 15 mL of THB, unless otherwise stated, and incubated overnight at 37°C in 5% CO2. Lipid extractions were performed as described above.

Growth curves.

Individual colonies were incubated overnight in 5 mL of DM. Cultures were diluted to a starting OD600 of 0.05 in 20 mL of prewarmed DM, DM with 130 μM GPC, or THB. The OD600 was monitored every 1 h using a Thermo Scientific Genesys 30 spectrophotometer. Growth curves were performed in biological triplicates for each strain.

ANI analysis.

The ANI calculator (50, 51) was used with default parameters to analyze the following genomes: SM43, *S. oralis* ATCC 35037 (GenBank accession no. PRJNA38733), and *S. mitis* ATCC 49456 (GenBank accession no. PRJNA173).

Accession number(s).

The SM43 whole-genome sequence generated in this study has been deposited in GenBank under accession no. CP040231. Illumina and SMRT sequence reads generated in this study have been deposited in the Sequence Read Archive under accession no. PRJNA542100.

2.8 Acknowledgements

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2.9 References

1. Doern CD, Burnham CAD. 2010. It's not easy being green: The Viridans Group Streptococci, with a focus on pediatric clinical manifestations. J Clin Microbiol 48:3829–3835.
2. Mitchell J. 2011. *Streptococcus mitis*: Walking the line between commensalism and pathogenesis. Mol Oral Microbiol 26:89–98.
3. Kolenbrander PE, London J. 1993. Adhere today, here tomorrow: Oral bacterial adherence. J Bacteriol 175:3247–3252.
4. Murdoch DR, Corey GR, Hoen B, Miró JM, Fowler VG, Bayer AS, Karchmer AW, Olaison L, Pappas PA, Moreillon P, Chambers ST, Chu VH, Falcó V, Holland DJ, Jones P, Klein JL, Raymond NJ, Read KM, Tripodi MF, Utili R, Wang A, Woods CW, Cabell CH, Investigators TI. 2009. Clinical presentation, Etiology and Outcome of Infective Endocarditis in the 21st Century: The International Collaboration on Endocarditis-Prospective Cohort Study. Arch Intern Med 169:463–473.
5. Marín M, Gudiol C, Garcia-Vidal C, Ardanuy C, Carratalà J. 2014. Bloodstream infections in patients with solid tumors: Epidemiology, antibiotic therapy, and outcomes in 528 episodes in a single cancer center. Medicine (Baltimore) 93:143–149.

6. Gudiol C, Bodro M, Simonetti A, Tubau F, González-Barca E, Cisnal M, Domingo-Domenech E, Jiménez L, Carratalà J. 2013. Changing aetiology, clinical features, antimicrobial resistance, and outcomes of bloodstream infection in neutropenic cancer patients. Clin Microbiol Infect 19:474–479.

7. Shelburne SA, Sahasrabhojane P, Saldana M, Yao H, Su X, Horstmann N, Thompson E, Flores AR. 2014. *Streptococcus mitis* strains causing severe clinical disease in cancer patients. Emerg Infect Dis 20:762–771.

8. Kawamura Y, Hou X-G, Sultana F, Miura H, Ezaki T. 1995. Determination of 16S rRNA Sequences of *Streptococcus mitis* and *Streptococcus gordonii* and Phylogenetic Relationships among Members of the Genus *Streptococcus*. Int J Syst Bacteriol 45:406–408.

9. Scholz CFP, Poulsen K, Kilian M. 2012. Novel Molecular Method for Identification of *Streptococcus pneumoniae* Applicable to Clinical Microbiology and 16S rRNA Sequence-Based Microbiome Studies. J Clin Microbiol 50:1968–1973.

10. Kilian M, Riley DR, Jensen A, Brüggemann H, Tettelin H. 2014. Parallel Evolution of *Streptococcus pneumoniae* and *Streptococcus mitis* to Pathogenic and Mutualistic Lifestyles. MBio 5:e01490-14.

11. Skov Sørensen UB, Yao K, Yang Y, Tettelin H, Kilian M. 2016. Capsular Polysaccharide Expression in Commensal *Streptococcus* Species: Genetic and Antigenic Similarities to *Streptococcus pneumoniae*. MBio 7:e01844-16.

12. Jensen A, Valdórsson O, Frimodt-Møller N, Hollingshead S, Kilian M. 2015. Commensal Streptococci Serve as a Reservoir for β-Lactam Resistance Genes in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 59:3529–3540.

13. Shekhar S, Khan R, Ferreira DM, Mitsi E, German E, Rørvik GH, Berild D, Schenck K, Kwon K, Petersen F. 2018. Antibodies reactive to commensal *Streptococcus mitis* show cross-reactivity with virulent *Streptococcus pneumoniae* serotypes. Front Immunol 9:747.

14. Adams HM, Joyce LR, Guan Z, Akins RL, Palmer KL. 2017. *Streptococcus mitis* and *S. oralis* Lack a Requirement for CdsA, the Enzyme Required for Synthesis of Major Membrane Phospholipids in Bacteria. Antimicrob Agents Chemother 61.

15. Mishra NN, Tran TT, Seepersaud R, Miro JM, Rybak MJ, Bayer AS, Arias CA, Sullam PM. 2017. Perturbations of Phosphatidate Cytidylyltransferase (CdsA) Mediate Daptomycin Resistance in *Streptococcus mitis/oralis* by a Novel Mechanism. Antimicrob Agents Chemother 61:1–13.

16. Humphries RM, Pollett S, Sakoulas G. 2013. A current perspective on daptomycin for the clinical microbiologist. Clin Microbiol Rev 26:759–780.

17. Meiers M, Volz C, Eisel J, Maurer P, Henrich B, Hakenbeck R. 2014. Altered lipid composition in *Streptococcus pneumoniae cpoA* mutants. BMC Microbiol 14:1–12.

18. Trombe M-C, Lanéelle M-A, Lanéelle G. 1979. Lipid composition of aminopterin-resistant and sensitive strains of *Streptococcus pneumoniae*. Biochim Biophys Acta - Lipids Lipid Metab 574:290–300.

19. Brundish DE, Shaw N, Baddiley J. 1967. The phospholipids of Pneumococcus I-192R, A.T.C.C. 12213. Biochem J 104:205–11.

20. Custer JE, Goddard BD, Matter SF, Kaneshiro ES. 2014. The relative proportions of different lipid classes and their fatty acid compositions change with culture age in the cariogenic dental pathogen *Streptococcus mutans* UA159. Lipids 49:543–554.

21. Chiu T-H, Hung S-A. 1979. Effect of age on the membrane composition of *Streptococcus sanguis*. Biochim Biophys Acta 558:267–272.

22. Goldfine H, Guan Z. 2015. Lipidomic Analysis of Bacteria by Thin-Layer Chromatography and Liquid Chromatography/Mass Spectrometry, p. 125–139. *In* Hydrocarbon and Lipid Microbiology Protocols.

23. Sohlenkamp C, López-Lara IM, Geiger O. 2003. Biosynthesis of phosphatidylcholine in bacteria. Prog Lipid Res 42:115–162.

24. Bogdanov M, Sun J, Kaback HR, Dowhan W. 1996. A phospholipid acts as a chaperone in assembly of a membrane transport protein. J Biol Chem 271:11615–11618.

25. Bogdanov M, Umeda M, Dowhan W. 1999. Phospholipid-assisted Refolding of an Integral Membrane Protein. J Biol Chem 274:12339–12345.

26. Lysenko ES, Gould J, Bals R, Wilson JM, Weiser JN. 2000. Bacterial Phosphorylcholine Decreases Susceptibility to the Antimicrobial Peptide LL-37/hCAP18 Expressed in the Upper Respiratory Tract. Infect Immun 68:1664–1671.

27. Geiger O, López-Lara IM, Sohlenkamp C. 2013. Phosphatidylcholine biosynthesis and function in bacteria. Biochim Biophys Acta - Mol Cell Biol Lipids 1831:503–513.

28. Conover GM, Martinez-Morales F, Heidtman MI, Luo Z-Q, Tang M, Chen C, Geiger O, Isberg RR. 2008. Phosphatidylcholine synthesis is required for optimal function of *Legionella pneumophila* virulence determinants. Cell Microbiol 10:514–528.

29. Comerci DJ, Altabe S, De Mendoza D, Ugalde RA. 2006. *Brucella abortus* synthesizes phosphatidylcholine from choline provided by the host. J Bacteriol 188:1929–1934.

30. Aktas M, Danne L, Moller P, Narberhaus F. 2014. Membrane lipids in *Agrobacterium tumefaciens*: biosynthetic pathways and importance for pathogenesis. Front Plant Sci 5:109.

31. Aktas M, Wessel M, Hacker S, Klüsener S, Gleichenhagen J, Narberhaus F. 2010. Phosphatidylcholine biosynthesis and its significance in bacteria interacting with eukaryotic cells. Eur J Cell Biol 89:888–894.

32. Malek AA, Wargo MJ, Hogan DA. 2012. Absence of membrane phosphatidylcholine does not affect virulence and stress tolerance phenotypes in the opportunistic pathogen *Pseudomonas aeruginosa*. PLoS One 7:e30829.

33. Kaneshiro T, Law JH. 1964. Phosphatidylcholine Synthesis in *Agrobacterium tumefaciens*. J Biol Chem 239:1705–1713.

34. De Rudder KEE, Sohlenkamp C, Geiger O. 1999. Plant-exuded choline is used for rhizobial membrane lipid biosynthesis by phosphatidylcholine synthase. J Biol Chem 274:20011–20016.

35. Sohlenkamp C, De Rudder KEE, Röhrs V, López-Lara IM, Geiger O. 2000. Cloning and characterization of the gene for phosphatidylcholine synthase. J Biol Chem 275:18919–18925.

36. DeLong CJ, Shen Y-J, Thomas MJ, Cui Z. 1999. Molecular distinction of phosphatidylcholine synthesis between the CDP-choline pathway and phosphatidylethanolamine methylation pathway. J Biol Chem 274:29683–29688.

37. Vences-Guzmán MÁ, Paula Goetting-Minesky M, Guan Z, Castillo-Ramirez S, Córdoba-Castro LA, López-Lara IM, Geiger O, Sohlenkamp C, Christopher Fenno J. 2017. 1,2-Diacylglycerol choline phosphotransferase catalyzes the final step in the unique *Treponema denticola* phosphatidylcholine biosynthesis pathway. Mol Microbiol 103:896–912.

38. Gisch N, Schwudke D, Thomsen S, Heß N, Hakenbeck R, Denapaite D. 2015. Lipoteichoic acid of *Streptococcus oralis* Uo5: a novel biochemical structure comprising an unusual phosphorylcholine substitution pattern compared to *Streptococcus pneumoniae*. Sci Rep 5:16718.

39. Bergström N, Jansson PE, Kilian M, Skov Sørensen UB. 2000. Structures of two cell wall-associated polysaccharides of a *Streptococcus mitis* biovar 1 strain: A unique teichoic acid-like polysaccharide and the group O antigen which is a C-polysaccharide in common with pneumococci. Eur J Biochem 267:7147–7157.

40. Seong Seo H, Cartee RT, Pritchard DG, Nahm MH. 2008. A new model of pneumococcal lipoteichoic acid structure resolves biochemical, biosynthetic, and serologic inconsistencies of the current model. J Bacteriol 190:2379–2387.

41. Denapaite D, Brückner R, Hakenbeck R, Vollmer W. 2012. Biosynthesis of Teichoic Acids in *Streptococcus pneumoniae* and Closely Related Species: Lessons from Genomes. Microb Drug Resist 18:344–358.

42. Fischer W. 1997. Pneumococcal lipoteichoic and teichoic acid. Microb Drug Resist 3:309–325.

43. Moser R, Aktas M, Narberhaus F. 2014. Phosphatidylcholine biosynthesis in *Xanthomonas campestris* via a yeast-like acylation pathway. Mol Microbiol 91:736–750.

44. Stålberg K, Neal AC, Ronne H, Ståhl U. 2008. Identification of a novel GPCAT activity and a new pathway for phosphatidylcholine biosynthesis in *S. cerevisiae*. J Lipid Res 49:1794–1806.

45. Fisher E, Almaguer C, Holic R, Griac P, Patton-vogt J. 2005. Glycerophosphocholine-dependent Growth Requires Gde1p (YPL110c) and Git1p in *Saccharomyces cerevisiae*. J Biol Chem 280:36110–36117.

46. Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vázquez-Fresno R, Sajed T, Johnson D, Li C, Karu N, Sayeeda Z, Lo E, Assempour N, Berjanskii M, Singhal S, Arndt D, Liang Y, Badran H, Grant J, Serra-Cayuela A, Liu Y, Mandal R, Neveu V, Pon A, Knox C, Wilson M, Manach C, Scalbert A. 2018. HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res 46:D608–D617.

47. Guo L, Milburn M V., Ryals JA, Lonergan SC, Mitchell MW, Wulff JE, Alexander DC, Evans AM, Bridgewater B, Miller L, Gonzalez-Garay ML, Caskey CT. 2015. Plasma metabolomic profiles enhance precision medicine for volunteers of normal health. Proc Natl Acad Sci 112:E4901–E4910.

48. Głab B, Beganovic M, Anaokar S, Hao MS, Rasmusson AG, Patton-Vogt J, Banaś A, Stymne S, Lager I. 2016. Cloning of glycerophosphocholine acyltransferase (GPCAT) from fungi and plants: A novel enzyme in phosphatidylcholine synthesis. J Biol Chem 291:25066–25076.

49. Akins RL, Katz BD, Monahan C, Alexander D. 2015. Characterization of high-level daptomycin resistance in viridans group streptococci developed upon in vitro exposure to daptomycin. Antimicrob Agents Chemother 59:2102–2112.

50. Rodriguez-R LM, Konstantinidis KT. 2016. The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. PeerJ Prepr 4:e1900v1.

51. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM. 2007. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57:81–91.

52. Rodriguez-R LM, Konstantinidis KT. 2014. Bypassing Cultivation To Identify Bacterial Species. Microbe 9:111–118.

53. Konstantinidis KT, Tiedje JM. 2005. Genomic insights that advance the species definition for prokaryotes. Proc Natl Acad Sci U S A 102:2567–72.

54. Ciufo S, Kannan S, Sharma S, Badretdin A, Clark K, Turner S, Brover S, Schoch CL, Kimchi A, DiCuccio M. 2018. Using average nucleotide identity to improve taxonomic assignments in prokaryotic genomes at the NCBI. Int J Syst Evol Microbiol 68:2386–2392.

55. Gopalakrishnan AS, Chen Y-C, Temkin M, Dowhan W. 1986. Structure and expression of the gene locus encoding the phosphatidylglycerophosphate synthase of *Escherichia coli*. J Biol Chem 261:1329–1338.

56. van Beilen J, Blohmke CJ, Folkerts H, de Boer R, Zakrzewska A, Kulik W, Vaz FM, Brul S, Ter Beek A. 2016. RodZ and PgsA Play Intertwined Roles in Membrane Homeostasis of *Bacillus subtilis* and Resistance to Weak Organic Acid Stress. Front Microbiol 7:1633.

57. Tran TT, Mishra NN, Seepersaud R, Diaz L, Rios R, Dinh AQ, Garcia-de-la-Maria C, Rybak MJ, Miro JM, Shelburne SA, Sullam PM, Bayer AS, Arias CA. 2019. Mutations in cdsA and pgsA Correlate with Daptomycin Resistance in *Streptococcus mitis/oralis*. Antimicrob Agents Chemother 63.

58. Van De Rijn I, Kessler RE. 1980. Growth characteristics of Group A Streptococci in a new chemically defined medium. Infect Immun 27:444–448.

59. Chang JC, LaSarre B, Jimenez JC, Aggarwal C, Federle MJ. 2011. Two Group A Streptococcal peptide pheromones act through opposing rgg regulators to control biofilm development. PLoS Pathog 7:e1002190.

60. Ojala PJ, Hirvonen TE, Hermansson M, Somerharju P, Parkkinen J. 2007. Acyl chain-dependent effect of lysophosphatidylcholine on human neutrophils. J Leukoc Biol 82:1501–1509.

61. Jiang D, Zhang Q, Zheng Q, Zhou H, Jin J, Zhou W, Bartlam M, Rao Z. 2014. Structural analysis of *Mycobacterium tuberculosis* ATP-binding cassette transporter subunit UgpB reveals specificity for glycerophosphocholine. FEBS J 281:331–341.

62. Großhennig S, Schmidl SR, Schmeisky G, Busse J, Stülke J. 2013. Implication of glycerol and phospholipid transporters in *Mycoplasma pneumoniae* growth and virulence. Infect Immun 81:896–904.

63. Yang K, Wang M, Metcalf WW. 2009. Uptake of Glycerol-2-Phosphate via the ugp-Encoded Transporter in *Escherichia coli* K-12. J Bacteriol 191:4667–4670.

64. Gupta R, Shah P, Swiatlo E. 2009. Differential gene expression in *Streptococcus pneumoniae* in response to various iron sources. Microb Pathog 47:101–109.

65. Clinical and Laboratory Standards Institute. 2016. Performance standards for antimicrobial susceptibility testing, 26th ed. CLSI supplement M100SClinical and Laboratory Standards Institute, Wayne, PA.

66. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 13:e1005595.

67. Morrison DA, Khan R, Junges R, Åmdal HA, Petersen FC. 2015. Genome editing by natural genetic transformation in *Streptococcus mutans*. J Microbiol Methods 119:134–141.

68. Salvadori G, Junges R, Morrison DA, Petersen FC. 2016. Overcoming the barrier of low efficiency during genetic transformation of *Streptococcus mitis*. Front Microbiol 7:1009.

69. Biswas I, Gruss A, Ehrlich SD, Maguin E. 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J Bacteriol 175:3628–3635.

70. Tan BK, Bogdanov M, Zhao J, Dowhan W, Raetz CRH, Guan Z. 2012. Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. Proc Natl Acad Sci 109:16504–16509.

71. Li C, Tan BK, Zhao J, Guan Z. 2016. In vivo and in vitro synthesis of phosphatidylglycerol by an *Escherichia coli* cardiolipin synthase. J Biol Chem 291:25144–25153.

72. Lu W, Kimball E, Rabinowitz JD. 2006. A high-performance liquid chromatography-tandem mass spectrometry method for quantitation of nitrogen-containing intracellular metabolites. J Am Soc Mass Spectrom 17:37–50.

CHAPTER 3

EFFECTS OF HUMAN SERUM ON THE SYNTHESIS OF PHOSPHATIDYLCHOLINE IN *STREPTOCOCCUS PNEUMONIAE*, *S. PYOGENES*, AND *S. AGALACTIAE*

3.1 Author Contributions

The contents of this chapter were produced in collaboration with Ziqiang Guan (ZG) and Kelli Palmer (KP). Luke Joyce (LJ), ZG, and KP designed the experiments. LJ performed all experiments and lipidomic analyses were performed by ZG. The contents of this chapter were written by LJ and edited by LJ, ZG, and KP. Permission from ZG was obtained for use of this manuscript in this Dissertation.

3.2 Introduction

Streptococci are Gram-positive bacteria that natively colonize humans in niches from the oral cavity to the genitourinary tract (1). Three species of streptococci, *Streptococcus pneumoniae*, *S. pyogenes* (Group A *Streptococcus*; GAS), and *S. agalactiae* (Group B *Streptococcus*; GBS), are considered major human pathogens, resulting in over 1.5 million deaths each year around the world (2, 3). These pathogens cause a wide range of diseases of varying severity in all age groups, including streptococcal pharyngitis, soft tissue infections, pneumonia, meningitis, bacteremia, and necrotizing fasciitis (3–10).

Many studies into the pathogenicity of streptococci have focused on factors including secreted proteins, surface polymers and adhesins, and mechanisms for sensing the extracellular

environment (11–13). Little is known about the cellular membrane of these pathogens. Earlier studies mainly utilized thin layer chromatography (TLC) or column separation coupled with paper chromatography to study membrane lipids extracted from cultures in routine laboratory medium (14–19). However, these methods lacks the molecular specificity and sensitivity for the comprehensive identification of the cellular membrane lipids (20). The phospholipids phosphatidylglycerol (PG) and cardiolipin (CL), and the glycolipids monohexosyldiacylglycerol (MHDAG) and dihexosyldiacylglycerol (DHDAG) were previously detected in all three streptococcal species (14–19, 21, 22).

We recently utilized normal phase liquid chromatography coupled with electrospray ionization mass spectrometry (NPLC-ESI/MS) to characterize the lipid membranes of the Mitis group streptococci, which includes *S. pneumoniae*, and described for the first time the presence of the zwitterionic phospholipid phosphatidylcholine (PC) in these organisms (23, 24). We further showed that the biosynthesis of PC in these organisms occurs through the rare glycerophosphocholine (GPC) pathway which relies upon scavenging of the major human metabolites GPC and lysophosphatidylcholine (lysoPC) ([24], Chapter 2 of this dissertation).

Here we utilize whole cell lipid extraction coupled with NPLC-ESI/MS to characterize the cellular membrane lipids of the three major streptococcal pathogens. To mimic the human host environment and to assess the effects of human serum on the membrane lipid compositions, these streptococcal species were cultured in defined medium with or without human serum supplementation. We show that: 1) human serum provides the substrates required for *S.*

pneumoniae to synthesize PC; 2) the GAS scavenge the human metabolite lysoPC to synthesize PC via an abbreviated GPC pathway; and, 3) the GBS also scavenge lysoPC, and the unique plasmalogen lipid, plasmanyl-PC (pPC) is present in the membrane when GBS are cultured in the presence of human serum.

3.3 Results

Pathogenic streptococci remodel their membrane phospholipid composition in response to human serum

The major phospholipids present for each streptococcal species and strain used in this study, when cultured in the rich, undefined laboratory medium Todd-Hewitt Broth (supplemented with yeast extract where appropriate), and in streptococcal defined medium supplemented with or without 5% v/v human serum, are shown in Table 3.1. A figure describing the biosynthetic pathways for each lipid is shown in Figure 3.1. Growth curves for streptococci cultured with or without 5% v/v human serum are shown in Figure 3.2A-F. Human serum supplementation did not significantly alter the growth of the bacteria, except during late exponential and early stationary phase of *S. pneumoniae* D39 (Figure 3.2A).

The lipid profile of *S. pneumoniae* cultured in Todd-Hewitt Broth supplemented with 0.5% w/v yeast extract consists of the major anionic phospholipids PG and CL, the zwitterionic phospholipid PC, and the glycolipids MHDAG and DHDAG (24). Figure 3.3A displays the positive ESI mass

Table 3.1. Summary of major glycolipids and phospholipids detected in pathogenic streptococci.

Species	Strain	Medium[1]	Detection of[2]										
			DAG	MHDAG	DHDAG	PA	PG	CL	C_{55}-P	Lys-PG	?[4]	PC	pPC
S. pneumoniae	D39	THB+0.5Y	+	+	+	+	+	+	+	−	−	+	−
		DM	+	+	+	+	+	+	+	−	−	−	−
		DM-HS	+	+	+	+	+	+	+	−	−	+	−
	TIGR4	THB+0.5Y	+	+	+	+	+	+	+	−	−	+	−
		DM	+	+	+	+	+	+	+	−	−	−	−
		DM-HS	+	+	+	+	+	+	+	−	−	+	−
S. pyogenes (GAS)	NZ131	THB+0.2Y	+	+	+	+	+	+	+	−	−	−	−
		DM	+	+	+	+	+	+	+	−	−	−	−
		DM-HS	+	+	+	+	+	+	+	−	−	+	−
	MGAS 315	THB+0.2Y	+	+	+	+	+	+	+	−	−	−	−
		DM	+	+	+	+	+	+	+	−	−	−	−
		DM-HS	+	+	+	+	+	+	+	−	−	+	−
S. agalactiae (GBS)	COH1	THB	+	+	+	+	+	+	+	+	+	−	−
		DM	+	+	+	+	+	+	+	+	+	−	−
		DM-HS	+	+	+	+	+	+	+	+	+	−	+
	A909	THB	+	+	+	+	+	+	+	+	+	−	−
		DM	+	+	+	+	+	+	+	+	+	−	−
		DM-HS	+	+	+	+	+	+	+	+	+	−	+

[1]Todd-Hewitt Broth (THB); THB supplemented with 0.5% yeast extract (THB+0.5Y); THB supplemented with 0.2% yeast extract (THB+0.2Y); Streptococcal defined medium (DM), Streptococcal defined medium supplemented with 5% v/v human serum (DM-HS) .

[2]Abbreviations and denotations: + , detected; -, undetected; DAG, diacylglycerol; MHDAG, monohexosyldiacylglycerol; DHDAG, dihexosyldiacylglycerol; PA, phosphatidic acid; PG, phosphatidylglycerol; CL, cardiolipin; C_{55}-P, undecaprenyl phosphate; Lys-PG, lysyl-phosphatidylglycerol; PC, phosphatidylcholine; pPC, plasmanyl-PC.

[3]*S. pneumoniae* lipid profiles in THB+0.5Y and defined media originally described in Joyce *et al* 2019 (24).

[4]? - Unidentified lipid is described in Chapter 4 of this dissertation.

spectrum of PC (appearing at the retention time 19-20.5 min) in the membrane of *S. pneumoniae*. We previously determined that Todd-Hewitt Broth contains GPC, a substrate utilized by *S. pneumoniae* to synthesize PC (24). A similar phospholipid profile is observed for *S. pneumoniae* cultured in defined medium, except PC is absent, due to the lack of GPC and lysoPC substrates in

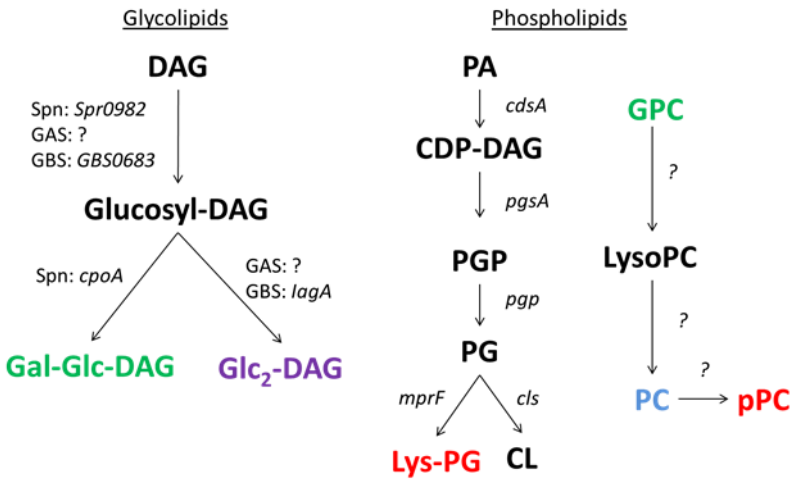

Figure 3.1. Glycolipid and phospholipid biosynthesis pathways in *S. pneumoniae*, *S. pyogenes* (GAS), and *S. agalactiae* (GBS). Genes of known or predicted function in each pathway are indicated. Lipids and substrates in black are common to all three species, in green are specific to *S. pneumoniae* (Spn), in red are specific to GBS, in blue are present in *S. pneumoniae* and GAS, and in purple are present in both GAS and GBS. DAG, diacylglycerol; Glc_2-DAG, diglucosyl-DAG; Gal-Glc-DAG, galactosyl-glucosyl-DAG; PA, phosphatidic acid; CDP-DAG, cytidine diphosphate-DAG; PGP, PG-3-phosphate; PG, phosphatidylglycerol; Lys-PG, lysyl-phosphatidylglycerol; CL, cardiolipin; GPC, glycerophosphocholine; lysoPC, lyso-phosphatidylcholine; PC, phosphatidylcholine; pPC, plasmanyl-PC. "?" denotes unidentified genes.

the medium (24). The total ion chromatogram (TIC) and mass spectrum (MS) of retention time 19-20.5 min of *S. pneumoniae* D39 grown in defined medium lacking human serum are shown in Figure 3.4A and 3.4B, respectively, and no PC is present. However, *S. pneumoniae* synthesizes PC when defined medium is supplemented with 5% v/v human serum (Figure 3.4C). Figure 3.4D shows the ESI mass spectrum of the major PC species. These data demonstrate that human serum provides the substrates required for PC biosynthesis via the GPC pathway in *S. pneumoniae*.

