THE ROAD TO RESILIENCY: UNDERSTANDING THE IMPACT OF INTRAPARTUM ANTIBIOTICS ON GROUP B *STREPTOCOCCUS*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Microbiology and Molecular Genetics – Doctor of Philosophy

2023

ABSTRACT

Streptococcus agalactiae (Group B *Streptococcus* or GBS) is an opportunistic bacterial pathogen that asymptomatically colonizes the recto-vaginal tract of up to 35% of pregnant people. GBS colonization during pregnancy is a risk factor for adverse pregnancy outcomes including chorioamnionitis, preterm and stillbirths, as well as severe neonatal disease. GBS disease in neonates has two presentations: early (EOD) and late onset disease (LOD), which occur within the first week or first three months after birth, respectively. Prevention protocols for GBS disease include screening for recto-vaginal colonization during late stages of pregnancy (35-37 weeks) and, if positive, administering intrapartum antibiotic prophylaxis (IAP) treatment. IAP has only been successful in reducing EOD and is not effective in preventing LOD, preterm births, or stillbirths. Importantly, there are increasing observations of persistent colonization after IAP intervention, indicating that the pathogen can survive and rebound following antibiotic exposure. Intriguingly, strains classified as a hypervirulent genotype are better able to withstand these stressors and persistently colonize the vaginal tract, while others are easily cleared by host immune cells or a course of antibiotics. The mechanisms by which these GBS strains avoid antibiotic-mediated killing to persistently colonize the vaginal tract, however, are poorly understood. I sought to investigate these mechanisms by examining the impact of IAP on 1) GBS genomic evolution and 2) the biogenesis of membrane vesicles (MVs). I employed whole genome sequencing analyses on 97 clinical GBS isolates previously obtained from the vaginal tracts of pregnant individuals before (35-37 weeks' gestation) and after (6 weeks postpartum) IAP and childbirth. One goal was to identify key genomic signatures associated with persistent colonization. Using both reads-based and assembly-based methods, I observed substantial evidence of genomic variation between pairs (prenatal-postpartum) of isolates, leading to the discovery of mutators among the postpartum isolates. These mutators have exceptionally high mutation rates due to disruptions in DNA mismatch repair systems and provide a reservoir of beneficial mutations that enhance fitness. Indeed, I observed evidence of genes under positive

selection in mutator isolates after IAP exposure, including those that encode attachment and regulatory proteins. Moreover, we observed stronger biofilms in most of the postpartum isolates compared to their respective prenatal isolates. These findings demonstrate that antibiotic treatment impacts GBS evolution *in* vivo by selecting for mutations that promote persistent colonization and survival. The presence of mutators may lead to the emergence of more resilient strains in the vaginal tract, increasing the risk of invasive GBS infection. To further elucidate mechanisms of survival in the presence of antibiotics, I examined the production and composition of MVs produced by a hypervirulent GBS strain that persistently colonized the vaginal tract after IAP. MVs are biologically active, lipid-enclosed entities that have been shown to play a role in bacterial persistence and survival of antibiotic stress in other species. No prior studies of GBS MVs in the context of antibiotics have been conducted and thus, I isolated MVs produced by GBS after exposure to β-lactam and macrolide antibiotics. Quantification of MVs revealed that antibiotic treatment significantly increases the abundance of MVs produced regardless of the antibiotic class. Using proteomics to characterize the proteins packaged within MVs revealed protein compositions that were distinct for each antibiotic treatment when compared to the untreated (control) group. Furthermore, increased abundances of antibiotic targets that were specific to each respective antibiotic treatment were detected, suggesting that GBS MVs protect the bacteria from antibiotic killing by disseminating decoy targets outside of the cell. Both the excess quantities and distinct compositions of MVs produced in the presence of antibiotics may enhance GBS survival and contribute to persistent colonization despite IAP intervention. Improving our understanding of GBS MVs and their role in persistent infections will aid in the development of more targeted and effective treatments for GBS disease during pregnancy. Together, this work considerably improves our understanding of persistent colonization despite antibiotic exposure and is a fundamental step towards improving GBS treatment and prevention strategies to effectively reduce the incidence of GBS disease.

To those who have unconditionally loved and supported me along the way.
This would not have been possible without you.

ACKNOWLEDGEMENTS

This dissertation would not have been possible without all the help and support from so many wonderful people and organizations, as they say; it truly takes a village. I would first like to thank my mentor, Dr. Shannon Manning, who has been an incredible source of guidance, encouragement, and support throughout my graduate career, believing in me when I often do not believe in myself. I knew Shannon was going to be my mentor the minute I met her. I recall the first time I walked into her office to inquire about rotating in her lab and I remember how she really listened to my thoughts and ideas in a way that made me feel confident and excited about them, which has been a recurring theme under her mentorship, allowing me to become a more confident and independent scientist. Shannon is not only a distinguished and well-accomplished scientist, but she is also an incredibly strong, intelligent, and kind person. Shannon has continually encouraged me to pursue opportunities that I'm passionate about and has connected me with many wonderful people and organizations. I am very grateful to be under the mentorship of such an incredible woman in science during my time here at Michigan State University (MSU) and beyond. I cannot thank her enough for being such an inspirational role model and mentor. I would also like to acknowledge the Manning lab as a whole. This lab has been truly the best to be a part of and has always felt like family from the very beginning. Many of the people I have met through this lab have become lifelong friends. Going to work every day never felt like a chore because of this wonderfully fun and supportive group. A special thank you to Samantha Carbonell, our lab manager, who continually keeps the lab running smoothly and has been an incredible source of support through listening to me vent about anything and everything and for always being there to lend a hand.

I am very grateful for the guidance received from my graduate committee members, Dr. Peggy Petroff, Dr. Chris Waters, and Dr. Jonathan Hardy, who have all been essential in helping to better my research as well as myself as a scientist. Thank you, Dr. Hardy, for always expressing such interest in my work and asking wonderfully thought-provoking questions.

Thanks to Dr. Petroff, not only for her knowledge of and expertise in human pregnancy and immunology, but for allowing use of her lab space and equipment needed for completion of my membrane vesicle studies. I am thankful for the Petroff lab, as a whole (Dr. Soo Ahn, Morgan Collins, and Geoffrey Grzesiak), for being so welcoming and inclusive of me and for essentially being my “adopted” lab over the years. Thank you to Dr. Waters, for introducing me to bacterial pathogenesis research and the wonderfully collaborative research community both within the Microbiology and Molecular Genetics (MMG) department and across MSU. I would also like to specifically thank Roseann Bills in the MMG department who keeps everything running so smoothly for the graduate students and has always been incredibly helpful, answering every question and working to solve any problems I have had along the way. Thank you, also, to the talented individuals at the MSU Core facilities; Carol Flegler, Amy Albin, and Dr. Alicia Withrow as well as Douglas Whitten for their expertise and assistance with all the microscopy and proteomics components of my work, respectively. A special thanks to our external collaborators Dr. H. Dele Davies and Dr. Heather Blankenship as well as Dr. David Aronoff and Dr. Jennifer Gaddy, who have provided valuable advice and expertise throughout the course of projects described in chapters 2 and 3 of this dissertation, respectively. Additionally, my completion of this degree certainly would not have been possible without the generous financial support. I am very grateful for the funding provided by the National Institutes of Health and am honored to have received the Ralph Evans and Gerhardt Awards from the MMG department, as well as the Graduate Continuation Fellowship and Outstanding Scholar Awards from the College of Natural Science at MSU.

I am very fortunate to be surrounded by wonderful friends who have been an integral part of my Ph.D. journey. I’d like to first acknowledge the national professional organization, Graduate Women in Science (GWIS), that I have been a part of since the beginning of my graduate career. This organization has helped me discover my passion of sharing science with the community and has introduced me to so many phenomenal women in science who have

really been an incredible network of support throughout my time here at MSU. Special thanks to Abby Sulesky-Grieb who led the GWIS Mid-Michigan team with me as co-presidents during my last year. Abby has been my dissertation buddy throughout the last several months and has become a very dear friend. Thank you to all the incredible people within my volleyball family, special shoutout to the founding members that brought me into the community: Zoe Hansen, Mike Pajkos, Nick Valverde, and Adam Kawash. I have had so much fun with the many teammates I have played with over the years and this community has been a great escape from the day-to-day stress of graduate school. Thank you to Bailey Bowcutt, a former undergraduate student in the Manning lab, for being my writing buddy and a great source of support at the start of my dissertation process. Thank you to the lifelong friends that I met through the Manning lab: Jose Rodrigues, Zoe Hansen, Sanjana Mukherjee, and Cole McCutcheon. I am incredibly lucky to have worked with some of my best friends over the years who have all made this journey so wonderful. Thank you, Jose, for always making us incredible food, sharing a glass of port wine, and being a good sport for our office pranks. Thank you, Zoe, for being my workout buddy, introducing me to GWIS and volleyball groups, and always being down for a vent session over a cup of earl grey tea. Thank you to Sanjana, for being so emotionally supportive over the years, always knowing the right thing to say at just the right time. And to Cole, who has shown me all there is to know about GBS and real housewives, for being an incredible friend and always being down for a Starbucks run or a glass of wine. I would also like to thank my incredible friends who have been with me from the very start of my microbiology journey at the University of Wisconsin-Madison (Go Badgers!). Thank you to Erin Ladd who made all the difficult science courses so much more bearable, especially organic chemistry! Thank you to Rachel Beaver, who has a heart of gold and is one of the kindest people I know, for always bringing such positivity and light to my life. Finally, thank you to my best friend and soul sister, Lauren Putnam. Lauren and I met as freshman roommates in college and have been best friends ever