The lipid profile of *S. pyogenes* (GAS) cultured in Todd-Hewitt Broth supplemented with 0.2% w/v yeast extract is similar to that of *S. pneumoniae*, with the exception that PC is not detected

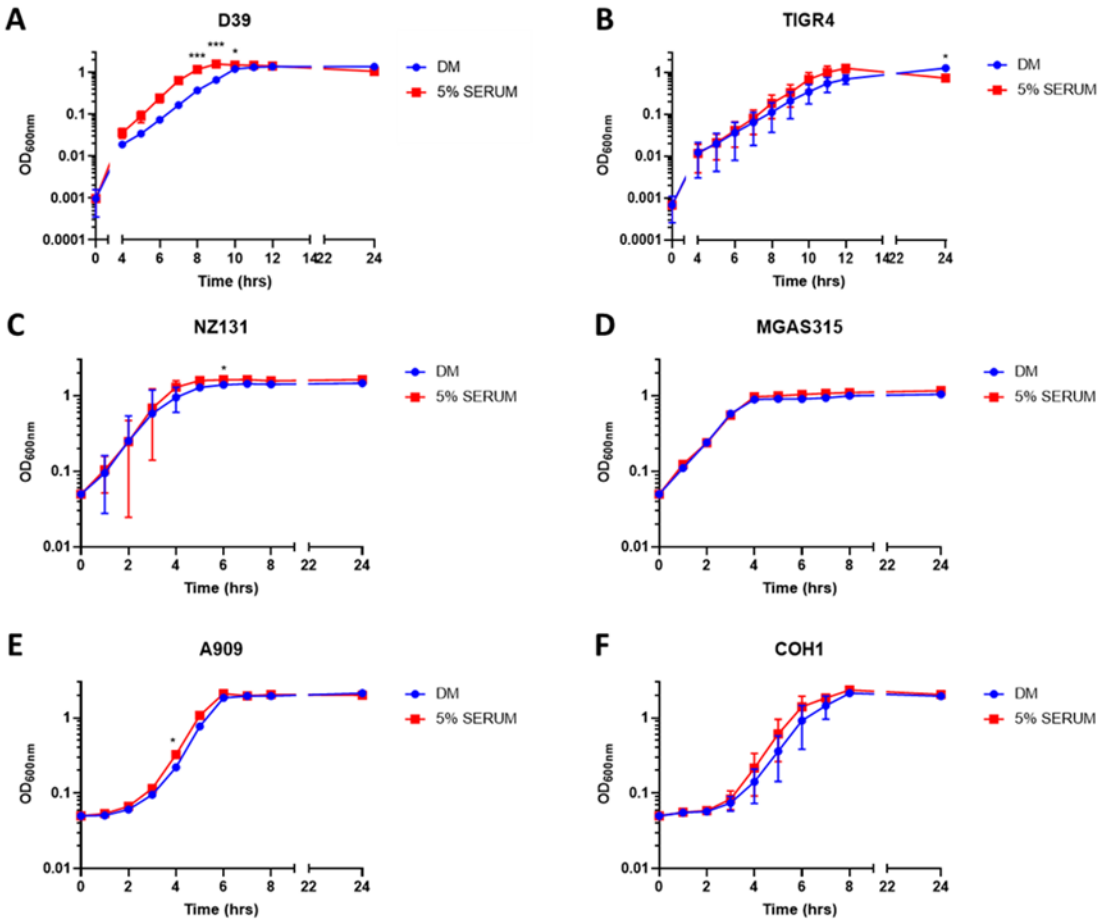

Figure 3.2. Growth characteristics of streptococci in the presence of 5% human serum. Growth curves in defined medium (DM) are shown in blue and defined media supplemented 5% v/v human serum are shown in red. A) *S. pneumoniae* D39, B) *S. pneumoniae* TIGR4, C) *S. pyognes* NZ131, D) *S. pyogenes* MGAS315, E) *S. agalactiae* A909, and F) *S. agalactiae* COH1. Manual OD_{600nm} readings were performed every hour. *S. pneumoniae* cultures were grown for 4 h before manual readings were performed. Growth curves were performed in biological triplicate. Mean and SD are indicated. Statistical analysis: A-F) repeated measures two-way ANOVA with Bonferroni's multiple comparisons test. * denotes p-value <0.05. *** denotes p-value <0.001.

(Table 3.1, Figure 3.3B). The lipid profile remains unchanged in defined medium, and no PC is detected (Figure 3.5A and 3.5B). Interestingly, PC is observed in the membrane of GAS when defined medium is supplemented with 5% v/v human serum, indicating that human serum, but not Todd-Hewitt Broth or defined medium, provide substrates required for PC biosynthesis (Figure

3.5C). The ESI/MS of the major PC species identified in the GAS membrane is shown in Figure 3.5D. The chemical structure of PC (16:0/18:2) (with the M^+ ion at *m/z* 758) is shown in Figure 3.6A and the supporting tandem mass spectrometry (MS/MS) fragmentation of *m/z* 758 is shown in Figure 3.6B.

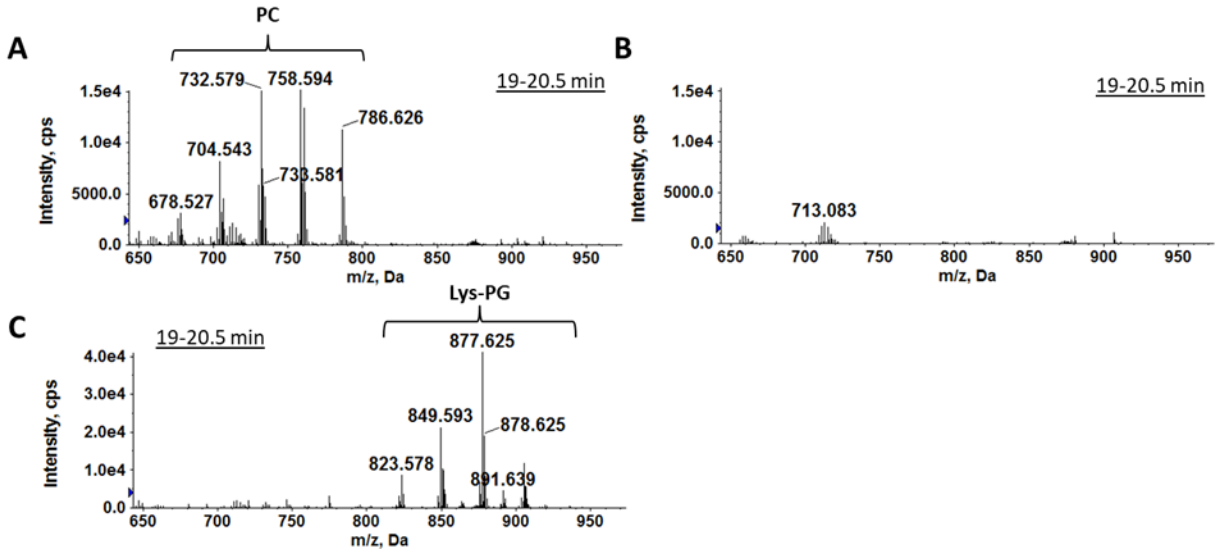

Figure 3.3. Presence and absence of PC in the membrane of streptococci when cultured in routine laboratory medium. Shown are positive ESI mass spectra obtained during the LC retention time of 19 – 20.5 min indicating the presence and absence of PC in the membrane of streptococci when cultured in Todd-Hewitt Broth. A) *S. pneumoniae* TIGR4 indicating PC is present in the membrane, as described in (24), B) *S. pyogenes* MGAS315 indicating PC is absent from the membrane, and C) *S. agalactiae* COH1 indicating PC is absent from the membrane but Lys-PG, which coelutes, is present. Only *S. pneumoniae* synthesizes PC when cultured in Todd-Hewitt Broth.

The lipid profile of *S. agalactiae* (GBS) cultured in Todd-Hewitt Broth consists of MHDAG, DHDAG, PG, and CL, as well as the aminoacylated PG molecule lysyl-PG (Lys-PG), and a novel, unidentified lipid that is characterized in Chapter 4 of this dissertation (Table 3.1, Figure 3.7A and 3.7C). The lipidomic profile of the GBS is unchanged when cultured in defined medium (Figure 3.7A and 3.7B). Like GAS, the GBS do not synthesize PC when cultured in Todd-Hewitt Broth or

defined medium (Figure 3.3C and Figure 3.7B). Also, like GAS, GBS synthesize PC when defined medium is supplemented with 5% v/v human serum (Figure 3.7C). However, ESI mass spectrum (Figure 3.7D) indicate a modified PC molecule that co-elute off the column at the same time as Lys-PG. The modified PC molecule was determined to be the plasmalogen, plasmanyl-PC (pPC) (Figure 3.6C), in which the *sn*-1 acyl chain linkage is modified to contain an ether bond, instead of the ester bond that is present in normal diacyl PC (Figure 3.6C). Figure 3.6D shows the MS/MS fragmentation of *m/z* 720 which supports the pPC structure, specifically the *m/z* 482 mass fragment compared to *m/z* 496 mass fragment for the diacyl PC in Figure 3.6B.

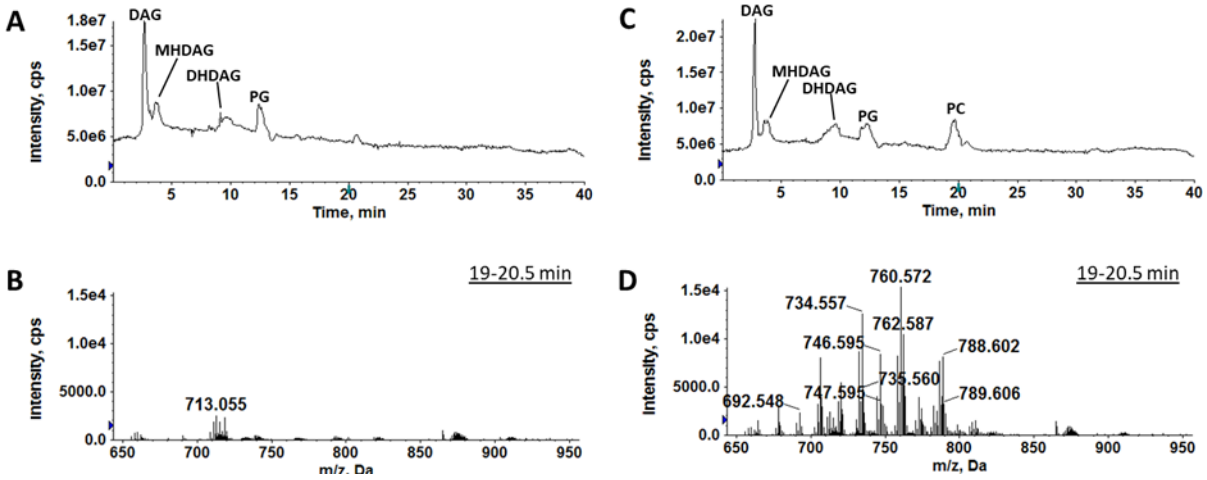

Figure 3.4. Lipidomic profiles of *S. pneumoniae* D39 cultured in defined medium with or without 5% v/v human serum supplementation. A) Positive ion mode total ion chromatogram (TIC) for *S. pneumoniae* D39 cultured in defined medium and B) mass spectrum (MS) of LC retention time 19-20.5 min indicating no PC is present in the membrane when cultured in defined medium, C) TIC of *S. pneumoniae* D39 cultured in defined medium supplemented with 5% v/v human serum, and D) MS of LC retention time 19-20.5 min indicating the presence of PC in the cellular membrane when grown in human serum-supplemented defined medium. Lipidomic analyses were performed in biological triplicate.

Taken together, these data demonstrate that major pathogenic streptococci remodel their membrane lipid composition by synthesizing PC in response to metabolites present in human

serum. Furthermore, plasmanyl-PC is uniquely observed in the membrane of the GBS during culture with human serum.

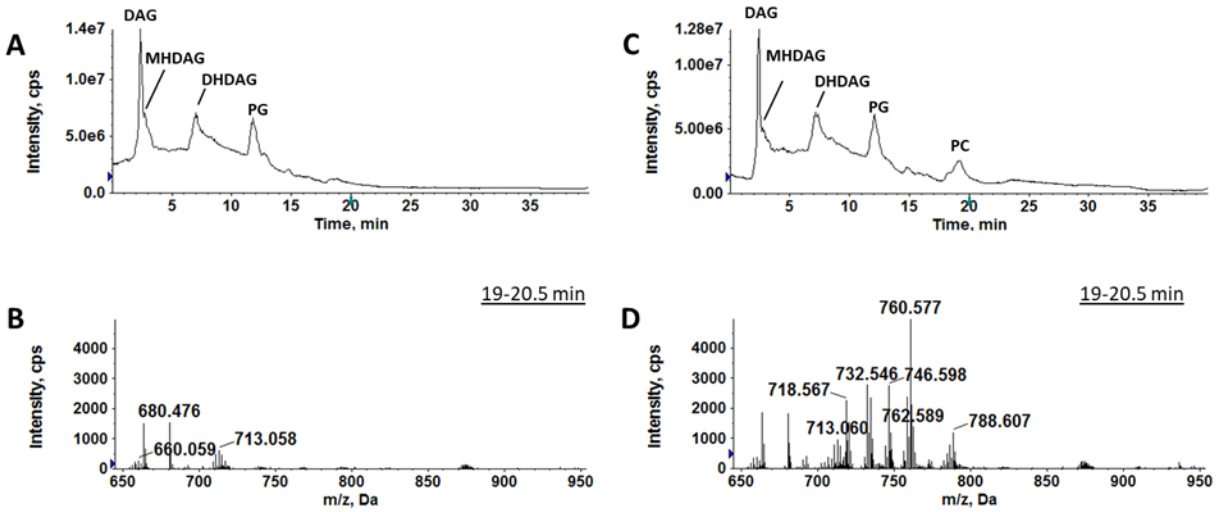

Figure 3.5. Lipidomic profiles of *S. pyogenes* MGAS315 cultured in defined medium with or without 5% v/v human serum supplementation. A) Positive ion mode total ion chromatogram (TIC) for *S. pyogenes* MGAS315 cultured in defined medium and B) mass spectrum (MS) of LC retention time 19-20.5 min indicating PC is absent from the membrane when cultured in defined medium, C) TIC of *S. pyogenes* MGAS315 cultured in defined medium supplemented with 5% v/v human serum, and D) MS of LC retention time 19-20.5 min indicating the presence of PC in the cellular membrane when grown in human serum-supplemented defined medium. Lipidomic analyses were performed in biological triplicate.

GAS and GBS scavenge lysoPC to form PC

Next, we sought to investigate if GAS and GBS utilize the GPC biosynthetic pathway previously identified in the Mitis group streptococci (24). GAS and GBS strains were cultured in defined medium supplemented with either 100 µM GPC or 100 µM lysoPC (20:0), a non-natural acyl chain length in these species, and lipidomic analysis was performed. These concentrations are physiologically relevant because lysoPC and GPC are present in varying concentrations

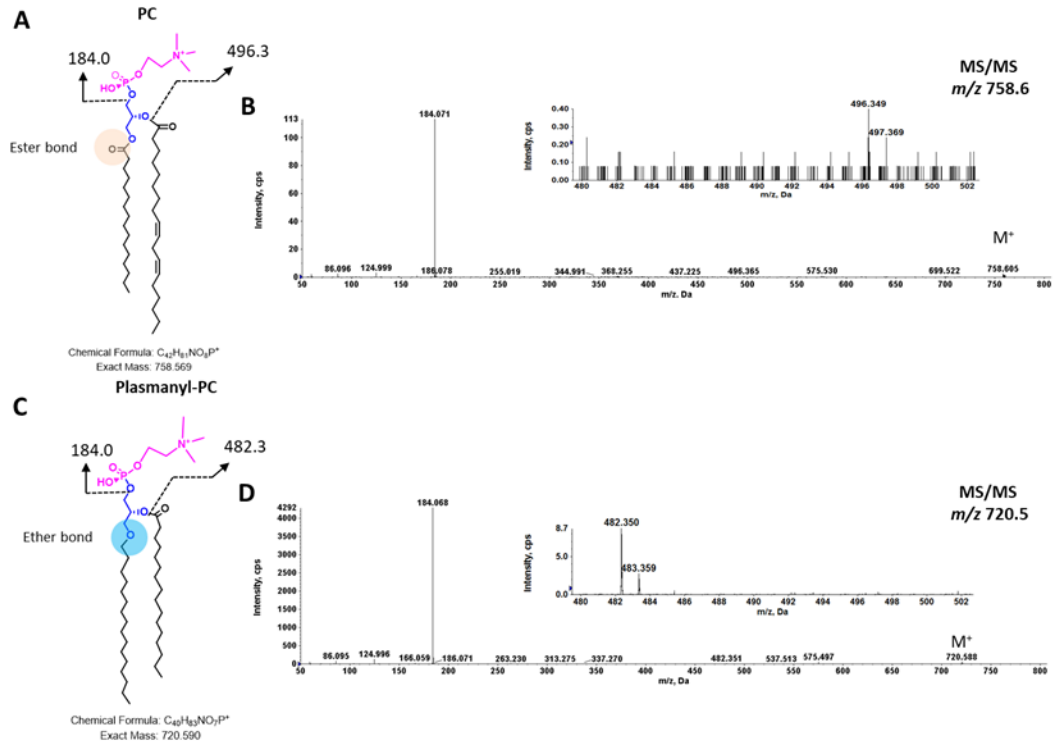

Figure 3.6. Confirmation of major PC and plasmanyl-PC species by MS/MS fragmentation. A) Chemical structure and fragmentation scheme of PC(16:0/18:2), B) MS/MS of *m/z* 758 for PC(16:0/18:2) , C) Chemical structure and fragmentation scheme of pPC(16:0/16:0), and D) MS/MS of *m/z* 720 for pPC(16:0/16:0). MS/MS fragmentation, specifically *m/z* 482 fragment, is consistent with the structure of pPC present in the GBS.

throughout the human body at ≤200 μM and ≤500 μM, respectively (25, 26). The ESI mass spectra are shown in Figure 3.8. When GAS is cultured in GPC-supplemented defined medium, very little PC is detected (Figure 3.8A). In defined medium supplemented with lysoPC (20:0), a substantial amount of PC is detected for GAS (Figure 3.8B). Similarly, when GBS is cultured in GPC-supplemented defined medium, no PC is detected (Figure 3.8C), and robust levels of PC are observed when GBS are cultured in defined medium supplemented with lysoPC (20:0) (Figure 3.8D). Taken together, these data identify lysoPC, not GPC, as the primary substrate scavenged by the GAS and GBS to synthesize PC.

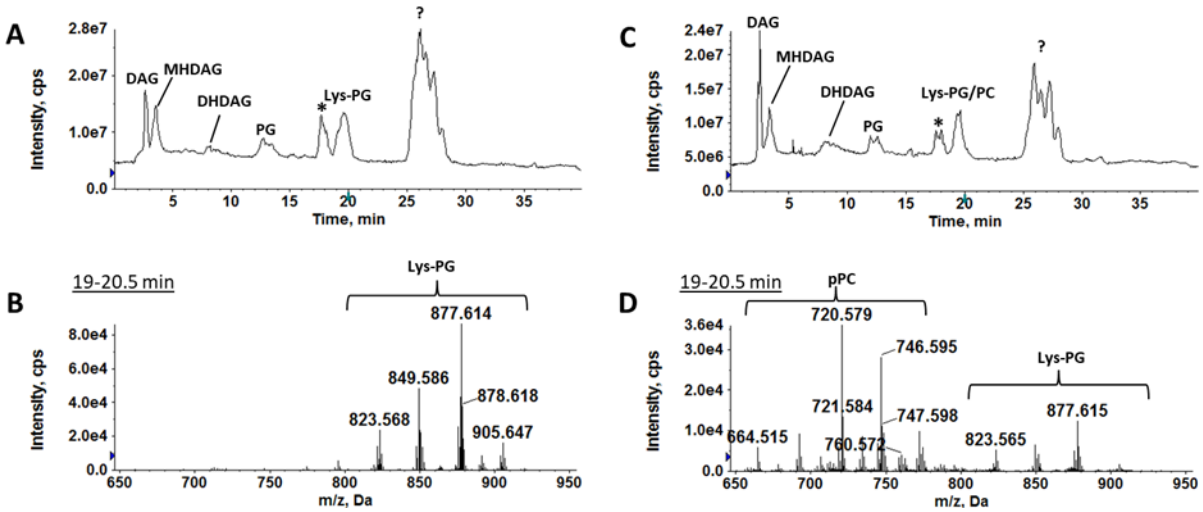

Figure 3.7. Lipidomic profiles of *S. agalactiae* COH1 cultured in defined medium with or without 5% v/v human serum supplementation. A) Positive ion mode total ion chromatogram (TIC) for *S. agalactiae* COH1 in defined medium, B) mass spectrum (MS) of LC retention time 19-20.5 min indicating no PC or pPC but Lys-PG is present in the membrane when cultured in defined medium, C) TIC of *S. agalactiae* COH1 cultured in defined medium supplemented with 5% v/v human serum, and D) MS of LC retention time 19-20.5 min indicating pPC and Lys-PG are present in the cellular membrane when cultured in human serum-supplemented defined medium. "?" indicates unidentified lipid and "*" indicates an extraction artifact of the unidentified lipid which is characterized in Chapter 4 of this dissertation. Lipidomic analyses were performed in biological triplicate.

3.4 Discussion

The cellular membrane is a critical and dynamic surface of bacterial cells that plays a role in many cellular processes throughout each stage of growth yet is largely understudied in streptococci. In this study we characterized the lipidome of the major streptococcal pathogens *S. pneumoniae*, *S. pyogenes* (GAS), and *S. agalactiae* (GBS) when grown in a standard rich laboratory medium (Todd-Hewitt Broth), and a defined medium supplemented with or without 5% v/v human serum. We show that all three streptococcal species remodel their cellular membrane in response to human metabolites, specifically, by synthesis of PC. To our knowledge, this is the first characterization

of PC and the plasmalogen pPC in the GAS and GBS, respectively. PC has been linked to virulence in certain bacteria (27, 28). Streptococci may incorporate PC into their membranes as a form of eukaryotic membrane mimicry to evade immune defense, although this must be investigated further.

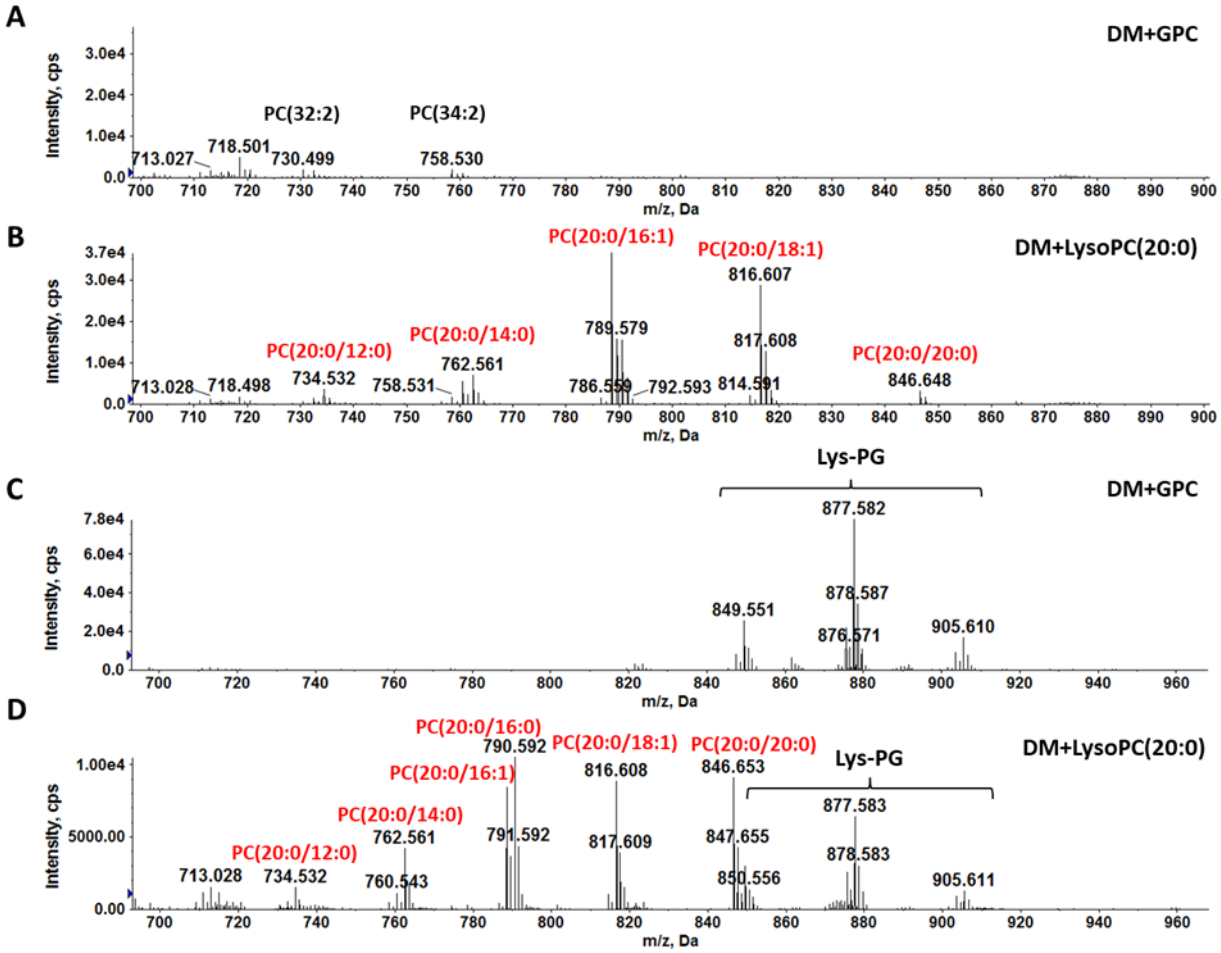

Figure 3.8. LysoPC is scavenged by both GAS and GBS to synthesize PC. A) GAS strain NZ131 grown in defined medium supplemented with 100 µM GPC synthesizes a very low level of PC. B) GAS strain NZ131 grown in defined medium supplemented with 100 µM lysoPC (20:0) synthesizes PC. C) No PC is detected for GBS strain COH1 grown in defined medium supplemented with 100 µM GPC. D) GBS strain COH1 grown in defined medium supplemented with 100 µM lysoPC (20:0) synthesizes PC. These data demonstrate that both GAS and GBS scavenge lysoPC from the environment for PC biosynthesis.

Human serum is a rich source of nutrients for streptococci. A major metabolite of human serum is lysoPC (25, 29). *S. pneumoniae* utilizes a full GPC pathway (24), scavenging metabolites from human serum to synthesize PC. However, the GAS and GBS predominantly synthesize PC using an abbreviated GPC pathway, by primarily scavenging lysoPC from the exogenous environment. This is likely due to either a missing transporter, or the inability to acylate GPC to form lysoPC. The ability of *S. pneumoniae* but not GAS or GBS to scavenge GPC could be capitalized upon in comparative genomic and laboratory experiments to help identify the GPC transporter, which is currently unknown.

To our knowledge, the presence of the plasmalogen pPC in the GBS membrane has not been described before. This is intriguing because to date, plasmalogens have only been observed in strict anaerobes such as *Clostridium* and *Bifidobacterium* (30–32). Biosynthesis of plasmalogens in bacteria is poorly understood, with the first bacterial desaturase, involved in plasmenyl *sn*-1 vinyl ether bond formation, only recently identified (33). However, these lipids are thought to promote oxidative stress survival in animal cells (34, 35) and may well play a role in colonization and pathogenesis of the GBS. The lack of mechanistic knowledge underlying plasmalogen synthesis in bacteria makes it difficult to confirm whether pPC is synthesized *de novo* by GBS or if it is derived from ether lipids present in human serum. The origin and roles of pPC in GBS in the presence of human serum is a subject of further investigation.

Overall, this work demonstrates that culture medium can significantly alter the membrane lipid composition of major human streptococcal pathogens. This supports the significance of culturing

pathogens in media that more closely represent the host environments in which the pathogens are found. Further investigation into the biosynthesis and mechanistic roles of PC in the membrane will provide novel insights into the pathogen-host interactions and pathogenesis of these streptococcal species.

3.5 Materials and Methods

Bacterial strains, media, and growth conditions

Culture media and bacterial strains used in this study are shown in Table 3.1 and Table 3.2, respectively. Routine laboratory culture conditions for each species were 37°C for *Streptococcus agalactiae* (GBS), and 37°C and 5% CO_2 for *S. pneumoniae* and *S. pyogenes* (GAS). Rich culture media used were Todd-Hewitt Broth for GBS and Todd-Hewitt Broth supplemented with yeast extract at 0.5% w/v for *S. pneumoniae* and 0.2% w/v for GAS. Streptococcal chemically defined medium (36) was diluted from stock as described (37) with 1% glucose, for GAS and GBS. *S. pneumoniae* defined medium was supplemented with 0.5 mM choline (24). Where appropriate, defined medium was supplemented with 5% v/v human serum (Sigma-Aldrich), 100 µM GPC (Sigma-Aldrich), or 100 µM lysoPC 20:0 (Avanti Polar Lipids).

Original cultures of GAS and GBS for lipidomic analysis

Single colonies of GAS and GBS were inoculated into 15 mL rich media, and incubated overnight as described above, until pelleting and storage for lipidomic analysis described below. For defined medium analysis, single colonies were cultured in defined medium overnight, diluted into 15 mL

pre-warmed defined medium supplemented with or without 5% v/v human serum to an OD_{600nm} of 0.05, and cultured for 8 h before pelleting. Cultures were performed in biological triplicate.

Table 3.2. Strains used in this study.

Organism	Strain	Description	Ref
S. pneumoniae	D39	Wild-type *S. pneumoniae* strain, capsule serotype 2. Obtained from Michael Federle, University of Illinois at Chicago	(38)
	ATCC BAA-334 (TIGR4)	Wild-type *S. pneumoniae* strain, capsule serotype 4	(39)
S. pyogenes (GAS)	ATCC BAA-595 (MGAS315)	Wild-type *S. pyogenes* strain, M3 serotype	(40)
	ATCC BAA-1633 (NZ131)	Wild-type *S. pyogenes* strain, M49 serotype	(41, 42)
S. agalactiae (GBS)	ATCC BAA-1176 (COH1)	Wild-type *S. agalactiae* strain, serotype III	(43)
	ATCC BAA-1138 (A909)	Wild-type *S. agalactiae* strain, serotype Ia	(44)

Original cultures of *S. pneumoniae* for lipidomic analysis

Single colonies of *S. pneumoniae* were inoculated into 6 mL rich media, serially diluted, and incubated overnight as described. Cultures in early exponential phase were inoculated into 15 mL pre-warmed rich media and grown until cultures reached early stationary phase. Overnight defined media cultures were inoculated straight from freezer stocks and serially diluted. Early exponential phase cultures were inoculated into defined media supplemented with or without 5% v/v human serum at an OD_{600nm} of 0.05 and incubated for 8 h until pelleting. Cultures were performed in biological triplicate.

Growth curves

GAS and GBS were cultured overnight in defined medium and diluted to a starting OD_{600nm} of 0.05 in 15 mL pre-warmed defined medium supplemented with or without 5% v/v human serum. The OD_{600nm} was monitored manually every hour using a Thermo Scientific Genesys 30 spectrophotometer. For *S. pneumoniae*, early exponential phase defined medium overnight cultures, as described above, were diluted 1:50 into pre-warmed defined medium supplemented with or without 5% v/v human serum. *S. pneumoniae* cultures were incubated for 4 h before the OD_{600nm} was monitored every hour. Growth curves were performed in biological triplicate. Repeated measures two-way ANOVA with Bonferroni's multiple comparisons test was performed in GraphPad Prism version 8 for windows, GraphPad software, San Diego, California, USA, www.graphpad.com

GPC and lysoPC supplementation experiments

Overnight defined medium cultures of GAS and GBS were diluted to a starting OD_{600nm} 0.05 in 15 mL defined medium supplemented with either 100 µM GPC or 100 µM lysoPC (20:0) and incubated for 8 h as described above. Cultures were pelleted and stored for lipidomic analysis. Cultures were performed in biological triplicate.

Acidic Bligh-Dyer extractions.

Centrifugation was performed using a Sorvall RC6+ centrifuge. Cultures were pelleted at 4,280 x *g* for 5 min at room temperature. The supernatants were removed and stored at -80°C until acidic Bligh-Dyer lipid extractions were performed as described (24, 38). Briefly, cell pellets were

resuspended in 1X PBS (Sigma-Aldrich) and transferred to Coring Pyrex glass tubes with PTFR-lined caps (VWR), followed by 1:2 vol:vol chloroform:methanol addition. Single phase extractions were vortexed periodically and incubated at room temperature for 15 minutes before 500 x *g* centrifugation for 10 min. A two-phase Bligh-Dyer system was achieved by addition of 100 µL 37% HCL, 1 mL $CHCl_3$, and 900 µL of 1X PBS, which was then vortexed and centrifuged for 5 min at 500 x *g*. The lower phase was removed to a new tube and dried under nitrogen before being stored at -80°C prior to lipidomic analysis.

Normal-Phase Liquid Chromatography-Electrospray Ionization/Mass Spectrometry.

Normal-phase LC-ESI/MS was performed on an Agilent 1200 quaternary LC system equipped with an Ascentis silica high-performance liquid chromatography (HPLC) column (5µm; 25 cm by 2.1 mm; Sigma-Aldrich) as described previously (45,46). Briefly, mobile phase A consisted of chloroform-methanol-aqueous ammonium hydroxide (800:195:5, vol/vol), mobile phase B consisted of chloroform-methanol-water-aqueous ammonium hydroxide (600: 340:50:5, vol/vol), and mobile phase C consisted of chloroform-methanol-water-aqueous ammonium hydroxide (450:450:95:5, vol/vol/vol/vol). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min, then linearly increased to 100% mobile phase B over 14 min, and held at 100% mobile phase B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min, held at 100% mobile phase C for 3 min, and, finally, returned to 100% mobile phase A over 0.5 min and held at 100% mobile phase A for 5 min. The LC eluent (with a total flow rate of 300 mL/min) was introduced into the ESI source of a high-resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). Instrumental settings for positive-

ion ESI and MS/MS analysis of lipid species were as follows: IS = 5,000 V, CUR = 20 psi, GSI = 20 psi, DP = +55 V, and FP = +150V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst TF1.5 software (Sciex, Framingham, MA).

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3.7 References

1. Facklam R. 2002. What happened to the streptococci: Overview of taxonomic and nomenclature changes. Clin Microbiol Rev 15:613–630.
2. Carapetis JR, Steer AC, Mulholland EK, Weber M. 2005. The global burden of group A streptococcal diseases. Lancet Infect Dis 5:685–94.
3. CDC. 2019. Antibiotic resistance threats in the United States, 2019. Atlanta, GA: US Department of Health and Human Services, CDC. Atlanta, Georgia.
4. Weiser JN, Ferreira DM, Paton JC. 2018. *Streptococcus pneumoniae*: transmission, colonization and invasion. Nat Rev Microbiol 16:355–367.

5. Kaplan EL, Top FH, Dudding BA, Wannamaker LW. 1971. Diagnosis of Streptococcal Pharyngitis: Differentiation of Active Infection from the Carrier State in the Symptomatic Child. J Infect Dis • 123:490–501.

6. Cunningham MW. 2000. Pathogenesis of Group A Streptococcal Infections. Clin Microbiol Rev 13:470–511.

7. Schuchat A. 1999. Group B *Streptococcus*. Lancet 353:51–56.

8. Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ. 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol 96:498–503.

9. Nandyal RR. 2008. Update on group B streptococcal infections: perinatal and neonatal periods. J Perinat Neonatal Nurs 22:230–7.

10. Edwards MS, Rench MA, Haffar AA, Murphy MA, Desmond MM, Baker CJ. 1985. Long-term sequelae of group B streptococcal meningitis in infants. J Pediatr 106:717–22.

11. Armistead B, Oler E, Adams Waldorf K, Rajagopal L. 2019. The Double Life of Group B *Streptococcus*: Asymptomatic Colonizer and Potent Pathogen. J Mol Biol 431:2914–2931.

12. Wilkening R V, Federle MJ. 2017. Evolutionary Constraints Shaping *Streptococcus pyogenes*-Host Interactions. Trends Microbiol 25:562–572.

13. Mitchell AM, Mitchell TJ. 2010. *Streptococcus pneumoniae*: Virulence factors and variation. Clin Microbiol Infect 16:411–418.

14. Cohen M, Panos C. 1966. Membrane Lipid Composition of *Streptococcus pyogenes* and Derived L Form. Biochemistry 5:2385–2392.

15. Meiers M, Volz C, Eisel J, Maurer P, Henrich B, Hakenbeck R. 2014. Altered lipid composition in *Streptococcus pneumoniae cpoA* mutants. BMC Microbiol 14:1–12.

16. Trombe M-C, Lanéelle M-A, Lanéelle G. 1979. Lipid composition of aminopterin-resistant and sensitive strains of *Streptococcus pneumoniae*. Biochim Biophys Acta - Lipids Lipid Metab 574:290–300.

17. Brundish DE, Shaw N, Baddiley J. 1967. The phospholipids of Pneumococcus I-192R, A.T.C.C. 12213. Biochem J 104:205–11.

18. Fischer W. 1977. The polar lipids of Group B Streptococci. II. Composition and positional distribution of fatty acids. Biochim Biophys Acta 487:89–104.

19. Curtis J, Kim G, Wehr NB, Levine RL. 2003. Group B Streptococcal phospholipid causes pulmonary hypertension. Proc Natl Acad Sci U S A 100:5087–90.

20. Goldfine H, Guan Z. 2015. Lipidomic Analysis of Bacteria by Thin-Layer Chromatography and Liquid Chromatography/Mass Spectrometry, p. 125–139. *In* Hydrocarbon and Lipid Microbiology Protocols.

21. Doran KS, Engelson EJ, Khosravi A, Maisey HC, Fedtke I, Equils O, Michelsen KS, Arditi M, Peschel A, Nizet V. 2005. Blood-brain barrier invasion by Group B *Streptococcus* depends upon proper cell-surface anchoring of lipoteichoic acid. J Clin Invest 115:2499–2507.

22. Schneewind O, Missiakas D. 2014. Lipoteichoic acids, phosphate-containing polymers in the envelope of gram-positive bacteria. J Bacteriol 196:1133–42.

23. Adams HM, Joyce LR, Guan Z, Akins RL, Palmer KL. 2017. *Streptococcus mitis* and *S. oralis* Lack a Requirement for CdsA, the Enzyme Required for Synthesis of Major Membrane Phospholipids in Bacteria. Antimicrob Agents Chemother 61:e02552-16.

24. Joyce LR, Guan Z, Palmer KL. 2019. Phosphatidylcholine Biosynthesis in Mitis Group Streptococci via Host Metabolite Scavenging. J Bacteriol 201:e00495-19.

25. Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vázquez-Fresno R, Sajed T, Johnson D, Li C, Karu N, Sayeeda Z, Lo E, Assempour N, Berjanskii M, Singhal S, Arndt D, Liang Y, Badran H, Grant J, Serra-Cayuela A, Liu Y, Mandal R, Neveu V, Pon A, Knox C, Wilson M, Manach C, Scalbert A. 2018. HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res 46:D608–D617.

26. Ojala PJ, Hirvonen TE, Hermansson M, Somerharju P, Parkkinen J. 2007. Acyl chain-dependent effect of lysophosphatidylcholine on human neutrophils. J Leukoc Biol 82:1501–1509.

27. Sohlenkamp C, Geiger O. 2016. Bacterial membrane lipids: Diversity in structures and pathways. FEMS Microbiol Rev 40:133–159.

28. Geiger O, López-Lara IM, Sohlenkamp C. 2013. Phosphatidylcholine biosynthesis and function in bacteria. Biochim Biophys Acta - Mol Cell Biol Lipids 1831:503–513.

29. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, Sinelnikov I, Krishnamurthy R, Eisner R, Gautam B, Young N, Xia J, Knox C, Dong E, Huang P, Hollander Z, Pedersen TL, Smith SR, Bamforth F, Greiner R, McManus B, Newman JW, Goodfriend T, Wishart DS. 2011. The human serum metabolome. PLoS One 6:e16957.

30. Goldfine H. 2017. The anaerobic biosynthesis of plasmalogens. FEBS Lett 591:2714–2719.

31. Mawatari S, Sasuga Y, Morisaki T, Okubo M, Emura T, Fujino T. 2020. Identification of plasmalogens in *Bifidobacterium longum*, but not in *Bifidobacterium animalis*. Sci Rep 10:1–10.

32. Guan Z, Katzianer D, Zhu J, Goldfine H. 2014. *Clostridium difficile* contains plasmalogen species of phospholipids and glycolipids.

33. Gallego-García A, Monera-Girona AJ, Pajares-Martínez E, Bastida-Martínez E, Pérez-Castaño R, Iniesta AA, Fontes M, Padmanabhan S, Elías-Arnanz M. 2019. A bacterial light response reveals an orphan desaturase for human plasmalogen synthesis. Science 366:128–132.

34. Zoeller RA, Morand OH, Raetz CRH. 1988. A possible role for plasmalogens in protecting animal cells against photosensitized killing. J Biol Chem 263:11590–11596.

35. Broniec A, Żądło A, Pawlak A, Fuchs B, Kłosiński R, Thompson D, Sarna T. 2017. Interaction of plasmenylcholine with free radicals in selected model systems.

36. Van De Rijn I, Kessler RE. 1980. Growth characteristics of Group A Streptococci in a new chemically defined medium. Infect Immun 27:444–448.

37. Chang JC, LaSarre B, Jimenez JC, Aggarwal C, Federle MJ. 2011. Two Group A Streptococcal peptide pheromones act through opposing rgg regulators to control biofilm development. PLoS Pathog 7:e1002190.

38. Lanie JA, Ng WL, Kazmierczak KM, Andrzejewski TM, Davidsen TM, Wayne KJ, Tettelin H, Glass JI, Winkler ME. 2007. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. J Bacteriol 189:38–51.

39. Tettelin H, Nelson KE, Paulsen IT, Eisen JA, Read TD, Peterson S, Heidelberg J, Deboy RT, Haft DH, Dodson RJ, Durkin AS, Gwinn M, Kolonay JF, Nelson WC, Peterson JD, Umayam LA, White O, Salzberg SL, Lewis MR, Radune D, Holtzapple E, Khouri H, Wolf AM, Utterback TR, Hansen CL, Mcdonald LA, Feldblyum T V, Angiuoli S, Dickinson T, Hickey EK, Holt IE, Loftus BJ, Yang F, Smith HO, Venter JC, Dougherty BA, Morrison DA, Hollingshead SK, M. FC. 2001. Complete Genome Sequence of a Virulent Isolate of *Streptococcus pneumoniae*. Science 293:498–507.

40. Musser JM, Hauser AR, Kim MH, Schlievert PM, Nelson K, Selander RK. 1991. *Streptococcus pyogenes* causing toxic-shock-like syndrome and other invasive diseases: clonal diversity and pyrogenic exotoxin expression. Proc Natl Acad Sci U S A 88:2668–72.

41. Simon D, Ferretti JJ. 1991. Electrotransformation of *Streptococcus pyogenes* with plasmid and linear DNA. FEMS Microbiol Lett 66:219–24.

42. McShan WM, Ferretti JJ, Karasawa T, Suvorov AN, Lin S, Qin B, Jia H, Kenton S, Najar F, Wu H, Scott J, Roe BA, Savic DJ. 2008. Genome sequence of a nephritogenic and highly transformable M49 strain of *Streptococcus pyogenes*. J Bacteriol 190:7773–85.

43. Kuypers JM, Heggen LM, Rubens CE. 1989. Molecular analysis of a region of the Group B *Streptococcus* chromosome involved in type III capsule expression. Infect Immun 57:3058–65.

44. Lancefield RC, McCarty M, Everly WN. 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J Exp Med 142:165–79.

45. Tan BK, Bogdanov M, Zhao J, Dowhan W, Raetz CRH, Guan Z. 2012. Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. Proc Natl Acad Sci 109:16504–16509.

46. Li C, Tan BK, Zhao J, Guan Z. 2016. In vivo and in vitro synthesis of phosphatidylglycerol by an *Escherichia coli* cardiolipin synthase. J Biol Chem 291:25144–25153.

CHAPTER 4

STREPTOCOCCUS AGALACTIAE MPRF SYNTHESIZES A NOVEL CATIONIC GLYCOLIPID, LYSYL-GLUCOSYL-DIACYLGLYCEROL

4.1 Author contributions

The contents of this chapter were produced in collaboration with Ziqiang Guan (ZG), Haider Manzer (HM), Kelly Doran (KD), and Kelli Palmer (KP). Luke Joyce (LJ), ZG, and KP designed the experiments, except for human cell line work designed by HM and KD. LJ performed all experiments, besides human cell line work performed by HM and lipidomic analyses performed by ZG. The contents of this chapter were written by LJ, with exception of the mass spectrometry identification section by ZG, and edited by LJ and KP. Permission from ZG, HM, and KD was obtained for use of this manuscript in this Dissertation.

4.2 Introduction

Streptococcus agalactiae (Group B *Streptococcus*; GBS) are Gram-positive, β-hemolytic bacteria that colonize the lower genital and gastrointestinal tracts of approximately 30% of healthy women (1, 2). The GBS opportunistically cause life-threatening neonatal infections such as meningitis, sepsis, and pneumonia (3–6). They can also cause disease in healthy adults and pregnant women (7). GBS can be transmitted from colonized mothers to newborns during natural childbirth, and due to the severity of the resulting diseases, intrapartum antibiotic prophylaxis is prescribed for colonized pregnant women (8, 9). Even with these measures, the GBS are still a major disease burden with potentially long-lasting neurological effects on babies (3–6).

Research on the pathogenesis of the GBS has mainly focused on cell wall-anchored or secreted proteins and polysaccharides that aid in the attachment to and invasion of host cells. The numerous attachment and virulence factors possessed by the GBS are summarized in a recent review by Armistead *et al* 2019 (10). Comparatively little is known about GBS cellular membrane lipids, even though the bacterial membrane is a critical site for host-pathogen interactions. To our knowledge, the only characterization of GBS phospholipids prior to our study here was the identification of the phospholipids phosphatidylglycerol (PG), cardiolipin (CL), and lysyl-phosphatidylglycerol (Lys-PG) (11, 12). Similarly, investigation into the glycolipids of the GBS membrane has focused on di-glucosyl-diacylglycerol (Glc_2-DAG), which is the lipid anchor of the Type I lipoteichoic acid, and its role in pathogenesis (13).

Previous genomic analyses of GBS have identified a homolog of MprF (multiple peptide resistance factor) (14), an enzyme which catalyzes the aminoacylation of the anionic phospholipid PG in a range of Gram-positive and Gram-negative bacteria (15, 16). MprF is a membrane-bound enzyme comprised of a N-terminal flippase domain (17) and a C-terminal catalytic domain that uses a charged tRNA as the amino acid donor and facilitates a nucleophilic attack to transfer the amino acid from the tRNA to the head group of PG (18, 19). An important function of aminoacylation, in particular lysynylation, of PG in the membranes of bacteria is to lower the net negative charge of their cellular envelope to protect them from cationic antimicrobial peptides (CAMPs) produced by host immune systems and bacteriocins produced by competitor bacteria (15, 16). A previous study by Saar-Dover *et al* 2012 performed an in-frame deletion of *mprF* from the model GBS

strain NEM316, which did not result in significant differences in CAMP and antibiotic susceptibility *in vitro* under the conditions tested (14).

In this study, we utilized normal phase liquid chromatography coupled with electrospray ionization high-resolution tandem mass spectrometry (LC-ESI/MS) to characterize the GBS membrane lipid composition, and identified a novel cationic glycolipid, lysyl-glucosyl-diacylglycerol (Lys-Glc-DAG), which comprises a major portion of the GBS total lipid extract. While Lys-PG has been reported in a range of bacterial species (20), Lys-Glc-DAG represents, to our knowledge, the first example of lysine modification of a neutral lipid. By gene deletion and heterologous expression, we show the GBS MprF enzyme is responsible for the biosynthesis of both the novel Lys-Glc-DAG lipid and Lys-PG. We hypothesize that this expanded substrate repertoire of MprF allows for more extensive masking of the negative charges of the cellular membrane, contributing to GBS pathogenesis.

4.3 Results

Identification of Lys-Glc-DAG, a novel cationic glycolipid in GBS

To characterize the membrane lipids of GBS, three commonly used strains of different serotypes (*S. agalactiae* COH1 (ATCC BAA-1176), *S. agalactiae* A909 (ATCC BAA-1138), and *S. agalactiae* CNCTC 10/84, Table 4.1) were cultured in Todd-Hewitt Broth (THB). Lipid extraction was performed using an acidic Bligh-Dyer method (21–23), followed by lipidomic analysis by normal phase LC-ESI/MS performed in both positive and negative ion modes.

Table 4.1. Summary of phospholipids and glycolipids present in *S. agalactiae* and expression strains used in this study[1].

Species	Strain	Lipids						
		PA	PG	CL	Lys-PG	Lys-Glc-DAG	MHDAG	DHDAG
GBS	COH1	+	+	+	+	+	+	+
	A909	+	+	+	+	+	+	+
	CNCTC 10/84	+	+	+	+	+	+	+
	COH1Δ*mprF*	+	+	+	-	-	+	+
	COH1Δ*mprF*(pGBSMprF)	+	+	+	+	+	+	+
	COH1Δ*mprF*(pABG5Δ*phoZ*)	+	+	+	-	-	+	+
S. mitis	ATCC 49456	+	+	+	-	-	+	+
	ATCC 49456(pGBSMprF)	+	+	+	+	+	+	+
	ATCC 49456(pEfmMprF1)	+	+	+	+	-	+	+
	ATCC 49456(pEfmMprF2)	+	+	+	-	-	+	+

[1]Abbreviations: GBS, Group B *Streptococcus*; PA, phosphatidic acid; PG, phosphatidylglycerol; CL, cardiolipin; Lys-PG, lysyl-phosphatidylglycerol; Lys-Glc-DAG, lysyl-glucosyl-diacylglycerol; MHDAG, monohexosyldiacylglycerol; DHDAG, dihexosyldiacylglycerol.

Figure 4.1A shows the total ion chromatogram (TIC) of LC-ESI/MS of the lipid extract of *S. agalactiae* COH1 in the negative mode. Common Gram-positive bacterial lipids were identified by negative ion ESI/MS/MS, including diacylglyercol (DAG), monohexosyldiacylglycerol (MHDAG), dihexosyldiacylglycerol (DHDAG), PG, lyso-phosphatidylglycerol (lyso-PG), CL, Lys-PG, phosphatidic acid (PA), and undecaprenyl phosphate (C_{55}-P) (Table 4.1).

Surprisingly, the positive TIC (Figure 4.1B) shows highly abundant peaks of unknown identity at the retention time ~25-29 min. These TIC peaks are derived from *m/z* 803.595, *m/z* 831.624, *m/z* 857.644, *m/z* 885.671 and *m/z* 913.703 ions (Figure 4.1C), which are indicative of a lipid with varying acyl chain lengths. These ions were interpreted as $[M+H]^+$, given their corresponding $[M-H]^-$ and $[M+Cl]^-$ ions were detected in the negative mode (not shown). The mass spectra and LC

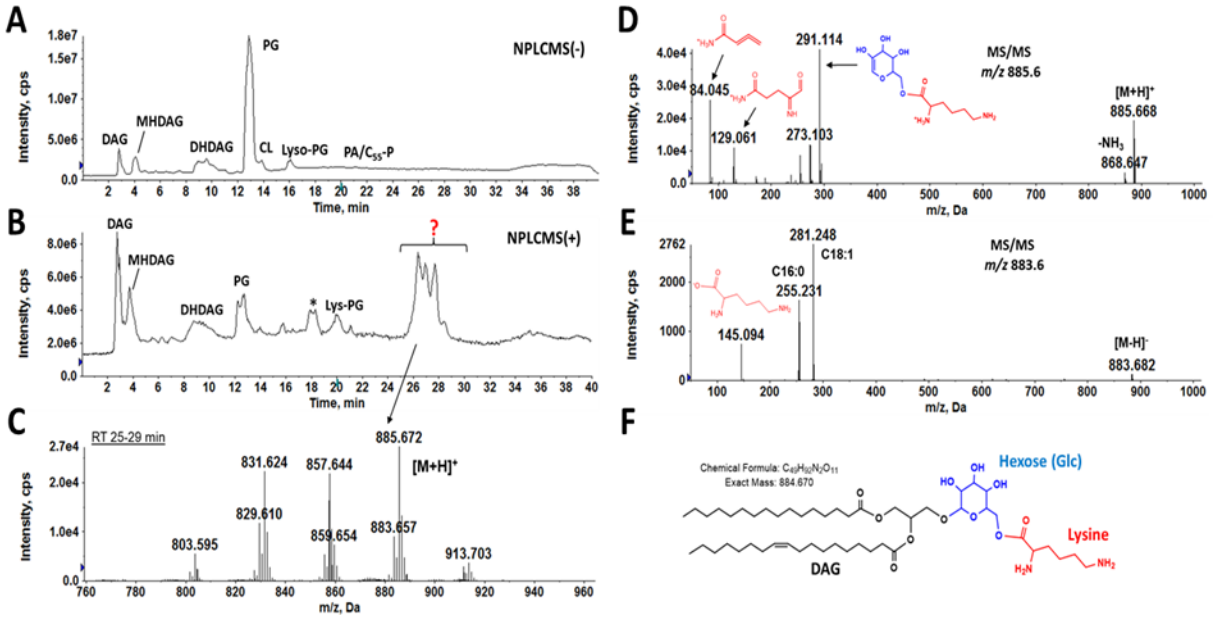

Figure 4.1. Lipidomic profiling identifies a major unknown lipid as Lysyl-Glucosyl-Diacylglycerol in GBS. The total lipid extract of wild-type *S. agalactiae* COH1 was analyzed by normal phase LC-ESI/MS in both negative and positive ion modes. A) Total ion chromatogram (TIC) of LC/MS in the negative ion mode shows common Gram-positive bacterial lipids. B) TIC of LC/MS in the positive ion mode shows a major unknown lipid eluting from the column at ~25 to 29 min. C) Positive ESI/MS showing the $[M+H]^+$ ions of the unknown lipid. The spectrum was averaged from those acquired during the retention time of 25 to 29 min. D) Positive ion MS/MS spectrum of $[M+H]^+$ at *m/z* 885.6 of the unknown lipid. E) Negative ion MS/MS spectrum of $[M-H]^-$ at *m/z* 883.6 of the unknown lipid. F) Lys-Glc-DAG (16:0/18:1) is proposed as the structure of the unknown lipid that gave rise to the $[M+H]^+$ at *m/z* 885.6 and $[M-H]^-$ at *m/z* 883.6. The hexose was assigned as glucose, because glucosyl-DAG is a major gram-positive membrane lipid and is supported by the isotopic labeling studies using a ^{13}C-labeled glucose shown below. The acyl chain compositions and exact masses of Lys-Glc-DAG molecular species observed in *S. agalactiae* COH1 are listed in Table 4.2.

retention times of this unknown lipid do not match with any other bacterial lipids we have analyzed. Searching lipidomic databases by exact masses did not result in any matches. The unknown lipid was also observed in A909 and CNCTC 10/84 strains (Figure 4.2A and 4.2B). To identify this unknown lipid, MS/MS was performed in both positive and negative ion modes.