since. Lauren is one of the strongest people I know and an incredibly loyal friend. Thank you, Lauren, for always looking out for me and being so supportive through everything.

I am also deeply grateful for my family. I have a wonderfully large family and with a big family comes big love and support. Thank you to my little sisters Emily, Sam, and Ava Pell for always making me laugh and feel so loved. Sam and Aves I am so proud of the young, intelligent women you have become, and I am so grateful and proud to be your big sister. Emily, my built-in best friend since day one, thank you for always being my strong Corinthian column, a steady source of support through anything and everything, and for being able to bring me out of my moments of worry and self-doubt. Thank you to Fred and Sherry Sevic, for being so incredibly loving and supportive over the last decade, I am truly grateful to have you both in my life. Thank you to my Omi and Grandpa for always reminding me of how proud you are of me. The two of you are always there when I need you and have always been there to support me, coming to every single tennis match, band concert, school event, you name it. Thank you to my parents; my Mom and Stepdad, Shannon and Ingolf Meijer, and my Dad and Stepmom, Dave and Michelle Pell. You have all helped raise me and have provided such a loving and supportive environment throughout my life and have all laid the foundation from which I have been able to succeed and achieve my dreams. Dad, thank you for, first of all your endless supply of dry and sarcastic humor which I may or may not have inherited, for always being my calm voice of reason, and reminding me of who I am and what I can accomplish. And mom, thank you for always anticipating my every need, being so thoughtful, and for raising me to be a strong, independent woman. Thank you to my cat and fur-child, Bubba, who we adopted when I started grad school. Thank you, Bubs for all the love and snuggles, and for always keeping me laughing.

Finally, thank you to my partner, Will Sevic, who is one of the most caring, hard-working, and selfless people I know. Will and I have been together since high-school and he has been my rock ever since. He knows how passionate I am about microbiology and what this Ph.D. truly

means to me. He has listened to every single practice talk, has brought me dinner when I'm stuck at the lab late at night doing a vesicle prep, and importantly, he has always given me a reason to come home. Thank you, Will, for being a constant source of unconditional love and support every single day, for always picking me up and taking care of me when I am too tired and stressed to take care of myself, and for taking care of our home and our life while I have been dedicating my time towards school for the past five and a half years. I seriously could not have done this without you and am so incredibly grateful to have you as my partner in life.

While earning my Ph.D. has been one of the most challenging processes I have encountered in my life, it has also been one of the most rewarding experiences. I knew I wanted to become a scientist since the day I learned about microorganisms in the 6th grade. Looking back, I never would have imagined how incredibly rewarding this journey has been, all of which is due to the people who have taught, mentored, supported, encouraged, and loved me along the way.

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CHAPTER 1:
LITERATURE REVIEW – GROUP B *STREPTOCOCCUS* DISEASE, PREVENTION, AND PERSISTENT COLONIZATION

GROUP B STREPTOCOCCAL DISEASE & BURDEN

Streptococcus agalactiae, or Group B *Streptococcus* (GBS), is a Gram-positive, opportunistic bacterial pathogen that can cause disease in pregnant people and their neonates, elderly adults, and a wide variety of animals including cattle and tilapia. GBS was first identified as a human pathogen in 1938 causing postpartum sepsis and has emerged as the leading cause of newborn infection in the United States starting since the 1970s [1,2]. Neonatal GBS disease presents as bacteremia, meningitis, pneumonia, and/or sepsis, and can occur in two onsets: early onset disease (EOD) which occurs within the first week after birth or late onset disease (LOD) which occurs within the first week to 3 months after birth [3]. EOD is thought to result from vertical transmission from the colonized pregnant person to the neonate during labor or after the rupture of placental membranes and presents mainly as bacteremia, sepsis, and pneumonia [4,5]. Studies have suggested that LOD results from infant pharyngeal or gastrointestinal colonization that is horizontally acquired through breast milk, but the pathogenesis of LOD remains elusive [6,7]. Neonatal meningitis is more commonly associated with LOD affecting 30% of cases compared to 10% of EOD cases. Moreover, LOD has been shown to result in invasive infection of bones, joints, and soft tissues [4]. In 2015, an estimated 319,000 cases of neonatal GBS disease were reported worldwide; most (64%, 205,000) were EOD cases but LOD accounted for the remaining 36% (114,000) [8].