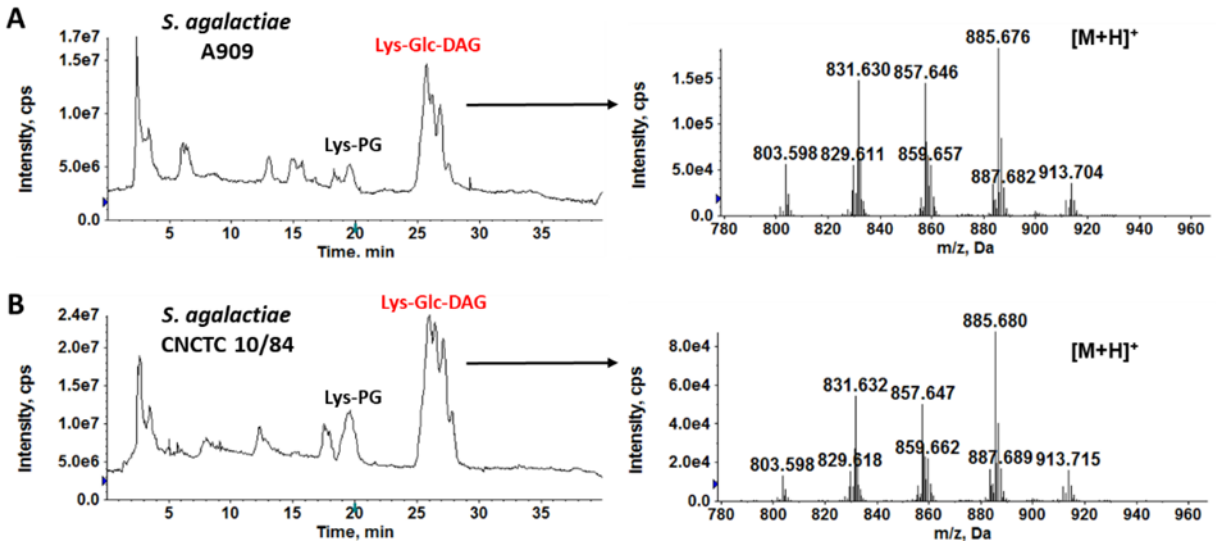

Figure 4.2. Detection of Lys-PG and Lys-Glc-DAG in *S. agalactiae* A909 and *S. agalactiae* CNCTC 10/84. Positive TICs (left) showing the presence of Lys-PG and Lys-Glc-DAG in S. *agalactiae* A909 and S. *agalactiae* CNCTC 10/84. Mass spectra (right) show the $[M+H]^+$ ions of Lys-Glc-DAG.

Positive ion MS/MS spectrum of *m/z* 885.6 (the most abundant species with a molecular mass of 884.6 Da) produced structurally informative fragments (Figure 4.1D). In particular, the low-mass fragments *m/z* 84.045 and *m/z* 129.061 are indicators of the presence of amino acids, with lysine being a strong possibility (24). The *m/z* 129 is proposed to be formed from lysine by elimination of H_2O; it fragments further to produce *m/z* 84, a low mass ion observed in the spectra of many lysine derivatives (24). The NH_3 loss peak observed at *m/z* 868.6 resulted from the elimination of an amino group of the lysine side chain. The accurate mass measurement of fragments indicates that lysine is linked to a hexose moiety. Specifically, the exact mass difference between *m/z* 291.114 and *m/z* 129.056 is 162.053, which is in agreement with the elemental composition of a hexose subunit ($C_6H_{10}O_5$, calculated mass 162.053).

Negative ion MS/MS provided further structural information. As shown in Figure 4.1E, MS/MS of the $[M-H]^-$ ion at *m/z* 883.6 produced a major fragment ion at *m/z* 145.08, which corresponds to a deprotonated lysine. The *m/z* 255.2 and 281.2 ions are the carboxylic anions of palmitic (C16:0) and oleic (C18:1) fatty acids, respectively, revealing the two acyl chains of the most abundant molecular species (MW 884.6).

Based on the above MS/MS and exact mass measurement, we propose lysyl-glucosyl-diacylglyerol (Lys-Glc-DAG) (Figure 4.1F) as the structure of this unknown lipid. The assignment of glucose was based on the observation that glucosyl-diacylglycerol (Glc-DAG), the precursor of Lys-Glc-DAG, is a major membrane component of GBS and other streptococci (13, 25), as well as the results from an isotopic labeling experiment using a ^{13}C-labeled glucose, described below.

The chemical structure of Lys-Glc-DAG is consistent with its chromatographic properties under normal phase LC conditions. Due to the presence of both polar (glucose) and charged (lysine) groups which bind strongly with the silica-based stationary phase, Lys-Glc-DAG has longer retention times than other major lipids in GBS. Also, Lys-Glc-DAG consists of several molecular species with different fatty acyl compositions. These different Lys-Glc-DAG molecular species have slightly different retention times, giving rise to multiple, unresolved TIC peaks (~25-29 min). The observed and calculated exact masses of major Lys-Glc-DAG molecular ion species are shown in Table 4.2.

Table 4.2. Observed and calculated exact masses of the $[M+H]^+$ ions of Lys-Glc-DAG molecular species in *S. agalactiae* COH1.

Lys-Glc-DAG	$[M+H]^+$	
	Observed mass	Exact mass
C28:1	801.575	801.583
C28:0	803.595	803.599
C30:1	829.610	829.615
C30:0	831.624	831.630
C32:2	855.623	855.630
C32:1	857.644	857.646
C34:2	883.657	883.622
C34:1	885.672	885.677
C36:2	911.686	911.693
C36:1	913.703	913.709

Isotopic incorporation of lysine and glucose into Lys-Glc-DAG

To further confirm the lysine and glucose moieties of Lys-Glc-DAG, we carried out stable isotope-labeling experiments by culturing *S. agalactiae* COH1 in streptococcal defined media (DM) supplemented with either deuterated lysine or ^{13}C-labeled glucose. In the deuterated lysine (lysine-*d4*), 4 hydrogen (1 Da) atoms across 2 of the carbon atoms are replaced with deuterium (2 Da) atoms, thereby increasing the mass of lysine by 4 Da. Figure 4.3A and 4.3B show the positive ion mass spectra of the $[M+H]^+$ ions of Lys-Glc-DAG species extracted from cultures in DM and in DM supplemented with lysine-*d4*, respectively. The incorporation of lysine-*d4* into Lys-Glc-DAG is reflected by a 4 Da mass shift between the $[M+H]^+$ ions of the unlabeled Lys-Glc-DAG (blue dots) and the lysine-*d4*-labeled Lys-Glc-DAG (red dots). The same 4 Da mass shift is observed in the negative ion mode: the $[M-H]^-$ ion of unlabeled Lys-Glc-DAG (16:0/18:1) is observed at *m/z* 883.6, while the lysine-*d4* containing Lys-Glc-DAG (16:0/18:1) is observed at *m/z* 887.6 (Figure B.1A in Appendix B). Furthermore, MS/MS of *m/z* 883.6 (Figure B.1B in Appendix B) and *m/z*

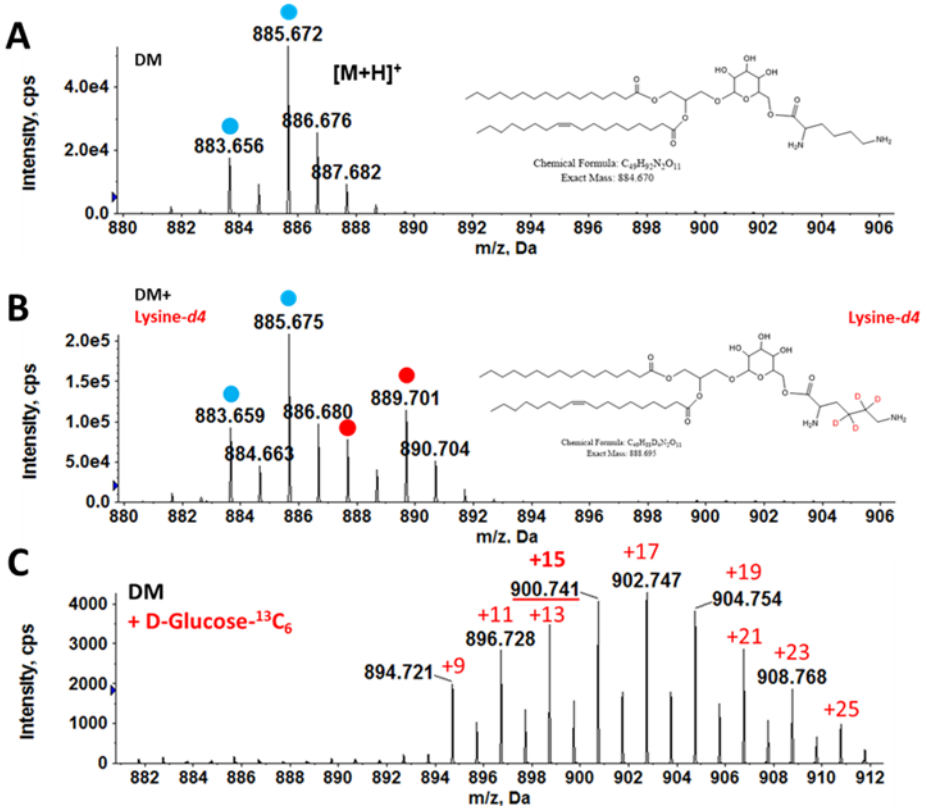

Figure 4.3. Isotopic incorporation of deuterated lysine and ^{13}C-labeled glucose into Lys-Glc-DAG. The lipid extracts of *S. agalactiae* COH1 cultured in DM, DM supplemented with 450 µM L-lysine-*d4* (4,4,5,5-D4), or in DM containing 0.5% w/v D-Glucose (U-$^{13}C_6$) were analyzed by LC-ESI/MS in the positive ion mode. A) $[M+H]^+$ ions of major Lys-Glc-DAG species in *S. agalactiae* COH1 cultured in DM. B) $[M+H]^+$ ions of major Lys-Glc-DAG species in *S. agalactiae* COH1 cultured in DM supplemented with lysine-*d4*. The incorporation of lysine-*d4* in Lys-Glc-DAG is evidenced by an upward *m/z* shift of 4 Da from unlabeled Lys-Glc-DAG (blue dots) to labeled Lys-Glc-DAG (red dots). C) $[M+H]^+$ ion of Lys-Glc-DAG (16:0/18:1) is observed at multiple peaks with higher m/z values (from m/z 894.7 to 910.8) when *S. agalactiae* COH1 is cultured in DM supplemented with D-Glucose-$^{13}C_6$. The mass shifts, ranging from +9 to +25 Da, indicate the numbers of ^{13}C atoms that were incorporated into Lys-Glc-DAG (16:0/18:1).

887.6 (Figure B.1C in Appendix B) produced fragment ions at *m/z* 145 and *m/z* 149, corresponding to the deprotonated lysine and lysine-*d4*, respectively. The molecular weight shift of lysine-*d4* incorporation is also observed in Lys-PG (Figure B.1D in Appendix B). The incorporation of

lysine-*d4* is shown by a 4 Da mass shift between the $[M+H]^+$ ions of *m/z* 887.6 unlabeled Lys-PG (blue dots) to the *m/z* 881.6 lysine-*d4*-labeled Lys-PG (red dots).

In D-Glucose (U-$^{13}C_6$), all six carbon (12 Da) atoms are replaced with carbon-13 atoms (^{13}C), thereby increasing the mass of glucose by 6 Da. In contrast to lysine-*d4* labeling, the molecular profile of Lys-Glc-DAG from ^{13}C-labeled glucose labeling is more complex. This is because ^{13}C-labeled glucose is utilized by GBS not only to replace the glucose moiety of Lys-Glc-DAG, but also to synthesize its fatty acyl chains via glycolysis. As shown in (Figure 4.3C), the *m/z* 885.6 $[M+H]^+$ ion of unlabeled Lys-Glc-DAG(16:0/18:1) is shifted to a broader distribution of ion peaks ranging from *m/z* 894.7 to 910.7. The mass shifts (numbers in red) indicate the numbers of total ^{13}C atoms being incorporated into the Lys-Glc-DAG (16:0/18:1) molecule. To locate the incorporated ^{13}C atoms on Lys-Glc-DAG, MS/MS was performed on one of the most abundant ion species. Figure B.1E in Appendix B shows the MS/MS spectrum of *m/z* 885.6 of Lys-Glc-DAG in THB and Figure B.1F in Appendix B shows the MS/MS spectrum of *m/z* 900.7 ion in DM supplemented with D-Glucose-$^{13}C_6$ (containing a total of fifteen ^{13}C atoms). The replacement of glucose by D-Glucose (U-$^{13}C_6$) in Lys-Glc-DAG is evidenced by a 6 Da mass shift of the lysyl-glucosyl moiety from *m/z* 291.1 (Figure B.1E in Appendix B) to *m/z* 297.1 (Figure B.1F in Appendix B). The other nine ^{13}C atoms must be distributed in the diacylglycerol portion of Lys-Glc-DAG. Furthermore, careful comparison of both MS/MS spectra of labeled and unlabeled Lys-Glc-DAG indicates that the lysine residue is linked to the 6-position of glucose; the fragmentation schemes for *m/z* 189.0 (no ^{13}C atoms) (Figure B.1E in Appendix B) and ^{13}C-labeled *m/z* 191.0 (two ^{13}C atoms) (Figure B.1F in Appendix B) are indicated.

Heterologous expression of GBS *mprF* in *S. mitis* results in the production of both Lys-Glc-DAG and Lys-PG.

GBS encode an ortholog of MprF (14). MprF catalyzes the aminoacylation of PG to form Lys-PG and other minor amino-PGs such as Ala-PG and Arg-PG in other bacterial species (15, 16). Our identification of Lys-PG in the cellular membrane of the GBS confirms an active MprF is present in GBS.

To ascertain if the GBS MprF is responsible for the addition of lysine to Glc-DAG to form Lys-Glc-DAG, a heterologous host expression system was designed in *Streptococcus mitis* ATCC 49456, the *S. mitis* type strain. We selected *S. mitis* ATCC 49456 because it does not encode *mprF* and it is naturally transformable. Moreover, our previous lipidomic analysis confirmed that it does not synthesize Lys-PG, but does synthesize Glc-DAG, the precursor of Lys-Glc-DAG, as a major membrane component (23, 26). The gene encoding GBS COH1 *mprF*, GBSCOH1_1931, was cloned into pABG5Δ*phoZ* (27) for expression (this vector is referred to as pABG5 hereafter). As controls, the two *mprF* genes of *Enterococcus faecium* strain 1,231,410, EFTG_00601 and EFTG_02430, were also cloned for expression in *S. mitis*. One *E. faecium mprF* allele (*mprF1*) has been experimentally verified to direct synthesis of Lys-PG, while the second one (*mprF2*) does not (18).

S. mitis strains were cultured overnight in THB before lipidomics was performed. Figure 4.4A-C shows the TIC lipidomic profile of *S. mitis* ATCC 49456(pABG5) empty vector control, ATCC 49456(pEfmMprF1), and ATCC 49456(pGBSMprF). Figure 4.4D-F and 4.4G-I show MS of the

retention times 19.5 – 21.5 min and 26 – 30 min, respectively. Phosphatidylcholine, a previously described membrane lipid in *S. mitis* (23), elutes off the column at roughly the same time as Lys-PG. The empty vector control strain does not synthesize Lys-PG (Figure 4.4D) or Lys-Glc-DAG (Figure 4.4G). In ATCC 49456(pEfmMprF1), expressing EFTG_00601, Lys-PG is observed in the membrane (Figure 4.4E) but no Lys-Glc-DAG is present (Figure 4.4H), which is consistent with previous findings in *E. faecium* (18). As expected, *S. mitis* ATCC 49456(pEfmMprF2) does not produce Lys-PG (Figure B.2 in Appendix B). Finally, ATCC 49456(pGBSMprF), shows a large TIC peak at 26 to 30 min (Figure 4.4C), consistent with Lys-Glc-DAG production. MS spectra indicate Lys-PG is present in the membrane (Figure 4.4F) and Lys-Glc-DAG is produced (Figure 4.4I) with major molecular species at *m/z* 857 and *m/z* 885, similar to what is detected in the GBS membrane. Taken together, these heterologous host expression data in *S. mitis* confirm that GBS MprF synthesizes Lys-PG and possesses the unique and novel activity of modifying Glc-DAG to form Lys-Glc-DAG.

Deletion of *mprF* in GBS results in the loss of synthesis of Lys-PG and Lys-Glc-DAG.

To validate the function of MprF in the synthesis of Lys-Glc-DAG *in vivo*, we deleted GBSCOH1_1931 from the COH1 genome using the sucrose sensitivity selection plasmid pMBSacB (28) and confirmed the deletion by whole genome sequencing. Figure 4.5A shows COH1 wildtype TIC and the abundance of Lys-PG (Figure 4.5D) and Lys-Glc-DAG (Figure 4.5G). The COH1Δ*mprF* TIC shows a complete loss of Lys-PG and Lys-Glc-DAG (Figure 4.5B),

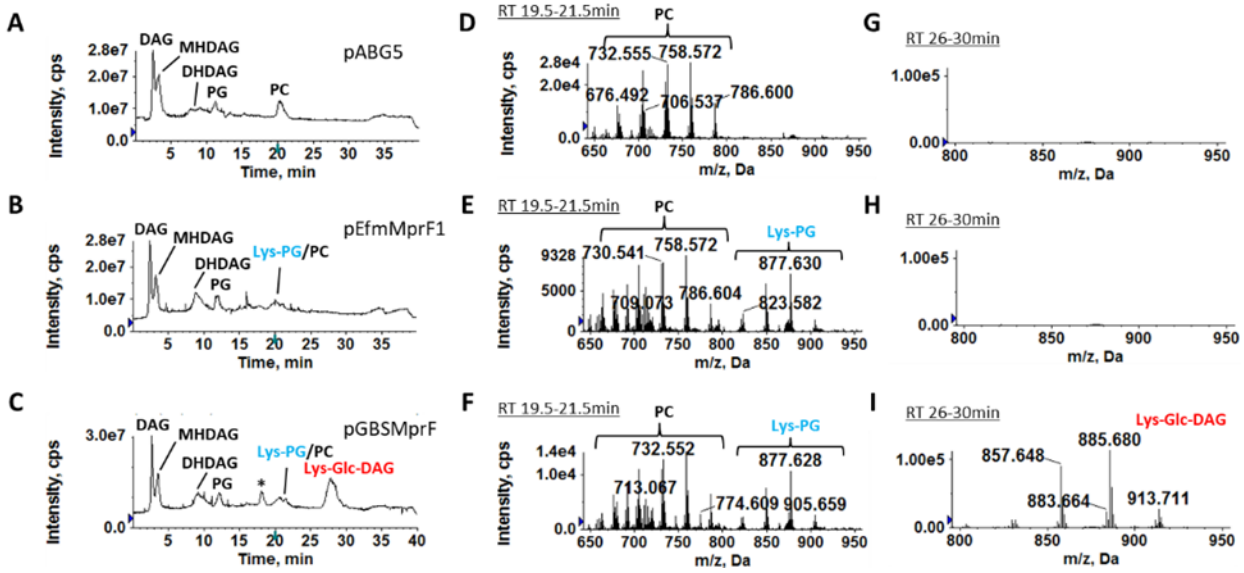

Figure 4.4. Expression of *E. faecium* MprF and GBS MprF in *S. mitis* ATCC 49456. The lipid extracts of *S. mitis* cells with empty vector control (pABG5) or expressing *E. faecium mprF1* (pEfmMprF1) or GBS *mprF* (pGBSMprF) were analyzed by LC-ESI/MS in the positive ion mode. Total ion chromatograms (TIC) of A) *S. mitis* ATCC 49456(pABG5), B) *S. mitis* ATCC 49456(pEfmMprF1), and C) *S. mitis* ATCC 49456(pGBSMprF) show the overall lipidomic profile of each strain. MS of retention time 19.5 – 21.5 min show the presence or absence of Lys-PG in D) *S. mitis* ATCC 49456(pABG5), E) *S. mitis* ATCC 49456(pEfmMprF1), and F) *S. mitis* ATCC 49456(pGBSMprF). Phosphatidylcholine elutes around the same retention time of Lys-PG. Lys-PG is present when *mprF* from either *E. faecium* or GBS are expressed. MS of retention time 26 – 30 min show the presence or absence of Lys-Glc-DAG in G) *S. mitis* ATCC 49456(pABG5), H) *S. mitis* ATCC 49456(pEfmMprF1), and I) *S. mitis* ATCC 49456(pGBSMprF). Lys-Glc-DAG is only synthesized by the GBS MprF, indicating two lipid substrates for the GBS MprF.

supported by loss of ESI/MS spectra of Lys-PG (Figure 4.5E) and Lys-Glc-DAG (Figure 4.5H). The complementation strain, COH1Δ*mprF*(pGBSmprF), has a TIC lipid profile similar to that of the COH1 wild type (Figure 4.5C) and shows reestablishment of both Lys-PG (Figure 4.5F) and Lys-Glc-DAG (Figure 4.5I). Together these data demonstrate that GBS MprF is responsible for synthesizing both Lys-PG and Lys-Glc-DAG *in vivo*. While MprF is well-known to modify anionic PG with lysine and other amino acids, to our knowledge aminoacylation of a neutral lipid

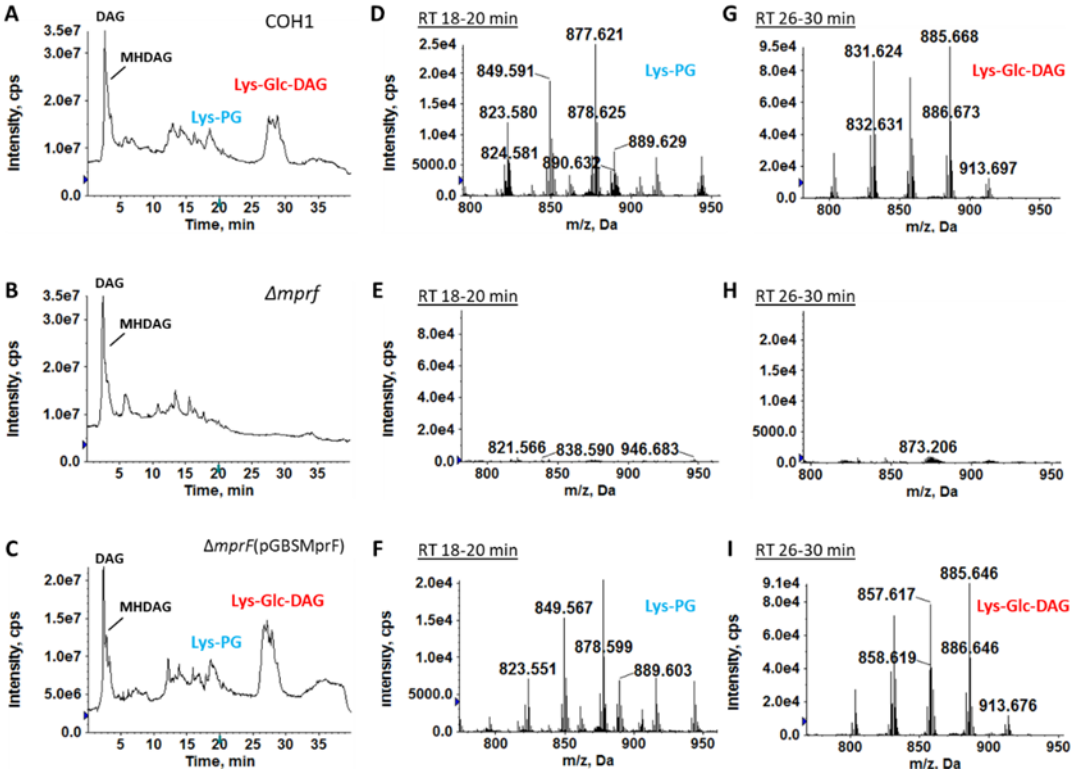

Figure 4.5. Deletion of *mprF* (GBSCOH1_1931) results in the loss of both Lys-PG and Lys-Glc-DAG from GBS. The lipid extracts of COH1 wild-type, Δ*mprF* mutant, and complement (pGBSMprF) strains of *S. agalactiae* COH1 were analyzed by LC-ESI/MS in the positive ion mode. Total ion chromatograms (TIC) of A) COH1 WT, B) Δ*mprF* mutant, and C) Δ*mprF*(pGBSMprF) complement show the overall lipidomic profile of each strain. MS of retention time 18-20 min show the presence or absence of Lys-PG in D) COH1 WT, E) Δ*mprF* mutant, and F) Δ*mprF*(pGBSMprF) complement. Lys-PG is only absent in the Δ*mprF* mutant. MS of retention time 26 – 30 min show the presence or absence of Lys-Glc-DAG in G) COH1 WT, H) Δ*mprF* mutant, and I) Δ*mprF*(pGBSMprF) complement. Lys-Glc-DAG is absent in the Δ*mprF* mutant and restored in the complement strain.

has not been reported. A model of the two biosynthetic pathways in the GBS is shown in Figure 4.6. Overall, these data conclusively demonstrate the novel function of GBS MprF. Moreover, results with the deletion mutant demonstrate that even though Lys-Glc-DAG makes up a relatively large proportion of the total lipid extract, it is non-essential for growth under the laboratory conditions tested here.

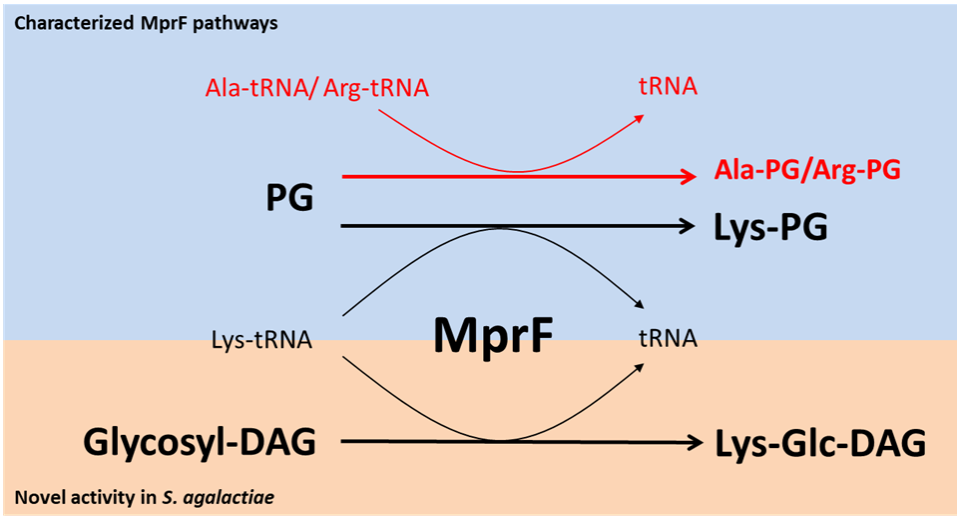

Figure 4.6. Biosynthetic pathways involving MprF. MprF catalyzes the formation of Ala-PG, Arg-PG, and Lys-PG in multiple different bacterial species (blue shading). In this manuscript, we characterize a novel function of MprF in *S. agalactiae* in the formation of Lys-Glc-DAG (orange shading). The biosynthetic pathways of MprF in *S. agalactiae* are denoted in black. Red denotation indicates pathway is not present in *S. agalactiae.*

A GBS *mprF* mutant has altered growth kinetics and grows in longer chains

To assess the impact of the loss of Lys-PG and Lys-Glc-DAG lipids on GBS, growth curves in THB were performed (Figure 4.7A). The COH1Δ*mprF* mutant displays a moderate defect in growth rate when compared to the wild type strain. The COH1Δ*mprF*(pGBSMprF) complement strain did not appear to revert to wild type growth and grew similarly to the Δ*mprF* mutant. A second growth curve experiments were performed that included the COH1(pABG5) empty vector control to assess if the plasmid backbone or kanamycin presence in the medium impacted the growth rate of the complement strain relative to the COH1 and Δ*mprF* strains (Figure 4.7B). There was no observable difference in growth between COH1(pABG5), COH1Δ*mprF*(pABG5), and COH1Δ*mprF*(pGBSMprF) strains, indicating that the presence of the pABG5 vector normalizes

growth kinetics among these strains. The maximum growth yield based on the highest optical density recorded during the growth curves was the same for each strain (Figure 4.7A and 4.7B).

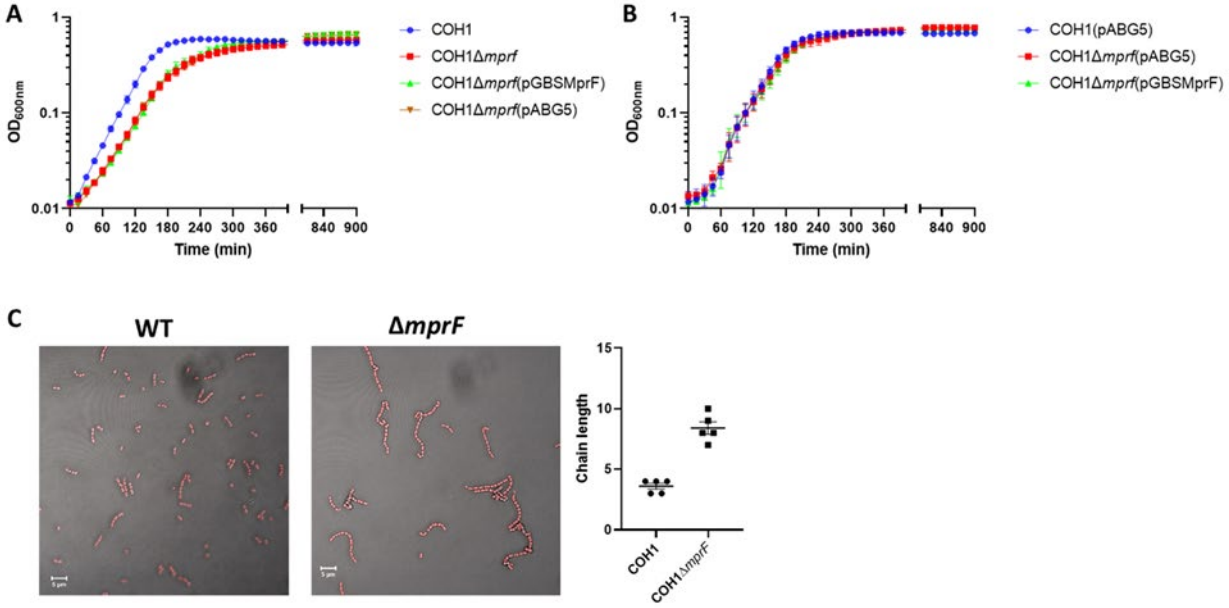

Figure 4.7. Growth curves and chain length. A) Growth curves of WT (blue) COH1Δ*mprF* mutant (red), COH1Δ*mprF*(pGBSMprF) complement (green), and COH1Δ*mprF* mutant empty vector (orange). The Δ*mprF* mutant exhibits a growth defect compared to the WT. B) Growth curves of COH1(pABG5) (blue), COH1Δ*mprF*(pABG5) (red), and COH1Δ*mprF*(pGBSMprF) (green). The empty vector and complement strain had no observable growth defect. C) Representative image of stationary phase COH1 WT and Δ*mprF* mutant stained with membrane dye FM4-64 and manual counts of the number of diplococci in each chain imaged in the 5 images taken from each slide. Scale bar is 5 μm. A) and B) mean with SD, and C) mean with SEM.

Confocal imaging of the COH1Δ*mprF* mutant and wild type after overnight growth, stained with the membrane dye FM4-64, revealed that the Δ*mprF* mutant grows in chains that are approximately twice as long compared to the wild type (Figure 4.7C). Chain length has been documented to alter virulence potential in other streptococci and could have an impact on GBS (29, 30). The chain length of streptococci is often controlled by cell wall and capsule biosynthesis enzymes (31–33), and additional unidentified factors (34). The increased chain lengths observed

in the Δ*mprF* mutant is like that seen in a GBS Δ*cpsA* mutant; *cpsA* encodes a membrane-bound transcriptional regulator involved in capsule biosynthesis (33). These data suggest that the disruption of the cellular membrane homeostasis by the removal of Lys-PG and Lys-Glc-DAG has a physiological effect on the cell by potentially displacing, directly or indirectly, enzymes associated with broader cell processes such as cell division.

The GBS lysine lipids mask negative charges and impact acid stress survival

The addition of lysine to PG masks negative membrane charges to help prevent bacterial killing by CAMPs and antibiotics (15). The loss of Lys-PG and Lys-Glc-DAG from the GBS membrane is likely to alter the net charge of the outer surface of GBS cells. A Cytochrome C binding assay was performed as a measure of the overall net charge of the outer surface of the cells (Figure 4.8A). Cytochrome C is positively charged, and more Cytochrome C bound to cells indicates a more negatively charged outer surface. COH1Δ*mprF* cells have a significantly increased net negative charge, with ~61% Cytochrome C bound, compared to COH1 wild type cells with ~47% Cytochrome C bound. When the *mprF* deletion is complemented, the net outer surface charge is similar to wild type levels, with ~50% Cytochrome C bound. The data indicate that the Lys-PG and/or the novel Lys-Glc-DAG lipid alter the outer surface charge of the GBS cell, making the outer surface more positively charged.

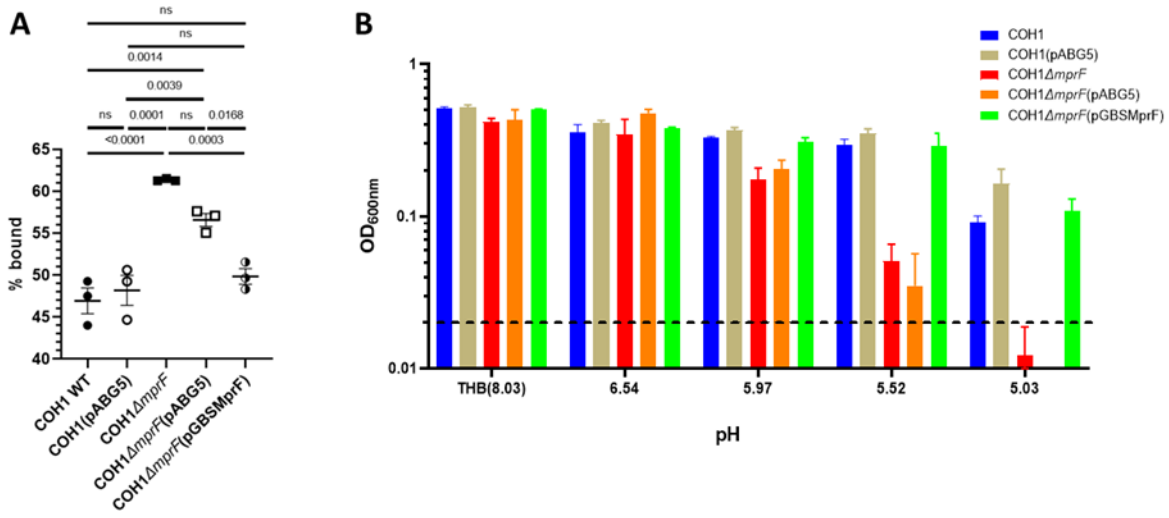

Figure 4.8. Physiological analyses of the GBS *mprF* mutant. A) Mean percent Cytochrome C bound to COH1 WT, WT containing the empty vector pABG5, Δ*mprF* mutant, Δ*mprF* mutant containing the empty vector, and the pGBSMprF complement strains. The Δ*mprF* mutant displays significantly more Cytochrome C bound to the cells, indicating a more net negative outer surface charge. One-way ANOVA with Tukey's multiple comparisons test, p-values indicated, mean and SEM shown. B) Growth yields (OD_{600nm}) after overnight culture in pH-adjusted THB broth. The Δ*mprF* mutant is more susceptible to the lower pH and fails to grow at pH 5 compared to the WT and complement. Mean with SD indicated. Dashed line represents starting inoculum of ~OD_{600nm} 0.02.

The human vaginal tract usually is pH 4.5 - 5.2, and protective vaginal commensals such as lactobacilli acidify their surrounding environment (35, 36). Lys-PG aids in acidic stress survival in *E. faecium* (37, 38). To investigate if Lys-PG and/or the novel Lys-Glc-DAG lipid aid survival in acid stress environments, COH1, COH1(pABG5), COH1Δ*mprF*, COH1Δ*mprF*(pABG5), and COH1Δ*mprF*(pGBSMprF) strains were grown in pH-adjusted THB (Figure 4.8B). The COH1Δ*mprF* and COH1Δ*mprF*(pABG5) strains begin to show growth inhibition at pH 6, and a pronounced growth inhibition at pH 5.5 compared to the wild type and complement strains. No growth was observed at pH 5 for COH1Δ*mprF* and COH1Δ*mprF*(pABG5); however, the wild

type and complement strains grew. Taken together, these data indicate that lysine lipids aid GBS survival at lower pH, and therefore may impact their ability to colonize and compete in the vaginal tract.

Lysine lipids are involved in GBS interactions with human cells *in vitro*

GBS are lysine auxotrophs (39). In the human host, L-lysine has been quantified in varying concentrations throughout the body, from ~43 μM in cerebrospinal fluid to ~441 μM in blood (40). To confirm the production of Lys-PG and Lys-Glc-DAG under low lysine concentrations, a lysine titration was performed in DM. At 50 μM lysine concentration, the GBS growth was reduced and reached an overnight OD_{600nm} of ~0.36, much lower compared to normal overnight growth to ~OD_{600nm} 1.75 with 600 μM lysine. Lys-PG and Lys-Glc-DAG were always present in the cellular membrane, but their levels varied in a lysine-dependent manner at concentrations ranging from 50 μM to 600 μM (Figure 4.9A-E). These data demonstrate that, even in low lysine conditions in the human body, we expect Lys-PG and Lys-Glc-DAG to be present and have a physiological role in the GBS membrane.

Next, we determined whether loss of Lys-PG and Lys-Glc-DAG altered human cell adherence and invasion. To mimic vaginal tract colonization, the vaginal epithelial cell line VK2/E6E7 was used. Similarly, to mimic the human blood-brain barrier, the human cerebral microvascular endothelial cell line hCMEC/D3 was utilized. *In vitro* assays for adherence and invasion were performed using pre-established protocols (Figure 4.10) (41, 42). To limit the number of strains used and to control

for the presence of the plasmid backbone in the complement strain, COH1(pABG5), COH1Δ*mprF*(pABG5), and COH1Δ*mprF*(pGBSMprF) were used. Significant decreases in

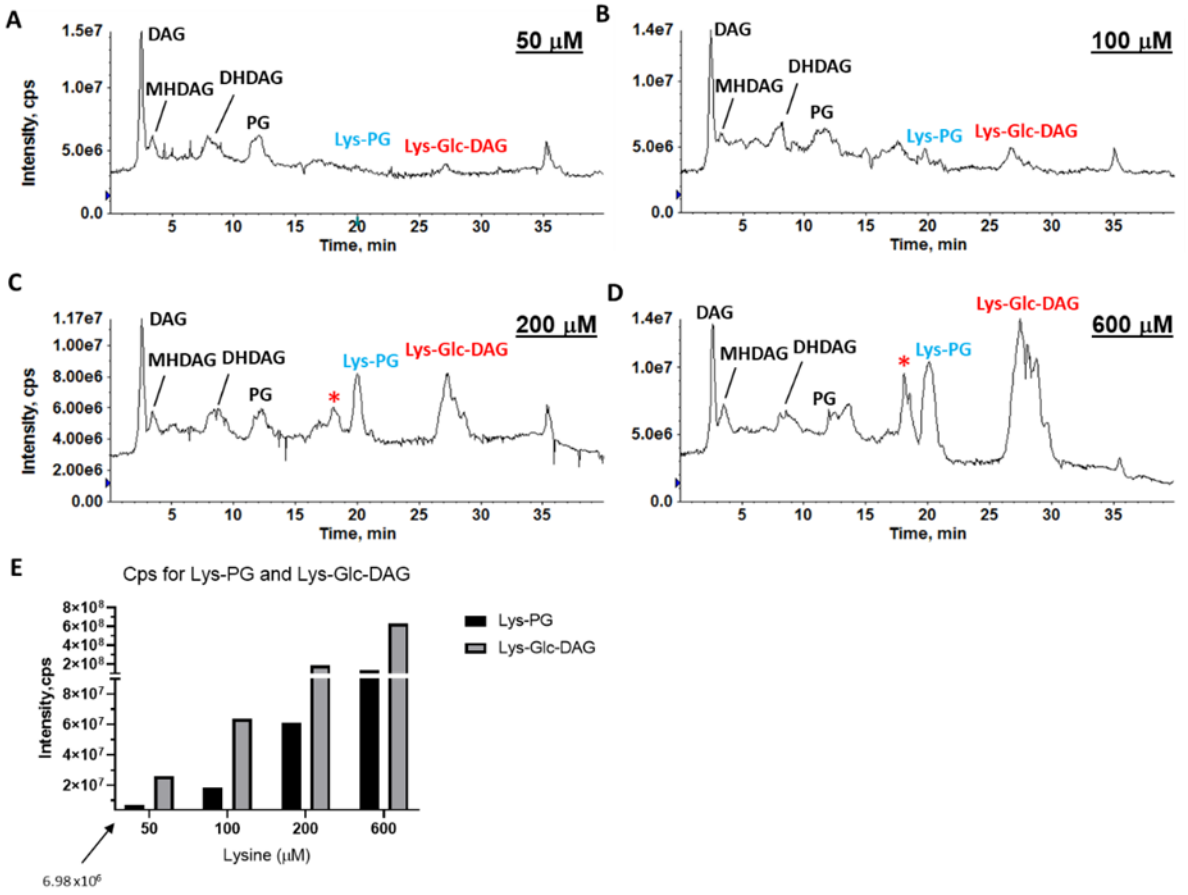

Figure 4.9. Effect of lysine concentration on Lys-Glc-DAG and Lys-PG synthesis by GBS. Shown are positive TICs of LC-ESI/MS of lipid extracts of *S. agalactiae* COH1 cultured in defined growth media containing A) 50 µM lysine, B) 100 µM lysine, C) 200 µM lysine and D) 600 µM lysine. Lys-Glc-DAG and Lys-PG levels in *S. agalactiae* COH1 positively correlate with lysine concentration in the growth medium. "*" denotes methylcarbamate, an extraction artifact of Lys-Glc-DAG. E) CPS counts from mass spectra for Lys-PG and Lys-Glc-DAG molecular species at each concentration.

adherence to the VK2 vaginal epithelial cells (Figure 4.10A) and invasion of both VK2 (Figure 4.10B) and hCMEC microvascular endothelial cells (Figure 4.10C) were observed for the Δ*mprF*

mutant. Hemolytic activity of the GBS is a known virulence factor, and no difference in hemolytic activity was observed between COH1 and COH1Δ*mprF* (Figure 4.10D). Taken together, these data indicate that the lysine lipids influence the ability of GBS to attach to epithelial cells of the vaginal tract and invade host cells.

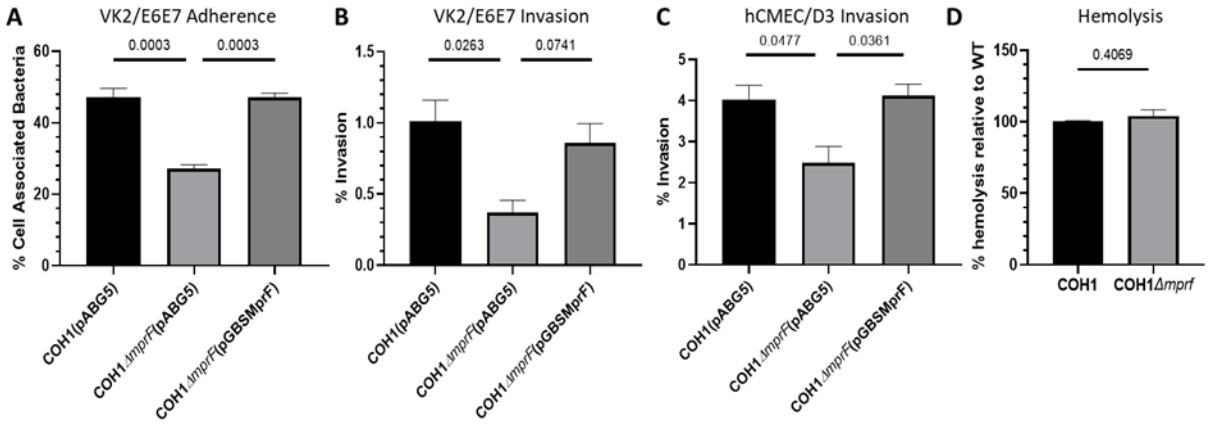

Figure 4.10. *In vitro* adherence and invasion of vaginal and endovascular cell lines, and hemolytic activity of Δ*mprF* mutant. COH1(pABG5), Δ*mprF*(pABG5), and complement strain Δ*mprF*(pGBSMprF) were tested for A) adherence and B) invasion of the vaginal epithelial VK2/E6E7 cell line, and C) invasion of cerebral microvascular endothelial hCMEC/D3 cells. D) Hemolytic activity of wild type and Δ*mprF* mutant. The Δ*mprF*(pABG5) strain displays a significant decrease of adherence to VK2/E6E7 cells and significant decrease in invasion in both VK2/E6E7 and hCMEC/D3 cell lines. *In vitro* tissue culture assay results are the mean of three replicate experiments, each with four technical replicates. Hemolytic assay was performed in biological triplicate, each with three technical replicates. A-D) Mean and SEM indicated. Statistical analyses: A-C) One-way ANOVA with Tukey's multiple comparisons test, D) unpaired two-tailed t-test, p-values indicated on figures.

4.4 Discussion

The cellular membrane is a critically understudied site of interaction between Gram-positive pathogens and their host. In this study, we used LC-ESI/MS to characterize the lipid membrane of varying serotypes of the GBS and identified Lys-Glc-DAG, a novel lysine modification on the

glycolipid Glc-DAG, which comprises a relatively large proportion of the cellular lipid extract. We show that MprF is responsible for the addition of lysine to both Glc-DAG and PG, thus establishing a novel lipid substrate for MprF. We identified physiological roles these lysine lipids, Lys-PG and novel Lys-Glc-DAG, play in the cellular membrane by masking negative charges and their contribution to tolerance of acidic environments which is relevant to vaginal colonization. Furthermore, using tissue culture adherence and invasion assays, we show that the lipids contribute to the colonization and virulence potential of the GBS within the human host. To our knowledge, this is the first description of the lysynylated glycolipid, Lys-Glc-DAG, and a glucosyl-DAG substrate for an MprF enzyme.

Synthesis of Lys-PG and Lys-Glc-DAG by MprF indicates a complex balance of interconnected substrate usage including amino acid, glycolipid, and phospholipid substrates. Lys-tRNAs are required by translational machinery for protein synthesis and are essential for survival; unlike *S. aureus*, a canonical organism for studying MprF function, GBS are lysine auxotrophs. Therefore, in GBS, both MprF and the ribosome would compete for lysine scavenged from the environment. Moreover, the Glc-DAG lipid, which is a substrate for GBS MprF, is also required for biosynthesis of the LTA anchor Glc_2-DAG; LTA is a critical polymer for binding host cells, repulsion of CAMPs, and is required for invasion of the blood-brain barrier (2, 13, 43). Finally, GBS MprF acts on two substrates, PG and Glc-DAG, to synthesize two distinct lysine-modified lipids, the levels of all of which must be balanced to maintain appropriate membrane charge. In *E. faecium*, the hydrolase encoded immediately upstream of *mprF* is responsible for the turnover of Lys-PG in the membrane, thus balancing the amount of Lys-PG present (37). A similar mechanism is likely

present in GBS to maintain the balance between both Lys-PG and Lys-Glc-DAG and their respective non-lysine decorated lipids. GBSCOH1_1932, immediately upstream of the GBS *mprF*, contains the hydrolase domain, MhpC (COG0596), indicating that it may regulate Lys-PG levels in the membrane; its activity on Lys-Glc-DAG is unknown. Overall, much remains to be determined about MprF activity and regulation in GBS.

Currently, we are unable to decouple the functional roles of the two lysine-modified lipids as we show they are both synthesized by the GBS MprF. We have previously analyzed *E. faecium* lipids using the same methods here, and we experimentally confirmed that *E. faecium* synthesizes Lys-PG but not Lys-Glc-DAG (44). Swapping domains and regions between *E. faecium* MprF1 and GBS MprF may help to localize the region of GBS MprF responsible for binding the Glc-DAG substrate and/or generate variants of GBS MprF that synthesize only one of the two lysine-modified lipids. Decoupling the synthesis of Lys-PG and Lys-Glc-DAG will allow further identification of the specific roles each lipid plays in membrane function and pathogenesis. More broadly, *mprF* is not widely distributed among the *Streptococcus* genus, with only three other species encoding it. This suggests that MprF and the lysine-modified lipids it synthesizes are especially important in the niches GBS colonizes.

Why did the GBS evolve to use MprF to lysinylate Glc-DAG? The GBS are lysine auxotrophs. The use of such an important substrate, required by GBS for protein synthesis, to decorate the extracellular cell surface is intriguing, especially under low lysine conditions *in vivo*. One possible explanation is to mask the recognition of Glc-DAG, an experimentally verified microbial antigen,

from natural killer T cells (45, 46). A second possible explanation is to increase resistance to neutrophil killing, as Lys-PG confers resistance to CAMPs in *S. aureus* (15); a role for GBS MprF in CAMP resistance was not observed in a previous study *in vitro* (14) but it may very well contribute under *in vivo* conditions. Finally, acid stress response that is greater with both lipids verses Lys-PG alone may not only aid in colonization but also increase the survival time in phagosomes where acidification is critical for bacterial killing (47).

The fact that Lys-Glc-DAG comprises a relatively large proportion of the GBS cellular lipid extract, and the unique signature of the Lys-Glc-DAG lipid head group, hold promise that Lys-Glc-DAG could be an efficient molecular target for a rapid diagnostic test that does not rely on culturing techniques or nucleic acid amplification.

Overall, our work shows the importance of the lipidome during pathogen-host interactions. Our identification of the novel Lys-Glc-DAG glycolipid and the joint role it plays with Lys-PG in GBS colonization and pathogenesis rationalizes further study of the lipidomes of human pathogens.

4.5 Materials and methods

Bacterial strains, media, and growth conditions.

Strains and plasmids used in this study are shown in Table 4.3. *Streptococcus agalactiae* strains were grown statically at 37°C in Todd-Hewitt Broth (THB) and *S. mitis* strains were grown statically at 37°C and 5% CO_2, unless otherwise stated. Streptococcal chemically defined medium (48) was diluted from stock as described (49) with 1% w/v glucose (referred to as DM), slightly

modified from (50), unless otherwise stated. *Escherichia coli* strains were grown in Lysogeny Broth (LB) at 37°C with rotation at 225 rpm. Kanamycin and erythromycin (Sigma-Aldrich) were supplemented to media at 50 µg/mL and 300 µg/mL for *E. coli,* respectively, or 300 µg/mL and 5 µg/mL, respectively, for streptococcal strains.

Table 4.3. Strains and plasmids used in this study.

Organism	Strain	Description	Ref
S. agalactiae	ATCC BAA-1176 (COH1)	Wild-type *S. agalactiae* strain, serotype III	(51)
	COH1Δ*mprF*	*mprF* (GBSCOH1_1931) deletion strain	This work
	COH1Δ*mprF(*pABG5)	Empty vector control strain	This work
	COH1Δ*mprF*(pGBSMprf)	Expresses GBS *mprF* from P_{prtF} in pABG5Δ*phoZ*	This work
	COH1(pABG5)	Empty vector control	This work
	ATCC BAA-1138 (A909)	Wild-type *S. agalactiae* strain, serotype Ia	(52)
	CNCTC 10/84	Wild-type *S. agalactiae* strain, serotype V. Obtained from Dr. K Patras, UCSD	(53, 54)
S. mitis	ATCC 49456	Wild-type *S. mitis* type strain, also known as NCTC 12261	(55)
	ATCC 49456(pABG5)	Empty vector control	This work
	ATCC 49456(pGBSMprF)	Expresses GBS *mprF* from P_{prtF} in pABG5Δ*phoZ*	This work
	ATCC 49456(pEfmMprF1)	Expresses *E. faecium mprF1* from P_{prtF} in pABG5Δ*phoZ*	This work
	ATCC 49456(pEfmMprF2)	Expresses *E. faecium mprF1* from P_{prtF} in pABG5Δ*phoZ*	This work
E. coli	DH5α	Plasmid cloning host; F^-, φ80*lacZ*ΔM15, *rec*A1, *end*A1, *hsd*R17, *pho*A, *sup*E 44, λ^- *thi*-1, *gyr*A96, *rel*A1	(56)
	DH5α(pABG5)	Empty vector control	This work
	DH5α(pGBSMprF)	Expresses COH1 *mprF* (GBSCOH1_1931) from P_{prtF} in pABG5Δ*phoZ*	This work
	DH5α(pEfmMprF1)	Expresses *E. faecium mprF1* from P_{prtF} in pABG5Δ*phoZ*	This work
	DH5α(pEfmMprF2)	Expresses *E. faecium mprF1* from P_{prtF} in pABG5Δ*phoZ*	This work
	DH5α(pMBMprFKO)	Allelic exchange plasmid containing ~2 kb sequence flanking GBSCOH1_1931	This work
E. faecium	1,231,410	Wild type *E. faecium* strain	(57)

Plasmid	Description	Ref
pABG5Δ*phoZ*	Constitutive expression vector for streptococci with the P_{prtF} promoter. Confers kanamycin resistance. Referred to as pABG5 throughout the text	(27)
pGBSMprF	pABG5Δ*phoZ* expressing COH1 *mprF* (GBSCOH1_1931) from P_{prtF}	This work
pEfmMprF1	pABG5Δ*phoZ* expressing *E. faecium* 1,231,410 *mprF1* (EFTG_00601) from P_{prtF}	This work
pEfmMprF2	pABG5Δ*phoZ* expressing *E. faecium* 1,231,410 *mprF2* (EFTG_02430) from P_{prtF}	This work
pMBSacB	Allelic exchange plasmid for *S. agalactiae*. Confers erythromycin resistance and sucrose sensitivity	(28)

Routine molecular biology techniques

All PCR reactions utilized Phusion polymerase (Thermo Fisher) and all primers are shown in Table B1 in Appendix B. PCR products and restriction digest products were purified using GeneJET PCR purification kit (Thermo Fisher) per manufacturer protocols. Plasmids were extracted using GeneJET plasmid miniprep kit (Thermo Fisher) per manufacturer protocols. Restriction enzyme digests utilized XbaI, XhoI, and PstI (New England Biolabs) for 3 h at 37°C in a water bath. Ligations utilized T4 DNA ligase (New England Biolabs) at 16°C overnight or Gibson Assembly Master Mix (New England Biolabs) per manufacturer protocols where stated. All plasmid constructs were sequence confirmed by Sanger sequencing (Massachusetts General Hospital DNA Core).

Deuterated lysine and C^{13}-D-glucose isotope tracking

A *S. agalactiae* COH1 colony was inoculated into 15 mL of DM containing 450 µM lysine-*d4* (Cambridge Isotopes Laboratories) or a single COH1 colony was inoculated into 10 mL DM supplemented with 0.5% w/v C^{13}-D-glucose (Cambridge Isotopes Laboratories) for overnight growth for lipidomic analysis described below.

Construction of MprF expression plasmids

Genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue kit per the manufacturer's protocol with the exception that cells were pre-treated with 180 µL 50 mg/mL lysozyme, 25 µL 2500 U/mL mutanolysin, and 15 µL 20 mg/mL pre-boiled RNase A and incubated at 37°C for 2 h. The *mprF* genes from *S. agalactiae* COH1, (GBSCOH1_1931), and *E. faecium* 1,231,410

(EFTG_00601 and EFTG_02430) were amplified and Gibson ligated into pABG5Δ*phoZ* (27). Plasmid constructs were transformed into chemical competent *E. coli* DH5α cells. Briefly, chemically competent cells were incubated for 10 min on ice with 5 µL of Gibson reaction before heat shock at 42°C for 70 sec, then placed on ice for 2 min before 900 µL of cold SOC medium was added. Outgrowth was performed at 37°C, with shaking at 225 rpm, for 1 h. Cultures were plated on LB agar plates containing 50 µg/mL kanamycin. Colonies were screened by PCR for presence of the *mprF* insert.

Expression of *mprF* in *S. mitis*

Natural transformation was performed as previously described (23). Briefly, precultures were thawed at room temperature, diluted in 900 µL of THB, further diluted 1:50 in prewarmed 5 mL THB, and incubated for 45 min at 37°C. 500 µL of culture was then aliquoted with 1 µL of 1 mg/mL competence-stimulating peptide (EIRQTHNIFFNFFKRR) and 1 µg/mL plasmid. Transformation reaction mixtures were cultured for 2 h at 37°C in microcentrifuge tubes before being plated on THB agar supplemented with 300 µg/mL kanamycin. Single transformant colonies were cultured in 15 mL THB overnight. PCR was used to confirm the presence of the *mprF* insert on the plasmid. Plasmids were extracted and sequence confirmed as described above. Lipidomics was performed as described below in biological triplicate.

Construction of *mprF* deletion plasmid.