Together, GBS disease of neonates and infants contributed to an estimated 90,000 deaths, including 57,000 GBS-associated stillbirths, which provides a minimum estimate of GBS fetal infections worldwide [8]. This estimate is higher than the number of neonatal deaths caused by human immunodeficiency virus (HIV), tetanus, pertussis, and respiratory syncytial virus (RSV) combined [9–11]. Moreover, GBS is the leading cause of neonatal bacterial meningitis in the United Kingdom and the United States [12,13]. Survivors of neonatal GBS disease are at risk of neurodevelopmental impairment as a result of GBS meningitis or sepsis [14]. On average, 10,000 new cases of neurodevelopmental impairment each year was

estimated for survivors of GBS neonatal meningitis [8].These frequencies vary by geographic location and are much higher in countries where treatment methods are not implemented. In Nigeria, for example, the rates of GBS colonization (34.2%) and transmission to the neonate (19%) are much higher, with a neonatal GBS disease incidence of 2.0 per 1,000 live births, compared to the average global incidence 0.49 per 1,000 live births [15,16].

Because vertical transmission is so important for neonatal and infant infections, numerous studies have been conducted to understand GBS colonization during pregnancy. In humans, GBS exists commensally in the gastrointestinal tract from which the urogenital tract may become colonized. Recent estimates indicate that 11-35% of pregnant patients are colonized [17], and both transient and persistent colonization has been observed during pregnancy [18]. Although GBS vaginal colonization is often asymptomatic, it is the main risk factor for invasive GBS disease during pregnancy, as it can be vertically transferred to the neonate either *in utero* via ascending infection or by aspiration of infected fluid while moving through the birth canal [2,5]. It was previously estimated that ~50% of GBS-colonized patients will transmit the bacterium to their newborns with 1-2% developing EOD [19,20]. The prevalence of GBS vaginal colonization varies by geographical region with concentrations ranging from 3.0 x 10^1 to 1.4 x 10^7 CFU/mL [8,19,21]. Heavy maternal colonization was identified as an important risk factor for neonatal infection along with gestational age <37 weeks, very low birthweight, intrapartum fever, prolonged rupture of membranes, intraamniotic infection, as well as young age and black race of the pregnant person [4,22–27].

Additionally, GBS colonization can lead to other adverse pregnancy outcomes including preterm births, stillbirths, and sepsis of the pregnant person [4,8,24,28]. Studies suggest that GBS likely accounts for more deaths *in utero* than after delivery due to its estimated association with up to 3.5 million preterm births and 1-4% of stillbirths, each year [8,24,29]. Intrauterine infections occur via ascension of GBS from the vagina and into the uterus where it can contaminate the amniotic fluid. Ascending infection greatly increases the risk of preterm labor

through GBS invading and rupturing the placental membranes [30,31]. Given the importance of GBS colonization of the vaginal tract, screening for and assessing risk factors of GBS colonization during pregnancy have become the focal point for GBS treatment and prevention practices.

TREATMENT & PREVENTION OF GBS DISEASE

Detecting and treating GBS colonization during pregnancy

In 1996, the Centers for Disease Control and Prevention (CDC) published the first set of guidelines for the treatment of perinatal GBS that consisted of two approaches: an antenatal screening method and a risk-assessment based approach [32]. The antenatal screening method involved universal screening of pregnant patients for rectal-vaginal GBS colonization between 35- and 37-weeks' gestation, while the risk-based approach relied on the use of clinical factors for perinatal GBS disease. After 6 years of GBS surveillance in the context of pregnancy, the antenatal screening method was found to be more effective in reducing neonatal disease [5,33–36]. Due to the intermittent nature of GBS colonization [18], obtaining vaginal-rectal culture results as close to the expected date of delivery as possible is important to prevent unnecessary administration of antibiotics. Hence, updated guidelines recommended antenatal screening between 36-37 weeks' gestation or upon hospital admission for individuals with known risk factors for preterm delivery and/or unknown colonization status [35].

Intrapartum antibiotic prophylaxis (IAP) for GBS prevention

When a pregnant patient tests positive for GBS at their antenatal screening, IAP is recommended during labor. IAP is also recommended as a precautionary measure if colonization status is unknown at the time of labor or if the patient has a history of an infant with GBS disease. Due to the increased risk of neonatal disease in preterm infants, IAP is also recommended for patients with premature rupture of membranes regardless of GBS culture status as ~50% of pregnant people delivering preterm lack an antenatal screening result. Additionally, in heavily colonized patients, GBS can be detected in urine cultures (bacteriuria),

and therefore, IAP is recommended for individuals presenting with GBS bacteriuria at any point during the pregnancy as well [2,5].