Regions ~2 kb upstream and downstream of the *S. agalactiae* COH1 *mprF* (GBSCOH1_1931) were amplified using PCR (Table B.1 in Appendix B). Plasmid, pMBSacB (28), and the PCR

products were digested using appropriate restriction enzymes and ligated overnight. 7 µL of the ligation reaction was transformed into chemically competent *E. coli* DH5α as described above, except that outgrowth was performed at 28°C with shaking at 225 rpm for 90 min prior to plating on LB agar supplemented with 300 µg/mL erythromycin. Plates were incubated at 28°C for 72 h. Colonies were screened by PCR for correct plasmid construction. Positive colonies were inoculated into 50 mL LB media containing antibiotic and incubated at 28°C with rotation at 225 rpm for 72 h. Cultures were pelleted using a Sorvall RC6+ centrifuge at 4,280 x *g* for 6 min at room temperature. Plasmid was extracted as described above except the cell pellet was split into 5 columns to prevent overloading and serial eluted into 50 µL. Plasmid construction was confirmed via restriction digest using XhoI and XbaI, and the insert was PCR amplified and sequence-verified.

Generation of electrocompetent *S. agalactiae* COH1 cells for knockout.

Electrocompetent cells were generated as described (28) with minor modifications. Briefly, a *S. agalactiae* COH1 colony was inoculated in 5 mL M17 medium (BD Bacto) with 0.5% glucose and grown overnight at 37°C. The 5 mL was used to inoculate a second overnight culture of 50 mL pre-warmed filter-sterilized M17 medium containing 0.5% glucose, 0.6% glycine, and 25% PEG 8000. The second overnight was added to 130 mL of the same medium and grown for 1 h at 37°C. Cells were pelleted at 3,200 x *g* in a Sorvall RC6+ at 4°C for 10 min. Cells were washed twice with 25 mL cold filter-sterilized GBS wash buffer containing 25% PEG 8000 and 10% glycerol in water, and pelleted as above. Cell pellets were re-suspended in 1 mL GBS wash buffer and either used immediately for transformation or stored in 100 µL aliquots at -80°C until use.

Deletion of *S. agalactiae* COH1 *mprF*.

The double crossover homologous recombination knockout strategy was performed as described previously (28, 41, 58) with minor modifications. 1 µg of plasmid was added to electrocompetent COH1 cells and transferred to a cold 1 mm cuvette (Fisher). Electroporation was carried out at 2.5 kV on an Eppendorf eporator. 1 mL of THB containing 12.5% PEG 8000, 20 mM $MgCl_2$, and 2 mM $CaCl_2$ was immediately added and then the entire reaction was transferred to a glass culture tube. Outgrowth was at 28°C for 2 h followed by plating on THB agar supplemented with 5 µg/mL erythromycin. Plates were incubated for 48 h at 28°C. A single colony was cultured overnight in 5 mL THB with 5 µg/mL erythromycin at 28°C. The culture was screened via PCR for the plasmid insert with the initial denaturing step extended to 10 min. The overnight culture was diluted 1:1000 THB containing 5 µg/mL erythromycin and incubated overnight at 37°C to promote single cross over events. The culture was then serial diluted and plated on THB agar plates with antibiotic and incubated at 37°C overnight. Colonies were screened for single crossover events by PCR (Table B.1 in Appendix B). Single crossover colonies were inoculated in 5 mL THB at 28°C to promote double crossover events. Overnight cultures were diluted 1:1000 into 5 mL THB containing sterile 0.75 M sucrose and incubated at 37°C. Overnight cultures were serial diluted and plated on THB agar and incubated at 37°C overnight. Colonies were patched onto THB agar with and without 5 µg/mL erythromycin to confirm loss of plasmid. Colonies were also screened by PCR (Table B.1 in Appendix B) for the loss of *mprF*. Colonies positive for the loss of *mprF* were inoculated into 5 mL THB at 37°C. Cultures were stocked and gDNA extracted as described above, with minor modifications. Sequence confirmation of the *mprF* knockout was done via Sanger sequencing (Massachusetts General Hospital DNA Core) using primers in Table B.1 in Appendix B. The

mutant was grown overnight in 15 mL THB at 37°C and pelleted at 6,150 x *g* for 5 min in a Sorvall RC6+ centrifuge at room temperature for lipid extraction as described below. Genomic DNA of COH1Δ*mprF* was isolated as described above and whole genome sequencing was performed in paired-end reads (2 by 150 bp) on the Illumina NextSeq 550 platform at the Microbial Genome Sequencing Center (Pittsburgh, PA).

Complementation of *mprF* in COH1Δ*mprF*.

Electrocompetent COH1Δ*mprF* were generated as previously described (59). Briefly, COH1Δ*mprF* was inoculated into 5 mL THB with 0.6% glycine and grown overnight. The culture was expanded to 50 mL in pre-warmed THB with 0.6% glycine and grown to an OD_{600} nm of 0.3 and pelleted for 10 min at 3200 x *g* at 4°C in a Sorvall RC6+ floor centrifuge. The pellet was kept on ice through the remainder of the protocol. The pellet was washed twice with 25 mL and once with 10 mL of cold 0.625 M sucrose and pelleted as above. The cell pellet was resuspended in 400 µL of cold 20% glycerol, aliquoted in 50 µL aliquots, and used immediately or stored at -80°C until use. Electroporation was performed as described above, with recovery in THB supplemented with 0.25 M sucrose, and plated on THB agar with kanamycin at 300 µg/mL.

Acidic Bligh-Dyer extractions.

Centrifugation was performed using a Sorvall RC6+ centrifuge. Cultures were pelleted at 4,280 x *g* for 5 min at room temperature unless otherwise stated. The supernatants were removed, and cell pellets were stored at -80°C until acidic Bligh-Dyer lipid extractions were performed as described (23). Briefly, cell pellets were resuspended in 1X PBS (Sigma-Aldrich) and transferred to Coring

Pyrex glass tubes with PTFR-lined caps (VWR), followed by 1:2 vol:vol chloroform:methanol addition. Single phase extractions were vortexed periodically and incubated at room temperature for 15 minutes before 500 x *g* centrifugation for 10 min. A two-phase Bligh-Dyer was achieved by addition of 100 µL 37% HCl, 1 mL $CHCl_3$, and 900 µL of 1X PBS, which was then vortexed and centrifuged for 5 min at 500 x *g*. The lower phase was removed to a new tube and dried under nitrogen before being stored at -80°C prior to lipidomic analysis.

Liquid Chromatography/Electrospray Ionization Mass Spectrometry.

Normal phase LC-ESI/MS was performed on an Agilent 1200 quaternary LC system equipped with an Ascentis silica high-performance liquid chromatography (HPLC) column (5 µm; 25 cm by 2.1 mm; Sigma-Aldrich) as described previously (22, 60). Briefly, mobile phase A consisted of chloroform-methanol-aqueous ammonium hydroxide (800:195:5, vol/vol), mobile phase B consisted of chloroform-methanol-water-aqueous ammonium hydroxide (600: 340:50:5, vol/vol), and mobile phase C consisted of chloroform-methanol-water-aqueous ammonium hydroxide (450:450:95:5, vol/vol/vol/vol). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min, then linearly increased to 100% mobile phase B over 14 min, and held at 100% mobile phase B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min, held at 100% mobile phase C for 3 min, and, finally, returned to 100% mobile phase A over 0.5 min and held at 100% mobile phase A for 5 min. The LC eluent (with a total flow rate of 300 mL/min) was introduced into the ESI source of a high-resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). Instrumental settings for negative-ion ESI and MS/MS analysis of lipid species were as follows: IS = -4,500 V, CUR = 20 psi, GSI

= 20 psi, DP = -55 V, and FP = -150V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst TF1.5 software (Sciex, Framingham, MA).

Growth curves

Overnight cultures were used to inoculate a 96 well flat-bottom plate (Falcon) at an OD_{600nm} of ~0.02 in a final volume of 200 µL THB. Plates were grown in a Biotek MX Synergy 2.0 plate reader at 37°C with OD_{600nm} readings every 15 minutes for 15 h. Growth curves were performed in biological triplicate.

pH-adjusted THB growth

Approximately 30 mL of fresh THB were adjusted to different pH values, measured using a Mettler Toledo FiveEasy pH/MV meter, and sterile-filtered using 0.22 µM syringe filters. A final volume of 200 µL culture medium was aliquoted per well in a flat-bottom 96 well plate (Falcon); culture media were not supplemented with antibiotics. Overnight cultures of GBS strains were used to inoculate the wells to a starting OD_{600nm} 0.02 per well. Plates were incubated for 24 h at 37°C before OD_{600nm} was read using a BioTek MX Synergy 2 plate reader. This experiment was performed in biological triplicate.

Hemolytic assay

Hemolytic assays were performed as described (42) with minor modifications. Briefly, strains were grown to an OD_{600nm} of ~0.4, pelleted at 16,000 x *g* in an Eppendorf 5415D tabletop centrifuge, and resuspended in PBS. ~8-9 x 10^7 CFU in 20 µL PBS was added to 230 µL fresh

sheep blood (VWR) in U-bottom 96-well plates (Corning) for a final volume of 250 µL per well. Control wells were treated with 20 µL sterile water. The plates were sealed and incubated at 37°C with rotation at 100 rpm for 1 h. The plates were centrifuged at 400 x g for 10 min in an Eppendorf 5810 tabletop centrifuge, and 100 µL of the supernatant was transferred to a flat-bottom 96-well plate (Falcon). 50 µL was diluted 1:2 in PBS and the absorbance at 541 nm (A_{541}) was read using a BioTek MX Synergy 2 plate reader. Percent hemolysis was calculated by comparing the A_{541} of GBS treated wells to those of wells treated with water. Biological and technical replicates were performed for each strain.

Cytochrome C assay

Cytochrome C assay was performed as described previously (61, 62) with minor modifications. Briefly, overnight cultures of strains were diluted to an OD_{600nm} of 0.05 in 20 mL prewarmed THB, with no antibiotic, and grown to mid exponential phase (OD_{600nm} between 0.48 – 0.6) before cells were pelleted to an OD_{600nm} of 7 in 1 mL and washed twice with morpholinepropanesulfonic acid (MOPS) at pH 7. Cells were resuspended in 1 mg/mL Cytochrome C (Sigma) in MOPS buffer and incubated for 10 min at room temperature. Cells were pelleted at 16,000 x *g* for 3 min in a tabletop centrifuge, supernatant transferred to disposable spectrophotometer cuvette (Fisher), and absorbance read at 535nm (A_{535}) using a Thermo Scientific Genesys 30 spectrophotometer. The control A_{535} was 1 mg/mL Cytochrome C in MOPS buffer with no cells present, which was also incubated and centrifuged. % bound was calculated using the following formula: (100 – (A_{535}/Control A_{535})) * 100. Biological triplicate was used for each strain.

hCMEC and VK2 cell adherence and invasion assay

Human Cerebral Microvascular Endothelial cells hCMEC/D3 (obtained from Millipore) were grown in EndoGRO-MV complete media (Millipore, SCME004) supplemented with 5% fetal bovine serum (FBS) and 1 ng/mL fibroblast growth factor-2 (FGF-2; Millipore). Human vaginal epithelial VK2/E6E7 (obtained from American Type Culture Collection (ATCC) (CRL-2616)) cells were grown in keratinocyte serum-free medium (KSFM) (Gibco) supplemented with 0.5 ng/mL human recombinant epidermal growth factor and 0.05 mg/mL bovine pituitary extract. Cells were grown in tissue culture treated 24 well plates and 5% CO_2 at 37°C.

Assays to determine the total number of bacteria adhered to host cells or intracellular bacteria were performed as described previously (41, 42). Briefly, bacteria were grown to mid log phase (OD_{600nm} 0.4-0.5) and normalized to 1 x 10^8 to infect cell monolayers at a multiplicity of infection (MOI) of 1 (1 x 10^5 CFU per well). The total cell-associated GBS were recovered after 30 min incubation. Cells were washed slowly five times with 500 µL 1X PBS (Sigma) and detached by addition of 100 µL of 0.25% trypsin-EDTA solution (Gibco) and incubation for 5 min before lysing the eukaryotic cells with the addition of 400 µL of 0.025% Triton X-100 (Sigma) and vigorous pipetting. The lysates were then serially diluted and plated on THB agar and incubated overnight to determine CFU. Bacterial invasion assays were performed as described above except infection plates were incubated for 2 h before incubation with 100 µg gentamicin (Sigma) and 5 µg penicillin (Sigma) supplemented media for an additional 2 h to kill all extracellular bacteria, prior to being trypsinized, lysed, and plated as described. Experiments were performed in biological triplicate with four technical replicates per experiment.

Membrane dye and imaging of GBS

Overnight cultures were gently resuspended 5 times by pipette. 19 µL culture was removed into sterile Eppendorf tubes. 1 µL of 1 µg/mL FM4-64 (N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide) membrane dye (Invitrogen) was added to each tube. The culture and dye were dropped onto a 0.75 mm 0.75% agarose pad and allowed to stand for 5 min. Cover slips were placed and sealed. A Zeiss LSM 880 confocal microscope was used for imaging with 100X objective oil immersion and laser excitation at 561 nm. Settings of laser power 1%, gain 750, digital offset 0 and digital gain 1 were used for each image. Five representative images were taken from each slide and the number of diplococci occurring in each chain was manually counted.

4.6 Acknowledgements

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4.7 References

1. Wilkinson HW. 1978. Group B Streptococcal Infection in Humans. Annu Rev Microbiol 32:41–57.

2. Doran KS, Nizet V. 2004. Molecular pathogenesis of neonatal Group B Streptococcal infection: No longer in its infancy. Mol Microbiol 54:23–31.

3. Schuchat A. 1999. Group B *Streptococcus*. Lancet 353:51–56.

4. Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ. 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol 96:498–503.

5. Nandyal RR. 2008. Update on group B streptococcal infections: perinatal and neonatal periods. J Perinat Neonatal Nurs 22:230–7.

6. Edwards MS, Rench MA, Haffar AA, Murphy MA, Desmond MM, Baker CJ. 1985. Long-term sequelae of group B streptococcal meningitis in infants. J Pediatr 106:717–22.

7. Hall J, Adams NH, Bartlett L, Seale AC, Lamagni T, Bianchi-Jassir F, Lawn JE, Baker CJ, Cutland C, Heath PT, Ip M, Le Doare K, Madhi SA, Rubens CE, Saha SK, Schrag S, Sobanjo-Ter Meulen A, Vekemans J, Gravett MG. 2017. Maternal Disease With Group B *Streptococcus* and Serotype Distribution Worldwide: Systematic Review and Meta-analyses. Clin Infect Dis 65:S112–S124.

8. Schuchat A. 1998. Epidemiology of Group B Streptococcal disease in the United States: shifting paradigms. Clin Microbiol Rev 11:497–513.

9. Ohlsson A, Shah VS. 2014. Intrapartum antibiotics for known maternal Group B Streptococcal colonization. Cochrane database Syst Rev CD007467.

10. Armistead B, Oler E, Adams Waldorf K, Rajagopal L. 2019. The Double Life of Group B *Streptococcus*: Asymptomatic Colonizer and Potent Pathogen. J Mol Biol 431:2914–2931.

11. Curtis J, Kim G, Wehr NB, Levine RL. 2003. Group B Streptococcal phospholipid causes pulmonary hypertension. Proc Natl Acad Sci U S A 100:5087–90.

12. Fischer W. 1977. The polar lipids of Group B Streptococci. II. Composition and positional distribution of fatty acids. Biochim Biophys Acta 487:89–104.

13. Doran KS, Engelson EJ, Khosravi A, Maisey HC, Fedtke I, Equils O, Michelsen KS, Arditi M, Peschel A, Nizet V. 2005. Blood-brain barrier invasion by Group B *Streptococcus* depends upon proper cell-surface anchoring of lipoteichoic acid. J Clin Invest 115:2499–2507.

14. Saar-Dover R, Bitler A, Nezer R, Shmuel-Galia L, Firon A, Shimoni E, Trieu-Cuot P, Shai Y. 2012. D-alanylation of lipoteichoic acids confers resistance to cationic peptides in Group B *Streptococcus* by increasing the cell wall density. PLoS Pathog 8:e1002891.

15. Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, Kalbacher H, Nieuwenhuizen WF, Jung G, Tarkowski A, Van Kessel KPM, Van Strijp JAG. 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. J Exp Med 193:1067–1076.

16. Roy H, Ibba M. 2008. RNA-dependent lipid remodeling by bacterial multiple peptide resistance factors. Proc Natl Acad Sci U S A 105:4667–4672.

17. Ernst CM, Kuhn S, Slavetinsky CJ, Krismer BB, Heilbronner S, Gekeler C, Kraus D, Wagner S, Peschel A. 2015. The Lipid-Modifying Multiple Peptide Resistance Factor Is an Oligomer Consisting of Distinct Interacting Synthase and Flippase subunits. MBio 6:1–9.

18. Roy H, Ibba M. 2009. Broad range amino acid specificity of RNA-dependent lipid remodeling by multiple peptide resistance factors. J Biol Chem 284:29677–29683.

19. Hebecker S, Krausze J, Hasenkampf T, Schneider J, Groenewold M, Reichelt J, Jahn D, Heinz DW, Moser J. 2015. Structures of two bacterial resistance factors mediating tRNA-dependent aminoacylation of phosphatidylglycerol with lysine or alanine. Proc Natl Acad Sci U S A 112:10691–10696.

20. Slavetinsky C, Kuhn S, Peschel A. 2017. Bacterial aminoacyl phospholipids - Biosynthesis and role in basic cellular processes and pathogenicity. Biochim Biophys acta Mol cell Biol lipids 1862:1310–1318.

21. Bligh EG, Dyer WJ. 1959. A Rapid Method of Total Lipid Extraction and Purification. Can J Biochem Physiol 37:911–917.

22. Tan BK, Bogdanov M, Zhao J, Dowhan W, Raetz CRH, Guan Z. 2012. Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. Proc Natl Acad Sci 109:16504–16509.

23. Joyce LR, Guan Z, Palmer KL. 2019. Phosphatidylcholine Biosynthesis in Mitis Group Streptococci via Host Metabolite Scavenging. J Bacteriol 201:e00495-19.

24. Yalcin T, Harrison AG. 1996. Ion chemistry of protonated lysine derivatives. J Mass Spectrom 31:1237–43.

25. Meiers M, Volz C, Eisel J, Maurer P, Henrich B, Hakenbeck R. 2014. Altered lipid composition in *Streptococcus pneumoniae cpoA* mutants. BMC Microbiol 14:1–12.

26. Adams HM, Joyce LR, Guan Z, Akins RL, Palmer KL. 2017. *Streptococcus mitis* and *S. oralis* Lack a Requirement for CdsA, the Enzyme Required for Synthesis of Major Membrane Phospholipids in Bacteria. Antimicrob Agents Chemother 61:e02552-16.

27. Granok AB, Parsonage D, Ross RP, Caparon MG. 2000. The RofA binding site in *Streptococcus pyogenes* is utilized in multiple transcriptional pathways. J Bacteriol 182:1529–40.

28. Hooven TA, Bonakdar M, Chamby AB, Ratner AJ. 2019. A Counterselectable Sucrose Sensitivity Marker Permits Efficient and Flexible Mutagenesis in *Streptococcus agalactiae*. Appl Environ Microbiol 85:1–13.

29. Turner LS, Kanamoto T, Unoki T, Munro CL, Wu H, Kitten T. 2009. Comprehensive evaluation of *Streptococcus sanguinis* cell wall-anchored proteins in early infective endocarditis. Infect Immun 77:4966–75.

30. Dalia AB, Weiser JN. 2011. Minimization of bacterial size allows for complement evasion and is overcome by the agglutinating effect of antibody. Cell Host Microbe 10:486–96.

31. Zapun A, Vernet T, Pinho MG. 2008. The different shapes of cocci. FEMS Microbiol Rev 32:345–60.

32. Chatfield CH, Koo H, Quivey RG. 2005. The putative autolysin regulator LytR in *Streptococcus mutans* plays a role in cell division and is growth-phase regulated. Microbiology 151:625–631.

33. Hanson BR, Runft DL, Streeter C, Kumar A, Carion TW, Neely MN. 2012. Functional analysis of the CpsA protein of *Streptococcus agalactiae*. J Bacteriol 194:1668–78.

34. Evans K, Stone V, Chen L, Ge X, Xu P. 2014. Systematic study of genes influencing cellular chain length in *Streptococcus sanguinis*. Microbiology 160:307–315.

35. Neugent ML, Hulyalkar N V., Nguyen VH, Zimmern PE, De Nisco NJ. 2020. Advances in Understanding the Human Urinary Microbiome and Its Potential Role in Urinary Tract Infection. MBio 11.

36. Miller EA, Beasley DAE, Dunn RR, Archie EA. 2016. Lactobacilli dominance and vaginal pH: Why is the human vaginal microbiome unique? Front Microbiol 7.

37. Smith AM, Harrison JS, Sprague KM, Roy H. 2013. A conserved hydrolase responsible for the cleavage of aminoacylphosphatidylglycerol in the membrane of *Enterococcus faecium*. J Biol Chem 288:22768–22776.

38. Roy H. 2009. Tuning the properties of the bacterial membrane with aminoacylated phosphatidylglycerol. IUBMB Life 61:940–953.

39. Milligan TW, Doran TI, Straus DC, Mattingly SJ. 1978. Growth and amino acid requirements of various strains of Group B Streptococci. J Clin Microbiol 7:28–33.

40. Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vázquez-Fresno R, Sajed T, Johnson D, Li C, Karu N, Sayeeda Z, Lo E, Assempour N, Berjanskii M, Singhal S, Arndt D, Liang Y, Badran H, Grant J, Serra-Cayuela A, Liu Y, Mandal R, Neveu V, Pon A, Knox C, Wilson M, Manach C, Scalbert A. 2018. HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res 46:D608–D617.

41. Spencer BL, Deng L, Patras KA, Burcham ZM, Sanches GF, Nagao PE, Doran KS. 2019. Cas9 contributes to Group B Streptococcal colonization and disease. Front Microbiol 10:1–15.

42. Deng L, Spencer BL, Holmes JA, Mu R, Rego S, Weston TA, Hu Y, Sanches GF, Yoon S, Park N, Nagao PE, Jenkinson HF, Thornton JA, Seo KS, Nobbs AH, Doran KS. 2019. The Group B Streptococcal surface antigen I/II protein, BspC, interacts with host vimentin to promote adherence to brain endothelium and inflammation during the pathogenesis of meningitis. PLoS Pathog 15:1–30.

43. Poyart C, Pellegrini E, Marceau M, Baptista M, Jaubert F, Lamy MC, Trieu-Cuot P. 2003. Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells. Mol Microbiol 49:1615–1625.

44. Bhardwaj P, Hans A, Ruikar K, Guan Z, Palmer KL. 2018. Reduced Chlorhexidine and Daptomycin Susceptibility in Vancomycin-Resistant *Enterococcus faecium* after Serial Chlorhexidine Exposure. Antimicrob Agents Chemother 62.

45. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li X, Li Y, Imamura M, Kaneko Y, Okawara A, Miyazaki Y, Gómez-Velasco A, Rogers P, Dahesh S, Uchiyama S, Khurana A, Kawahara K, Yesilkaya H, Andrew PW, Wong C-H, Kawakami K, Nizet V, Besra GS, Tsuji M, Zajonc DM, Kronenberg M. 2011. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. Nat Immunol 12:966–74.

46. Zajonc DM, Girardi E. 2015. Recognition of microbial glycolipids by Natural Killer T cells. Front Immunol 6:1–11.

47. Flannagan RS, Cosío G, Grinstein S. 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat Rev Microbiol 7:355–66.

48. Van De Rijn I, Kessler RE. 1980. Growth characteristics of Group A Streptococci in a new chemically defined medium. Infect Immun 27:444–448.

49. Chang JC, LaSarre B, Jimenez JC, Aggarwal C, Federle MJ. 2011. Two Group A Streptococcal peptide pheromones act through opposing rgg regulators to control biofilm development. PLoS Pathog 7:e1002190.

50. Gupta R, Shah P, Swiatlo E. 2009. Differential gene expression in *Streptococcus pneumoniae* in response to various iron sources. Microb Pathog 47:101–109.

51. Kuypers JM, Heggen LM, Rubens CE. 1989. Molecular analysis of a region of the Group B *Streptococcus* chromosome involved in type III capsule expression. Infect Immun 57:3058–65.

52. Lancefield RC, McCarty M, Everly WN. 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J Exp Med 142:165–79.

53. Wilkinson HW. 1977. Nontypable group B streptococci isolated from human sources. J Clin Microbiol 6:183–4.

54. Hooven TA, Randis TM, Daugherty SC, Narechania A, Planet PJ, Tettelin H, Ratner AJ. 2014. Complete Genome Sequence of *Streptococcus agalactiae* CNCTC 10/84, a Hypervirulent Sequence Type 26 Strain. Genome Announc 2.

55. Kilian M, Mikkelsen L, Henrichsen J. 1989. Taxonomic Study of Viridans Streptococci: Description of *Streptococcus gordonii* sp. nov. and Emended Descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). Int J Syst Bacteriol 39:471–484.

56. Taylor RG, Walker DC, Mclnnes RR. 1993. *E. coli* host strains significantly affect the quality of small scale plasmid DNA preparations used for sequencing. Nucleic Acids Res 21:1677–1678.

57. Palmer KL, Carniol K, Manson JM, Heiman D, Shea T, Young S, Zeng Q, Gevers D, Feldgarden M, Birren B, Gilmore MS. 2010. High-quality draft genome sequences of 28 *Enterococcus* sp. isolates. J Bacteriol 192:2469–2470.

58. Holo H, Nes IF. 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl Environ Microbiol 55:3119–3123.

59. Framson PE, Nittayajarn A, Merry J, Youngman P, Rubens CE. 1997. New genetic techniques for Group B Streptococci: High-efficiency transformation, maintenance of temperature-sensitive pWV01 plasmids, and mutagenesis with Tn917. Appl Environ Microbiol 63:3539–3547.

60. Li C, Tan BK, Zhao J, Guan Z. 2016. In vivo and in vitro synthesis of phosphatidylglycerol by an *Escherichia coli* cardiolipin synthase. J Biol Chem 291:25144–25153.

61. Gogos A, Jimenez J, Chang J, Wilkening R, Federle M. 2018. A Quorum Sensing-Regulated Protein Binds Cell Wall *Streptococcus pyogenes*. J Bacteriol 200:1–16.

62. Kristian SA, Datta V, Weidenmaier C, Kansal R, Fedtke I, Peschel A, Gallo RL, Nizet V. 2005. D-alanylation of teichoic acids promotes Group A *Streptococcus* antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion. J Bacteriol 187:6719–25.

CHAPTER 5

SUMMARY AND DISCUSSION

5.1 Summary of findings

Streptococcus is a large genus of human commensal bacteria that cause a variety of diseases from minor infections to major invasive diseases with long lasting sequelae or death (1). Due to the disease burden of streptococci, significant time and resources have been dedicated to research of pathogenic mechanisms in streptococci. The various mechanisms identified range from proteins and polysaccharide polymers to gene regulation pathways, yet the cellular membrane, a major and important component of the cell, has been largely understudied (2–4). While investigating antibiotic resistance in a subset of Mitis group streptococci, Adams *et al* 2017 performed global lipidomics and identified a unique membrane composition in which the studied species were able to survive a loss-of-function mutation in the major phospholipid biosynthesis pathway which resulted in a membrane devoid of PG and CL (5). During the study, the observation was made that the Mitis group streptococci possessed the zwitterionic phospholipid PC. The work presented in this dissertation aimed to elucidate the biosynthetic pathway of PC within the streptococci and further characterize the cellular membrane of the major pathogenic streptococci in context of the human host.

The first study in this dissertation expanded the observation of PC in the Mitis group streptococci to the major human pathogen *S. pneumoniae*, and discovered that the rare GPC pathway is required for PC biosynthesis and that the scavenging of major human metabolites is required for the GPC

pathway. This was a novel finding in streptococci because PC had not been characterized in *S. pneumoniae*, and the GPC pathway had previously been characterized in only one other bacterium. Overall, this study demonstrated that a subset of streptococci remodel their cellular membrane in response to human metabolites, which could have implications on pathogenesis in context of the human host.

The observation of human metabolite scavenging and cellular membrane remodeling in the aforementioned study, rationalized expanding the characterization of the cellular membrane to the other major pathogenic streptococci *S. pyogenes* and *S. agalactiae*, in the context of the human host. To mimic the host, human serum was supplemented to a final volume of 5% in a defined medium before global lipidomics was performed. It was observed that *S. pneumoniae*, *S. pyogenes*, and *S. agalactiae* scavenged human metabolites from serum to remodel their cellular membrane by incorporating PC. This is the first time PC has been identified in the membrane of *S. pyogenes* and *S. agalactiae*. It was further elucidated that an abbreviated GPC pathway is utilized by *S. pyogenes* and *S. agalactiae* by only scavenging the intermediate lysoPC. Furthermore, *S. agalactiae* incorporated the plasmalogen plasmanyl-PC into their membrane, a novel finding for a facultative anaerobe.

The final study in this dissertation characterized lysyl-glucosyl-diacylglycerol, a novel amino acylated glycolipid in the cellular membrane of *S. agalactiae*, which was discovered during the lipidomic survey mentioned above. Furthermore, the final study identified MprF as the biosynthetic enzyme required for the addition of the amino acid lysine to the glycolipid, a novel

lipid substrate for MprF, forming Lys-Glc-DAG. The two lysine lipids, Lys-Glc-DAG and Lys-PG, were shown to alter the negative charge on the outer surface of the cell, aid in low pH tolerance, and be required for efficient cell division. Finally, it was shown that the two lysine lipids are required for efficient adhesion and invasion of mammalian cells *in vitro*. This is the first description of streptococcal lipids impacting colonization and pathogenesis and has the potential to be utilized as a possible target for patient screening and treatments.

5.2 Implications on streptococcal biology

The research presented in this dissertation has uncovered novel cellular membrane components and remodeling in the streptococci. PC is a major component of eukaryotic membranes and promotes bilayer formation, aids protein folding, as well as surviving environmental changes such as temperature shifts (6–9). PC is also required for virulence in certain bacteria (10, 11). While this work has broadened the knowledge of the streptococcal membrane and expanded the species utilizing the GPC pathway, many questions remain. The more obvious question is why are the streptococci incorporating human metabolites into their cellular membrane? This could be a strategy to preserve limited resources, an immune evasion technique via membrane mimicry, or to interfere with host immunity, as lysoPC can be an immune-modulating metabolite (12).

The streptococcal genes required for the GPC pathway must be identified so that isogenic mutants unable to produce PC can be generated. These mutants will be critical to future research on the physiological role of PC within the membrane and the role of PC in pathogenesis *in vivo*. Due to the small genome size of streptococci (~2 Mbp), one could hypothesize that individual enzymes

have evolved to have flexible functions. For example, the acyltransferase PlsC, which is present in streptococci, is well characterized in other species to acylate lysoPA to PA (13) which is a similar activity in the GPC pathway from lysoPC to PC. Likewise, a sugar-binding ABC transporter in *Mycobacterium* has been shown to have flexible substrate specificity and can transport GPC as well (14). Future research will be required to elucidate the streptococcal acyltransferases and transporters involved in this pathway.

While certain physiological roles of the two *S. agalactiae* lysine lipids have been elucidated through the work presented in this dissertation, future research will need to decouple the biosynthesis of both lipids to begin evaluating their individual roles in the membrane and to answer the question, why did the MprF of *S. agalactiae* evolve to produce both lysine lipids? Streptococcal glycolipids are known immune antigens (15, 16); could this be an immune evasion mechanism? Only future molecular investigations into the MprF of these streptococci coupled with *in vitro* and *in vivo* experimentation will answer this question. Likewise, further *in vivo* investigation of the lysine lipid mutants, for example in animal models of infection, are needed to understand the individual contributions of the lysine lipids during colonization and pathogenesis of *S. agalactiae*.

5.3 Concluding remarks

Streptococci are devasting human pathogens. The focus of this dissertation was to elucidate the PC biosynthetic pathway in streptococci and characterize the streptococcal cellular membrane in context of the human host. In summary, the work conducted here discovered the presence of PC in the membrane of all major pathogenic streptococci. Specifically, the GPC pathway is required

for biosynthesis of PC via scavenging of human metabolites, which highlights the importance of using growth media that mimics the native growth environment of pathogens. Furthermore, the identification of a novel glycolipid substrate of the *S. agalactiae* MprF, a novel lipid, and its physiological role in pathogenesis *in vitro* demonstrates the importance of investigating the role of cellular membranes in host-pathogen interactions.

5.4 References

1. Facklam R. 2002. What happened to the streptococci: Overview of taxonomic and nomenclature changes. Clin Microbiol Rev 15:613–630.

2. Armistead B, Oler E, Adams Waldorf K, Rajagopal L. 2019. The Double Life of Group B *Streptococcus*: Asymptomatic Colonizer and Potent Pathogen. J Mol Biol 431:2914–2931.

3. Wilkening R V, Federle MJ. 2017. Evolutionary Constraints Shaping *Streptococcus pyogenes*-Host Interactions. Trends Microbiol 25:562–572.

4. Weiser JN, Ferreira DM, Paton JC. 2018. *Streptococcus pneumoniae*: transmission, colonization and invasion. Nat Rev Microbiol 16:355–367.

5. Adams HM, Joyce LR, Guan Z, Akins RL, Palmer KL. 2017. *Streptococcus mitis* and *S. oralis* Lack a Requirement for CdsA, the Enzyme Required for Synthesis of Major Membrane Phospholipids in Bacteria. Antimicrob Agents Chemother 61:e02552-16.

6. Sohlenkamp C, López-Lara IM, Geiger O. 2003. Biosynthesis of phosphatidylcholine in bacteria. Prog Lipid Res 42:115–162.

7. Bogdanov M, Sun J, Kaback HR, Dowhan W. 1996. A phospholipid acts as a chaperone in assembly of a membrane transport protein. J Biol Chem 271:11615–11618.

8. Bogdanov M, Umeda M, Dowhan W. 1999. Phospholipid-assisted Refolding of an Integral Membrane Protein. J Biol Chem 274:12339–12345.

9. Geiger O, López-Lara IM, Sohlenkamp C. 2013. Phosphatidylcholine biosynthesis and function in bacteria. Biochim Biophys Acta - Mol Cell Biol Lipids 1831:503–513.

10. Conover GM, Martinez-Morales F, Heidtman MI, Luo Z-Q, Tang M, Chen C, Geiger O, Isberg RR. 2008. Phosphatidylcholine synthesis is required for optimal function of *Legionella pneumophila* virulence determinants. Cell Microbiol 10:514–528.

11. Comerci DJ, Altabe S, De Mendoza D, Ugalde RA. 2006. *Brucella abortus* synthesizes phosphatidylcholine from choline provided by the host. J Bacteriol 188:1929–1934.

12. Law S-H, Chan M-L, Marathe GK, Parveen F, Chen C-H, Ke L-Y. 2019. An Updated Review of Lysophosphatidylcholine Metabolism in Human Diseases. Int J Mol Sci 20.

13. Yao J, Rock CO. 2013. Phosphatidic acid synthesis in bacteria. Biochim Biophys Acta 1831:495–502.

14. Jiang D, Zhang Q, Zheng Q, Zhou H, Jin J, Zhou W, Bartlam M, Rao Z. 2014. Structural analysis of *Mycobacterium tuberculosis* ATP-binding cassette transporter subunit UgpB reveals specificity for glycerophosphocholine. FEBS J 281:331–341.

15. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li X, Li Y, Imamura M, Kaneko Y, Okawara A, Miyazaki Y, Gómez-Velasco A, Rogers P, Dahesh S, Uchiyama S, Khurana A, Kawahara K, Yesilkaya H, Andrew PW, Wong C-H, Kawakami K, Nizet V, Besra GS, Tsuji M, Zajonc DM, Kronenberg M. 2011. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. Nat Immunol 12:966–74.

16. Zajonc DM, Girardi E. 2015. Recognition of microbial glycolipids by Natural Killer T cells. Front Immunol 6:1–11.

APPENDIX A

CHAPTER 2 SUPPLEMENTAL TEXT, FIGURES, AND TABLES

Text A1. Supplementary Materials and Methods.

Acidic Bligh-Dyer extractions.

Centrifugation was performed using a Sorvall RC6+ centrifuge. Cultures were pelleted at 4,280 x *g* for 5 min at room temperature. The supernatants were removed and stored at -80°C until acidic Bligh-Dyer lipid extractions were performed as described (1) with minor modifications. Cell pellets were resuspended in 1X PBS (Sigma-Aldrich) and transferred to Coring Pyrex glass tubes with PTFR-lined caps (VWR), followed by 1:2 vol:vol chloroform:methanol addition. Single phase extractions were vortexed periodically and incubated at room temperature for 15 minutes before 500 x *g* centrifugation for 10 min. A two-phase Bligh-Dyer was achieved by addition of 100 µL 37% HCL, 1 mL $CHCl_3$, and 900 µL of 1X PBS, which was then vortexed and centrifuged for 5 min at 500 x *g*. The lower phase was removed to a new tube and dried under nitrogen before being stored at -80°C prior to lipidomic analysis.

Normal phase Liquid Chromatography/ Mass Spectrometry.

Normal-phase LC/MS was performed on an Agilent 1200 quaternary LC system equipped with an Ascentis silica high-performance liquid chromatography (HPLC) column (5µm; 25 cm by 2.1 mm; Sigma-Aldrich) as described previously (1, 2). Briefly, mobile phase A consisted of chloroform-methanol-aqueous ammonium hydroxide (800:195:5, vol/vol), mobile phase B consisted of chloroform-methanol-water-aqueous ammonium hydroxide (600: 340:50:5, vol/vol), and mobile phase C consisted of chloroform-methanol-water-aqueous ammonium hydroxide (450:450:95:5,

vol/vol/vol/vol). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min, then linearly increased to 100% mobile phase B over 14 min, and held at 100% mobile phase B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min, held at 100% mobile phase C for 3 min, and, finally, returned to 100% mobile phase A over 0.5 min and held at 100% mobile phase A for 5 min. The LC eluent (with a total flow rate of 300 mL/min) was introduced into the ESI source of a high-resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). Instrumental settings for negative-ion ESI and MS/MS analysis of lipid species were as follows: IS = -4,500 V, CUR = 20 psi, GSI = 20 psi, DP = -55 V, and FP = -150V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst TF1.5 software (Sciex, Framingham, MA).

Metabolite extractions.

Briefly, cell pellets were resuspended in 300 µL super-chilled methanol:water (vol:vol 4:1) via vortexing and pipetting, and stored on dry ice for 15 min. Suspensions were centrifuged at 17,136 x *g* at 4°C for 5 min in a tabletop centrifuge. The supernatant was removed to a pre-chilled Corning Pyrex glass tube and kept on dry ice. A further 2 resuspensions in 200 µL methanol:water (vol:vol 4:1) were performed. Pooled supernatants were dried under nitrogen at room temperature and stored at -80°C prior to analysis.

Reverse phase LC-ESI/MS analysis.

RPLC-ESI/MS analysis of water-soluble metabolites was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps and a SCL-10A system controller) coupled to

a TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). LC was operated at a flow rate of 200 µl/min with a linear gradient as follows: 100% of mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 4 min. Mobile phase A consisted of methanol/acetonitrile/aqueous 1mM ammonium acetate (60/20/20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 µm, 2.1 x 50 mm) was obtained from Agilent (Palo Alto, CA). The LC eluent was introduced into the ESI source of the mass spectrometer. Instrument settings for negative ion ESI/MS and MS/MS analysis of lipid species were as follows: Ion spray voltage (IS) = -4500 V; Curtain gas (CUR) = 20 psi; Ion source gas 1 (GS1) = 20 psi; De-clustering potential (DP) = -55 V; Focusing potential (FP) = -150 V. Data acquisition and analysis were performed using the Analyst TF1.5 software (Sciex, Framingham, MA).

Deletion of SM43 *cdsA*.

Primers used in this study are shown in Table A.2 in Appendix A. The *cdsA* deletion construct was designed essentially as previously described (3, 4). Approximately 2 Kb flanking regions on either side of the gene were amplified using Phusion polymerase (Thermo Fisher). PCR products were digested with restriction enzymes (New England Biolabs) and ligated using T4 DNA ligase (New England Biolabs). Ligations were amplified by PCR utilizing the 5' and 3' most primers with Phusion polymerase to obtain a linear product. The PCR product was analyzed on a 1% agarose gel and extracted using the QIAquick Gel Extraction Kit per the manufacturer's protocol. The linear construct was transformed into SM43 via natural transformation, described below. Transformation plates were incubated overnight. Putative transformant colonies were inoculated

into THB and screened via PCR for the clean deletion of *cdsA*. The *cdsA* regions of putative mutants were sequenced for confirmation (Massachusetts General Hospital DNA Core).

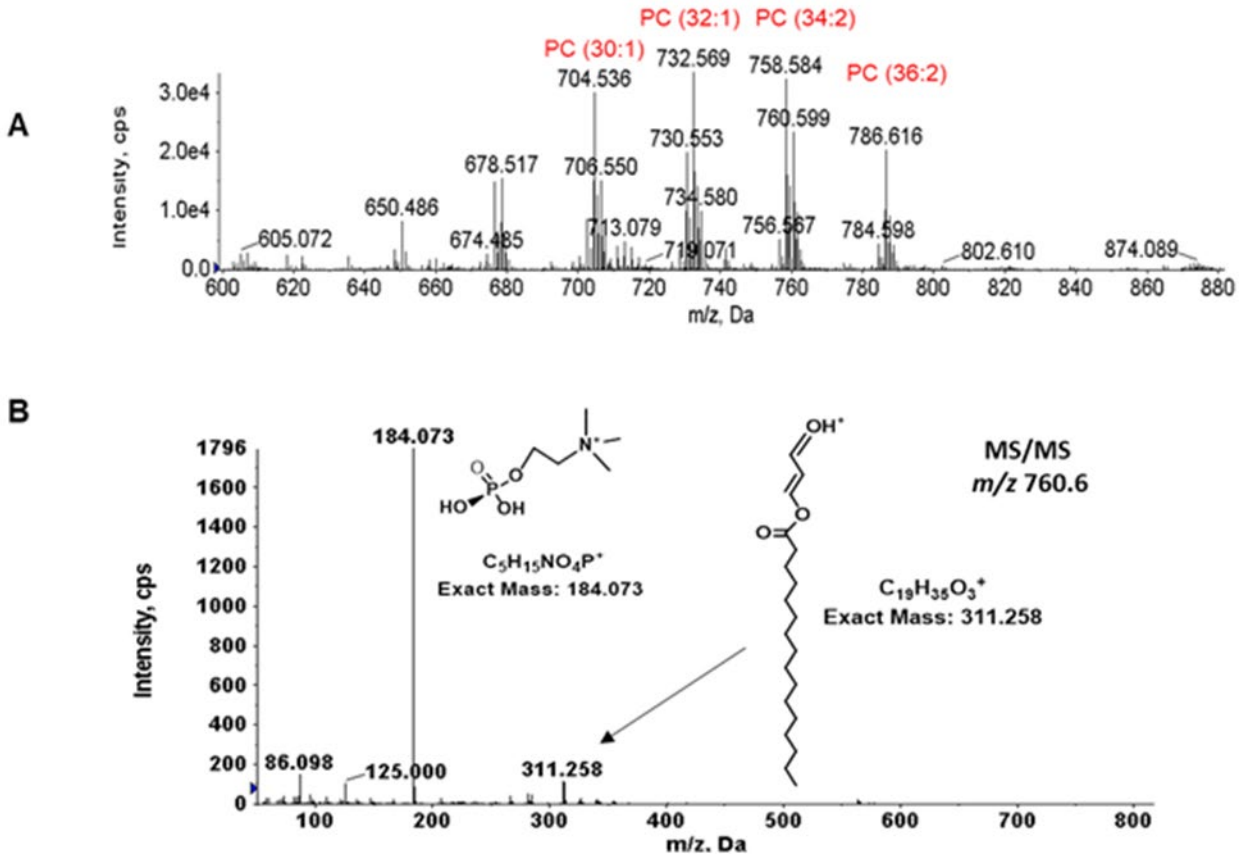

Figure A.1. Phosphatidylcholine (PC) species in *S. mitis* ATCC 49456. A) *S. mitis* ATCC 49456, B) MS/MS daughter ion fragmentation of PC (34:1) *m/z* 760.6 in SM43. The mass spectra shown are averaged from spectra acquired by NPLC-ESI/MS during the 20–21 min window. PC species are detected by positive ion ESI/MS as the M^+ ions.

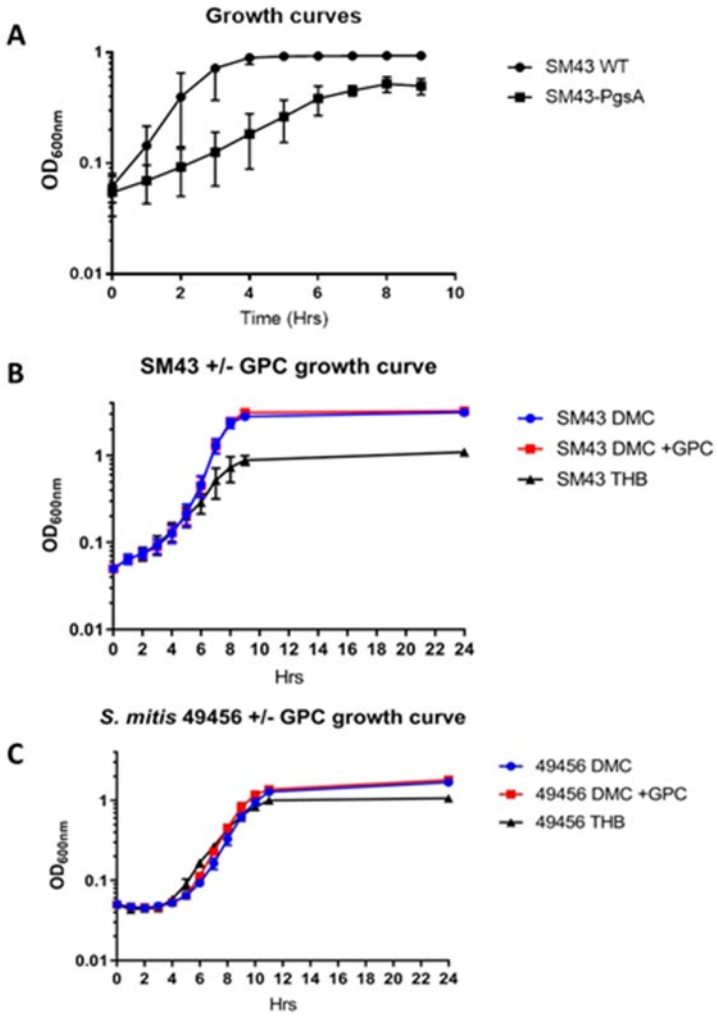

Figure A.2. **Growth curves.** A) Growth curves for SM43 and SM43Δ*pgsA* in THB. B) Growth curves of SM43 in THB, and in defined medium (DM) with and without GPC supplementation and C) Growth curves of ATCC49456 in THB, and in defined medium (DM) with and without GPC supplementation. OD_{600nm} readings were taken every hour, with a starting inoculum of OD_{600nm} 0.05. Growth curves were performed as biological triplicate. Error bars indicate standard deviation.

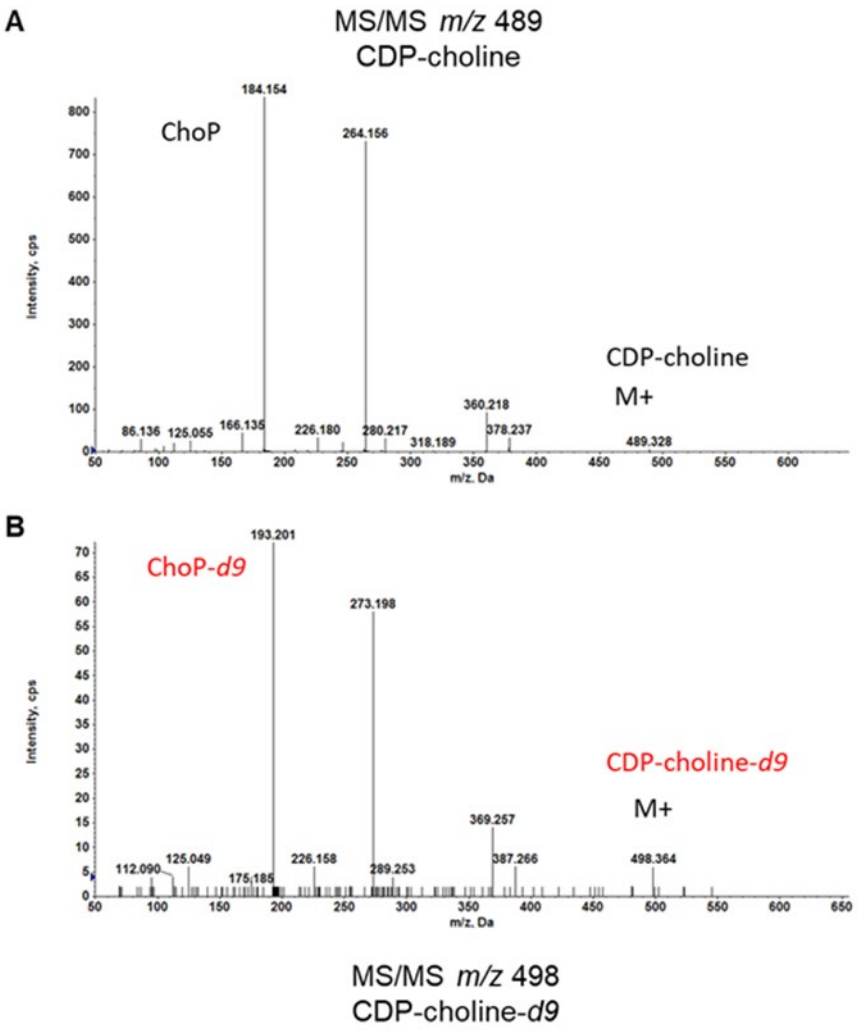

Figure A.3. MS/MS fragmentation of CDP-choline and CDP-choline-*d9*. A) MS/MS of CDP-choline (*m/z* 489) B) MS/MS of CDP-choline-*d9* (*m/z* 498). Note the mass shift of 9-*da* for all major MS/MS peaks in B.

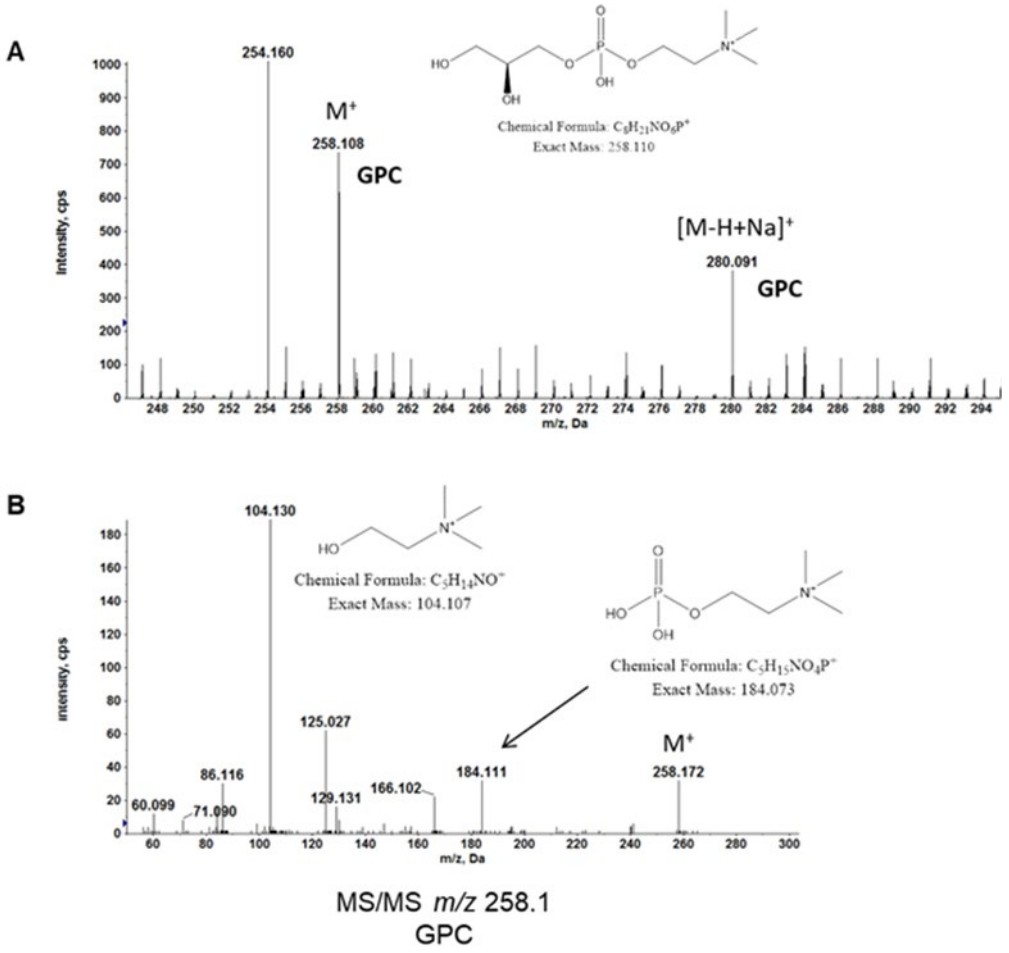

Figure A.4. MS detection of GPC in THB. The soluble-metabolite extract of THB was analyzed by reverse phase LC/MS. A) GPC is detected by positive ion ESI/MS as the M^+ ion at *m/z* 258.108 and $[M-H+Na]^+$ ion at *m/z* 280.091. B) MS/MS spectrum of the M^+ ion (*m/z* 258.1) of GPC. The chemical structures of major fragments are shown.

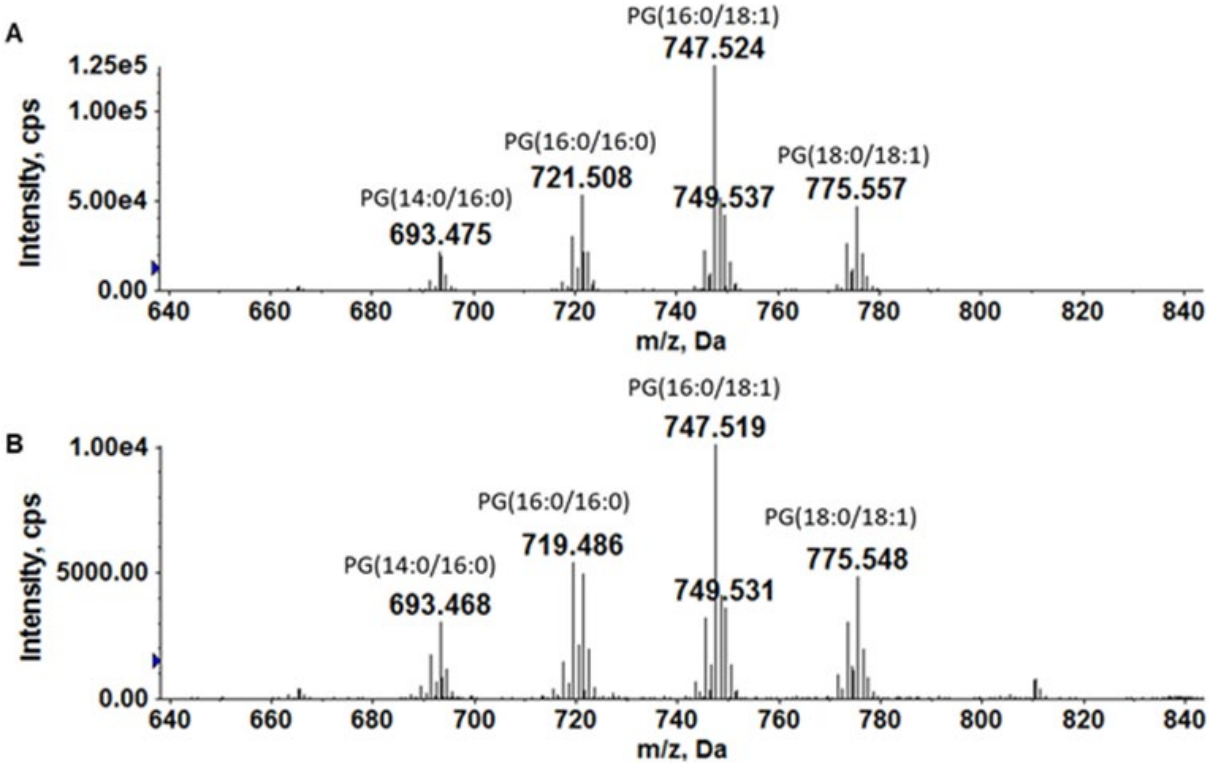

Figure A.5. ESI/MS of phosphatidylglycerol (PG) species of SM43 cultured in THB with and without lysoPC (20:0) addition. A) ESI/MS detection of PG species in SM43 cultured in THB. B) ESI/MS detection of PG species in SM43 cultured in THB in the presence of lysoPC (20:0). In sharp contrast to what is observed for PC, there is no appreciable incorporation of the 20:0-acyl chain into PG in the presence of lysoPC (20:0). The lack of incorporation of C20:0 into PG indicates the insignificant transacylation activity of SM43. PG species are detected by negative ion ESI/MS as the $[M-H]^-$ ions.

Table A.1. Strains and plasmids used in this study.

Organism	Strain	Description	Ref
Mitis group streptococci	1643 (SM43)	Wild-type infective endocarditis isolate	(5)
	1643Δ*cdsA*	*cdsA* deletion strain	This work
	1643Δ*pgsA::ermB*	*pgsA* deletion strain, replaced with *ermB*. Erm^r	This work
S. mitis	ATCC 49456 (NCTC 12261)	Wild-type *S. mitis* type strain from ATCC	(6)
S. oralis	ATCC 35037 (NCTC 11427)	Wild-type *S. oralis* type strain from ATCC	(7)
S. pneumoniae	D39	Wild type, historical strain	(8)
	TIGR4	Wild type, bloodstream isolate	(9)
Plasmid	**Description**		**Ref**
pG$^+$host 4	Encodes *ermB*		(10)

Table A.2. Primers used in this study.

Primer	Sequence (5'-3')	Use
L_Updel_F	TTTACGTGAATATATCGGT	SM43 upstream of *cdsA* region amplification
L_Updel_R PstI	ACGTCACTGCAGATCCTTGGTCATATCTTCTC	SM43 upstream of *cdsA* region amplification
L_Dwndel_F PstI	ACGTCACTGCAGCCAATGATGCACTTATTC	SM43 downstream of *cdsA* region amplification
L_Dwndel_R	CTTGATTTCTTCTTGCAC	SM43 downstream of *cdsA* region amplification
L1_R	CGTACATATCTTCGACTGTC	*cdsA* knockout sequencing with L_Updel_F
L2_F	CCTTCGTACCATGATTGAG	*cdsA* knockout sequencing
L2_R	GTCAGATTTTCCCATTTTTC	*cdsA* knockout sequencing
L3_F	TTTAGAGAATGAGGACCG	*cdsA* knockout sequencing
L3_R	CATATCTTCTGTGATGT	*cdsA* knockout sequencing
L4_F	ATGGTGGTCGTGCTGAA	*cdsA* knockout sequencing
L4_R	GATACTGTACATCCAAAGG	*cdsA* knockout sequencing
L5_F	CAGTAGATCACGATGCAAC	*cdsA* knockout sequencing
L5_R	GTCGCATAATGTCATTCC	*cdsA* knockout sequencing
L6_F	GTGTAGTTATCATGGTTG	*cdsA* knockout sequencing with L_Dwndel_R
L7F	CAAACGGCTCTTCCTATGC	*cdsA* knockout sequencing
L7R	CTGATAACTTGAGCATCG	*cdsA* knockout sequencing
SM43cdsA_reseq_F	TCAGGTCTATATGCATCTG	*cdsA* upstream resequence
SM43cdsA_reseq_R	GCTTCATCAAAATCAGG	*cdsA* upstream resequence
PgsA_updel_F	CTTTACTCCCTAAGCAAG	SM43 upstream of *pgsA* region amplification
PgsA_updel_R_PstI	ACGTCACTGCAGTCTACCAATAGTTAATACATTAGG	SM43 upstream of *pgsA* region amplification
PgsA_dwndel_F_XmaI	ACGTCACCCGGGTTGTTGCTTGTTGAGACG	SM43 downstream of *pgsA* region amplification
PgsA_dwndel_R	GATCAAGAAGAACATGGA	SM43 downstream of *pgsA* region amplification
Erm_F_XmaI	ACGTCACCCGGGTAACGATCACTCATCATG	*erm* amplification from pG^{+}host4 plasmid
Erm_R_PstI	ACGTCACTGCAGCAAGTTAAGGGATGCAGT	*erm* amplification from pG^{+}host4 plasmid

References

1. Tan BK, Bogdanov M, Zhao J, Dowhan W, Raetz CRH, Guan Z. 2012. Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. Proc Natl Acad Sci 109:16504–16509.

2. Li C, Tan BK, Zhao J, Guan Z. 2016. In vivo and in vitro synthesis of phosphatidylglycerol by an *Escherichia coli* cardiolipin synthase. J Biol Chem 291:25144–25153.

3. Morrison DA, Khan R, Junges R, Åmdal HA, Petersen FC. 2015. Genome editing by natural genetic transformation in *Streptococcus mutans*. J Microbiol Methods 119:134–141.

4. Salvadori G, Junges R, Morrison DA, Petersen FC. 2016. Overcoming the barrier of low efficiency during genetic transformation of *Streptococcus mitis*. Front Microbiol 7:1009.

5. Akins RL, Katz BD, Monahan C, Alexander D. 2015. Characterization of high-level daptomycin resistance in viridans group streptococci developed upon in vitro exposure to daptomycin. Antimicrob Agents Chemother 59:2102–2112.

6. Kilian M, Mikkelsen L, Henrichsen J. 1989. Taxonomic Study of Viridans Streptococci: Description of *Streptococcus gordonii* sp. nov. and Emended Descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). Int J Syst Bacteriol 39:471–484.

7. Bridge PD, Sneath PH. 1982. *Streptococcus gallinarum* sp. nov. and *Streptococcus oralis* sp. nov. Int J Syst Bacteriol 32:410–415.

8. Lanie JA, Ng WL, Kazmierczak KM, Andrzejewski TM, Davidsen TM, Wayne KJ, Tettelin H, Glass JI, Winkler ME. 2007. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. J Bacteriol 189:38–51.

9. Tettelin H, Nelson KE, Paulsen IT, Eisen JA, Read TD, Peterson S, Heidelberg J, Deboy RT, Haft DH, Dodson RJ, Durkin AS, Gwinn M, Kolonay JF, Nelson WC, Peterson JD, Umayam LA, White O, Salzberg SL, Lewis MR, Radune D, Holtzapple E, Khouri H, Wolf AM, Utterback TR, Hansen CL, Mcdonald LA, Feldblyum T V, Angiuoli S, Dickinson T, Hickey EK, Holt IE, Loftus BJ, Yang F, Smith HO, Venter JC, Dougherty BA, Morrison DA, Hollingshead SK, M. FC. 2001. Complete Genome Sequence of a Virulent Isolate of *Streptococcus pneumoniae*. Science 293:498–507.

10. Biswas I, Gruss A, Ehrlich SD, Maguin E. 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J Bacteriol 175:3628–3635.

APPENDIX B

CHAPTER 4 SUPPLEMENTAL FIGURES AND TABLE

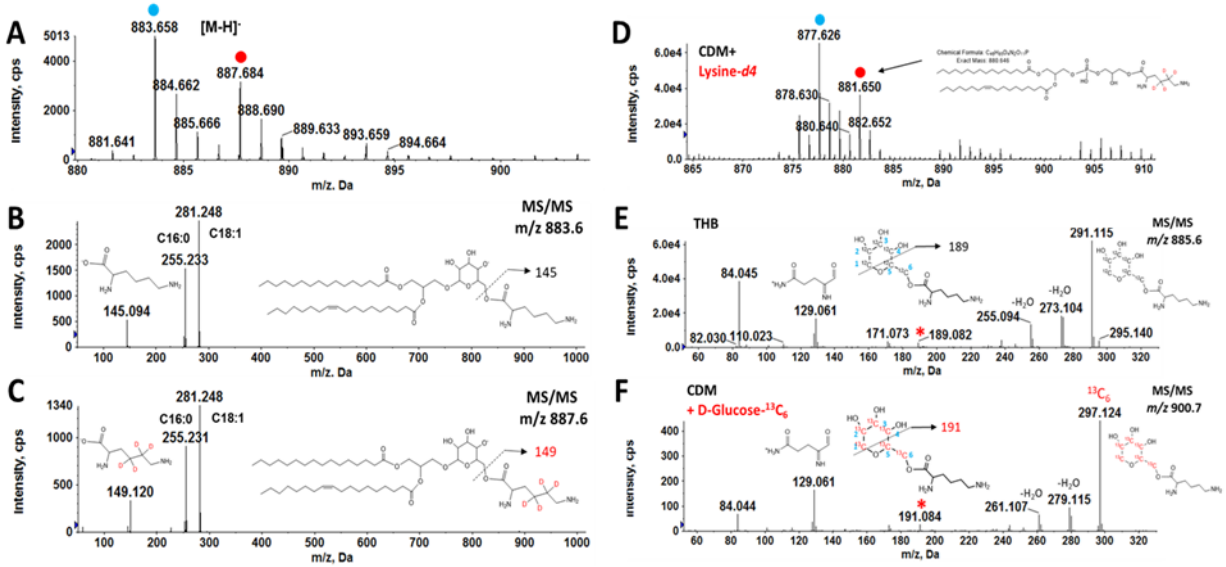

Figure B.1. Isotopic incorporation of deuterated lysine and MS/MS of ^{13}C-labeled glucose into Lys-Glc-DAG. The lipid extracts of *S. agalactiae* COH1 cultured in DM, DM supplemented with 450 µM L-lysine-*d4* (4,4,5,5-D4), or in DM containing 0.5% w/v D-Glucose (U-$^{13}C_6$) were analyzed by LC-ESI/MS in the positive ion mode. A) Negative ESI/MS of $[M-H]^-$ ions of major Lys-Glc-DAG species in *S. agalactiae* COH1 when cultured in DM supplemented with lysine-*d4*. The incorporation of lysine-*d4* into Lys-Glc-DAG is evidenced by an upward *m/z* shift of 4 Da of the $[M-H]^-$ ion (from *m/z* 883 to *m/z* 887). B) MS/MS of $[M-H]^-$ at *m/z* 883.6 produces a deprotonated lysine residue at *m/z* 145. C) MS/MS of $[M-H]^-$ at *m/z* 887.6 produces a deprotonated lysine-*d4* residue at *m/z* 149. D) $[M+H]^+$ ions of major Lys-PG species in *S. agalactiae* COH1 cultured in DM supplemented with lysine-*d4*. The incorporation of lysine-*d4* in Lys-PG is evidenced by an upward *m/z* shift of 4 Da from unlabeled Lys-PG (blue dots) to labeled Lys-PG (red dot). E) MS/MS of 885.6. A major product ion at *m/z* 291.1 is derived from glucose-lysine residue. F) MS/MS of *m/z* 900.7 (containing fifteen ^{13}C atoms). The presence of *m/z* 297.1 (with 6 Da shift) is consistent with glucose in Lys-Glc-DAG is replaced with D-Glucose (U-$^{13}C_6$). The other nine ^{13}C atoms are incorporated into the DAG portion of Lys-Glc-DAG. Furthermore, MS/MS data indicate that lysine is linked to the C6 position of glucose by the fragmentation schemes for forming *m/z* 189 ion from the unlabeled Lys-Glc-DAG and *m/z* 191 ion from the ^{13}C-labeled Lys-Glc-DAG.

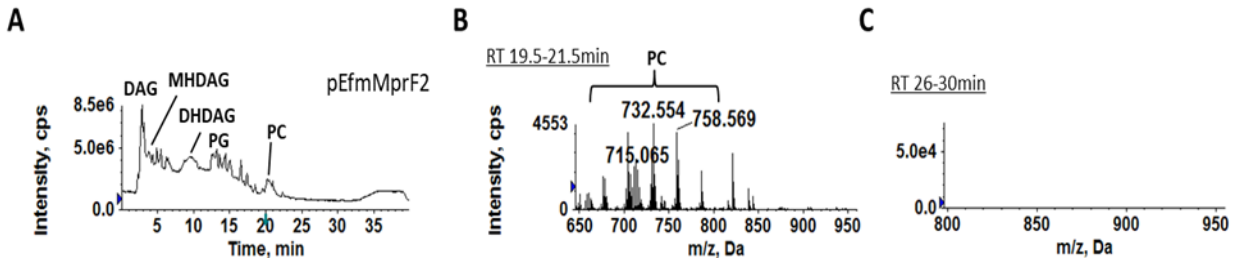

Figure B.2. Lipidomic analysis of *E. faecium mprF2* expression in *S. mitis* ATCC 49456. The lipid extracts of *S. mitis* cells expressing *E. faecium mprF2* (pEfmMprF2) was analyzed by LC-ESI/MS in the positive ion mode. A) TIC of 49456(pEfmMprF2) shows the whole lipidomic profile. B) MS of retention time 19.5 – 21.5 min showing the absence of Lys-PG in the strain. C) MS of retention time 26 – 30 min showing the absence of Lys-Glc-DAG. This data is consistent with previous literature that the *mprF2* allele in the *E. faecium* genome is inactive.

Table B.1. Primers used in this study.

Primer	5′ – 3′ sequence	Use
GBS_MprF_F	GAGAGGTCCCTTTCCTTGAAAAAGCTAATTGAAAAAGTC	Amplify GBSCOH1_1931 for Gibson assembly
GBS_MprF_R	ACCAATACCTTTATCTTATTTAACAATCTTAATTTTACTATC	Amplify GBSCOH1_1931 for Gibson assembly
Faec_MprF1_F	GAGAGGTCCCTTTCCTTGTTAAAAAATACCATACAATG	Amplify EFTG_00601 for Gibson assembly
Faec_MprF1_R	ACCAATACCTTTATCTTAATACTTTCTTCGTATCC	Amplify EFTG_00601 for Gibson assembly
Faec_MprF2_F	GAGAGGTCCCTTTCCGTGAAAAATAAACGATACTTG	Amplify EFTG_02430 for Gibson assembly
Faec_MprF2_R	ACCAATACCTTTATCTTATAATTCTGTGAGCACTTC	Amplify EFTG_02430 for Gibson assembly
pABG5-5'	GGAAAGGGACCTCTCTTCCTAAAC	Linearize pABG5Δ*phoZ* for Gibson assembly
pABG5-3'	GATAAAGGTATTGGTAAATAACAAA	Linearize pABG5Δ*phoZ* for Gibson assembly
	Expression plasmid sequencing	
GBS_S1	GAATGGAATAATATAGTAGGCT	For sequencing pGBSMprF, amplifies with pABG5_Fup2
GBS_S2	GATTGTATCCCTTATTCC	For sequencing pGBSMprF, amplifies with GBS_S3
GBS_S3	CGATTCAATAGCTTCAC	For sequencing pGBSMprF, amplifies with GBS_S2
GBS_S4	GATAAAAGGCTCTACTGG	For sequencing pGBSMprF, amplifies with pABG5_FDwn
pABG5_FDwn	CCAATAATAATGACTAGAGAAG	For pABG5 plasmid insert sequencing
pABG5_Fup2	CAAAAGGTTTCGACTTTTCACC	For pABG5 plasmid insert sequencing
EF1_S1	GAATAACGCTGATCAAAAGT	For sequencing pEfmMprF1, amplifies with pABG5_Fup2
EF1_S2	TGCCAAGAGAAATAGTC	For sequencing pEfmMprF1, amplifies with EF1_S3
EF1_S3	ACAATCTCTTCGCTTG	For sequencing pEfmMprF1, amplifies with EF1_S2
EF1_S4	CCAACTGTTCTTCTCCAA	For sequencing pEfmMprF1, amplifies with pABG5_FDwn
EF2_S1	CTCCTGTATGTGTGATGAAC	For sequencing pEfmMprF2, amplifies with pABG5_Fup2
EF2_S2	GGAATGGGGACTTTTGA	For sequencing pEfmMprF2, amplifies with EF2_S3
EF2_S3	ACTGATTCGTTGCTGC	For sequencing pEfmMprF2, amplifies with EF2_S2
EF2_S4	CTTCGCTTGTCAGGTTAC	For sequencing pEfmMprF2, amplifies with pABG5_FDwn
GBSCOH1_1931 knockout plasmid construction, sequencing, and integration screening		
Mp1F_PstI	ACGTCACTGCAGTTCAATTAGCTTTTTCAACAATTTC	Amplifies upstream fragment from within GBSCOH1_1931 leaving 6 codons, with Mp1R_XhoI
Mp1R_XhoI	ACGTCACTCGAGGCTGTTTATGGTGCTTTG	5′ most primer of upstream fragment, amplifies with Mp1F_PstI
Mp2F_XbaI	ACGTCATCTAGAGAAAAGGCTAGATTACGAAC	3′ most primer of downstream fragment, amplifies with Mp2R_PstI
Mp2R_PstI	ACGTCACTGCAGGTTAAATAAGCTTTATTTGGCA	Amplifies downstream fragment leaving 2 codons and stop codon of GBSCOH1_1931, with Mp2F_XbaI
T7 promoter	TAATACGACTCACTATAGGG	Amplifies with MpS5F below to sequence plasmid, amplifies with T3 promoter for insert screening and plasmid presence
T3 promoter	AATTAACCCTCACTAAAGGG	Amplifies with MpS3R below, amplifies with T7 promoter for insert screening and plasmid presence
Int_F	GCTAATTGAACTGCAGGTTAAATAAG	Anneals at *mprF* knockout site, amplifies with Out_R for single integration screening
Out_R	GCTATTATATTTAGTGGTTTAATTGG	Anneals outside recombination arms, amplifies with Int_F, for single integration screening
	Genomic knockout region sequencing	
MpS3F	CATTAGCTAGTCTTATCGGAG	Anneals outside integration arms, amplifies with MpS3R

MpS3R	ACAGCTACTTGGTAGTTCA	Amplifies with MpS3F
MpS4F	GCTACTAAGGCAAGATACG	Amplifies with MpS4R, knockout screening and plasmid sequencing primer
MpS4R	ATGGTCAGCGATGGTG	Amplifies with MpS4F, knockout screening and plasmid sequencing primer
MpS5F	CATAAGCGAAATAACTTGAG	Amplifies with MpS5R
MpS5R	GTATACAACGGCTTGATTGG	Anneals outside integration arms, amplifies with MpS5F

BIOGRAPHICAL SKETCH

Luke Rognvald Joyce was born in Johannesburg, South Africa in 1990 to Ian and Fiona Joyce. He graduated from I.R. Griffith Primary School in 2003 and attended Parktown Boys' High School, matriculating in 2008. In 2009, Luke began his undergraduate studies at Midwestern State University in Wichita Falls, Texas. He played collegiate tennis, earning numerous national rankings, played in the NCAA/ITA national quarterfinals, and was awarded the 2013 ITA National Arthur Ashe Jr. Award for Leadership and Sportsmanship. Luke graduated with his BSc in Molecular and Cellular Biology in 2014. After graduation, Luke joined The University of Texas at Dallas to begin his graduate studies in Molecular and Cell Biology. He joined the lab of Dr. Kelli Palmer in 2015 researching antibiotic resistance in the opportunistic pathogens, *Streptococcus mitis* and *S. oralis*. Luke received his MSc in 2016, joined the doctoral program at UT Dallas the same year, and began his research on the streptococcal cellular membrane.

CURRICULUM VITAE

Luke R. Joyce

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The University of Texas at Dallas
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EDUCATION

Ph.D.	Molecular and Cell Biology **The University of Texas at Dallas**, Richardson, TX Advisor: Dr. Kelli Palmer	2016-Present
M.Sc.	Molecular and Cell Biology **The University of Texas at Dallas**, Richardson, TX	2014-2016
B.Sc.	Molecular and Cellular Biology **Midwestern State University**, Wichita Falls, TX Minor: Chemistry	2009-2014

RESEARCH EXPERIENCE

Research Assistant, Dr. Kelli Palmer Lab 2015-Present
The University of Texas at Dallas, Richardson, TX

Projects:

1. Genome defense systems and gene essentiality in bloodstream infection-derived *Streptococcus mitis* and *S. oralis*
2. Lipidomic and lipoteichoic acid characterization in *Streptococcus mitis* and *S. oralis*
3. Lipidomic characterization of major streptococcal pathogens and novel lipid identification in *Streptococcus agalactiae*

Student researcher, Dr. Jung-Whan Kim Lab 2015
The University of Texas at Dallas, Richardson, TX

Project: Glycolytic flux in Squamous Cell Carcinomas

PUBLICATIONS (Reverse Chronological order)

1. **Joyce LR,** Guan Z, Palmer KL. 2019. Phosphatidylcholine biosynthesis in Mitis group streptococci via host metabolite scavenging. *J Bacteriol.* 201:e00495-19. PMC6805115
*Spotlighted by the editors of the Journal of Bacteriology as an article of significant interest

2. Adams HM, **Joyce LR**, Guan Z, Akins RL, Palmer KL. 2017. *Streptococcus mitis* and *S. oralis* Lack a Requirement for CdsA, the Enzyme Required for Synthesis of Major Membrane Phospholipids in Bacteria. *Antimicrob Agents Chemother* 61: e02552-16. PMC5404519

Oral presentations:

Virtual Streptococcal Trainee Symposium, 2020
Phosphatidylcholine biosynthesis in Mitis group streptococci via a host metabolite scavenging pathway
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

Prokaryotic Pathogens Online Seminar Series, 2020
Phosphatidylcholine biosynthesis in Mitis group streptococci via a host metabolite scavenging pathway
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

20th International Conference on Bacilli and Gram-Positive Bacteria, 2019
University of Maryland, Washington, DC
Phosphatidylcholine biosynthesis in Mitis group streptococci via a host metabolite scavenging pathway
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

American Society for Microbiology Texas Branch Meeting, 2019
University of Texas at San Antonio, San Antonio, TX
Phosphatidylcholine biosynthesis in Mitis group streptococci via a host metabolite scavenging pathway
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

Second UT Dallas/Texas A&M Agrilife Research Extension Symposium, 2019
Texas A&M Agrilife Center, Dallas, TX
Phosphatidylcholine biosynthesis in streptococci: you are what you eat
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

Understanding Bacterial Pathogenesis in the Host Environment, 2019
UT Southwestern, Dallas, TX
Streptococci scavenge human metabolites to synthesize membrane lipids
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

Wind River Conference on Prokaryotic Biology, 2018
Estes Park, CO
The curious case of phosphatidylcholine in *Streptococcus mitis*
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

Poster presentations:

Boston Bacterial Meeting (Virtual Meeting, Flash Talk), 2020
Boston, MA
Phosphatidylcholine biosynthesis in Mitis group streptococci via a host metabolite scavenging pathway
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

Cold Spring Harbor Laboratories: Microbial Pathogenesis and Host Response, 2019
Cold Spring Harbor, NY
Phosphatidylcholine biosynthesis in Mitis group streptococci via a host metabolite scavenging pathway
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

International Conference on Gram-Positive Pathogens, 2018
Omaha, NE
The curious case of phosphatidylcholine in *Streptococcus mitis*
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

Streptococcal Biology Gordon Research Conference, 2018
Newry, ME
The curious case of phosphatidylcholine in *Streptococcus mitis*
Luke R. Joyce, Ziqiang Guan, and Kelli Palmer

Wind River Conference on Prokaryotic Biology, 2017
Estes Park, CO
Streptococcus mitis and *Streptococcus oralis* mutate an 'essential' gene upon exposure to daptomycin
Hannah Adams, Luke R. Joyce, Ziqiang Guan, Ronda L. Akins, and Kelli Palmer

American Society for Microbiology Texas Branch Meeting, 2016
UT Dallas, Richardson, TX
Genome defense systems in *Streptococcus mitis* and *Streptococcus oralis* infective endocarditis isolates
Luke R. Joyce and Kelli Palmer

Collaboration posters and presentations (First author being the presenter):

Cold Spring Harbor Laboratories: Microbial Pathogenesis and Host Response, 2019
Cold Spring Harbor, NY
Lipoteichoic acid synthesis in *Streptococcus mitis*
Yahan Wei, Luke Joyce, Ashley Wall, Ziqiang Guan, Kelli Palmer

20th International Conference on Bacilli and Gram-Positive Bacteria, 2019
University of Maryland, Washington, DC
Lipoteichoic acid synthesis in *Streptococcus mitis*
Yahan Wei, Luke Joyce, Ashley Wall, Ziqiang Guan, Kelli Palmer

American Society for Microbiology Texas Branch Meeting, 2017
College Station, TX
Transposon Mutagenesis in *Streptococcus mitis* and *Streptococcus oralis*
Sai A. Kamma, Luke R. Joyce, and Kelli Palmer

American Society for Microbiology Streptococcal Genetics Meeting, 2016
Washington, DC
One-step daptomycin resistance: A widespread concern or a unique phenomenon
Hannah Adams, Luke R. Joyce, Ronda L. Akins and Kelli Palmer

Wind River Conference on Prokaryotic Biology, 2016
Estes Park, CO
One-step daptomycin resistance: A widespread concern or a unique phenomenon
Hannah Adams, Luke R. Joyce, Ronda L. Akins and Kelli Palmer

TEACHING EXPERIENCE

Teaching Assistant for UTD class BIOL3302, Fall 2015-Spring 2016
Undergraduate Molecular and Cell Biology class
The University of Texas at Dallas, Richardson, TX
Helped teach a workshop once a week, answering students' questions, reviewing material for exams, grading homework assignments, proctoring and grading exams for two class sections.

MENTORING EXPERIENCE

Supervised Harshini Cormaty (UTD undergraduate student) 2019-2020
Project: Antibiotic susceptibilities of Streptococci with phosphatidylcholine present in the lipid membrane.

Supervised Evelyn Gartstein (UTD honors undergraduate student) 2018-2019
Project: Identification and phenotypic characterization of clinical isolates of Viridans Group Streptococci

Supervised Lori Vu (UTD honors undergraduate student) 2018-2019
Project: Identifying *Streptococcus mitis* phage in human saliva

Supervised Rohit Badia (UTD honors undergraduate student) 2017-2018
Project: Restriction modification characterization in *Streptococcus mitis* and *Streptococcus oralis*

Supervised Sai Kamma (UTD undergraduate student) 2017-2018
Project: Transposon Mutagenesis in *Streptococcus mitis* and *Streptococcus oralis*

Supervised Katie Mulryan (UTD undergraduate student) Summer 2016
Project: Blood survival and growth rates of Viridans Group Streptococci

FELLOWSHIPS AND AWARDS

O. B. Williams Award 2019
American Society for Microbiology Texas Branch Meeting
Certificate of outstanding scientific achievement in general microbiology for first place in oral presentations by graduate students

International Conference of Gram-Positive Pathogens Travel Award 2018

Wind River Conference on Prokaryotic Biology Travel Award 2018

Wind River Conference on Prokaryotic Biology Travel Award 2017

Department of Biological Sciences Award 2017
The University of Texas at Dallas
Formerly the Harris Award, this is given to a doctoral student for the best research presentation

Samuel Kaplan Award 2016
American Society for Microbiology Texas Branch Meeting
Certificate of outstanding scientific achievement in genomic microbiology for first place in a poster presented by a graduate student

CERTIFICATIONS

Elsevier Research Academy Certified Peer Review Course 2019
Online course for training on effective and comprehensive reviews of manuscripts

Responsible Conduct of Research Seminar Professional Series 2017
Provided by the University of Texas at Dallas Office of Research to satisfy requirements by the NSF and NIH funding agencies. Topics covered: Introduction to Responsible Conduct of Research, Designing your Research, Conducting your Research, Reporting your Research, Responsibilities to Society, Research Ethics, Mentor and Mentee Responsibilities and Relationships, Human Subjects in Research, Data Management and Ownership

MEMBERSHIPS

Member of American Society for Microbiology 2016-Present

SERVICES TO THE FIELD

Journal of Emerging Investigators Reviewer 2020-Present

Biological Sciences Graduate Student representative at faculty meetings 2018-2020

Virtual Streptococcal Trainee Symposium Session Leader 2020

Royston C. Clowes Lecture Series 2018
Member of the Biology Graduate Student Committee at The University of Texas at Dallas to organize and run the *in-memoriam* Royston C. Clowes Lecture Series

Manuscript reviews

Journal of Emerging Investigators (2020)
Journal of Emerging Investigators (2020)
Journal of Emerging Investigators (2020)
Journal of Emerging Investigators (2020)
Journal of Emerging Investigators (2020)
BMC Microbiology (2020), in collaboration with graduate mentor
Journal of Emerging Investigators (2020)
Journal of Emerging Investigators (2020)