Prophylactic therapy is meant to prevent neonatal infections rather than treat active infections. Therefore, the goal of IAP is to decrease the level of GBS colonization in the pregnant person enough to prevent colonization of the fetus and limit transmission to the neonate during childbirth by reaching minimum inhibitory concentrations of antibiotics in the blood [37–39]. Studies show evidence of large decreases in the concentration of GBS in the vagina after 4 hours of IAP treatment [21,40–43] and since its implementation as a prevention strategy, IAP has successfully reduced the incidence of EOD [35,44–48]. In the United States, for example, the incidence of EOD decreased from 0.7 to 0.2 cases per 1,000 live births after IAP recommendations were implemented in the 1990s [49,50].

ANTIBIOTCS EFFECTIVE FOR GBS PREVENTION AND TREATMENT

Two main categories of antibiotics are available: bactericidal and bacteriostatic, which kill or prevent growth of bacteria, respectively. Both bactericidal antibiotics, including the β-lactams and glycopeptides, and bacteriostatic antibiotics, including macrolides and lincosamides, are used to treat and prevent GBS disease [5]. The effects of antibiotics are either concentration-dependent, meaning the degree of bacterial killing increases with increasing concentrations of the drug, or time-dependent, in which increased duration of treatment at the effective concentration increases bacterial killing [51]. To treat a given infection, the choice of antibiotic often depends on the level of information available regarding the pathogenic organism. In cases where this information is limited, broad-spectrum antibiotics that can target many different types of bacteria, are most helpful. Whereas directed antibiotic therapy can be implemented when the pathogen in question can be cultured and identified [37]. When possible, use of antibiotics with a narrower spectrum is the best practice to minimize the emergence and spread of antibiotic resistance and the negative impacts on commensal microbes and the microbiome in the body.

Penicillins: The gold standard for GBS treatment

The most effective antibiotics for preventing neonatal GBS disease are the penicillins representing the β-lactam antibiotic class. Penicillin G (benzylpenicillin), in particular, is the antibiotic of choice for IAP, but ampicillin is also used and can limit intrapartum transmission of GBS to the newborn while reducing the level of postpartum vaginal colonization [5,38,39]. Penicillin G, a natural penicillin, is a narrow spectrum antibiotic that is particularly effective against Gram-positive bacteria, whereas ampicillin is a broad-spectrum antibiotic that is effective against a wide range of Gram-negative and Gram-positive species [52–55]. In one study, intravenous penicillin G resulted in a 5- and 50-fold decrease in vaginal GBS colony counts after 2 and 4 hours of IAP, respectively [21]. Similarly, IAP with ampicillin decreased incidence of neonatal colonization to 46%, 28%, and between 1-3% after less than 1 hour, 2 hours, and 4 hours of antibiotics, respectively [42]. Consequently, the recommendation is for at-risk pregnant patients to receive 4 hours of intravenous penicillin before giving birth, if feasible [5].

As β-lactam antibiotics, both penicillin G and ampicillin act by inhibiting the final stages of bacterial cell wall synthesis: crosslinking of peptidoglycans. Penicillin-binding proteins (PBPs) are the enzymes that facilitate peptidoglycan crosslinking and are thus the target proteins of penicillins. The small size of penicillin allows it to access the entire depth of the cell wall and effectively bind PBPs located inside the cell wall. Specifically, the β-lactam ring of the penicillin molecule binds to and inactivates the PBPs. Without properly functioning PBPs and consequent crosslinking, the integrity of the cell wall is weakened and eventually lyses from osmotic pressure [54–56]. β-lactams are bactericidal antibiotics and their activity is time-dependent; therefore, using the recommended duration of antibiotic prophylaxis is crucial to achieve the maximum clinical benefit of the treatment [55,57,58].

Penicillin alternatives

Because ~10% of the United States population reports a penicillin allergy [59–61], alternative antibiotics are recommended for penicillin-allergic patients. Penicillin allergies are classified as low, moderate, or high risk based on the severity and types of symptoms. Low-risk allergy is the most common type and is based on nonallergic gastrointestinal symptoms, a family history of penicillin allergy, symptoms of itching without rash, or unknown reactions >10 years ago. Moderate-risk allergy is characterized by hives or other itchy rashes and IgE-mediated reactions, while high-risk allergies are considered if patients report anaphylaxis, recurrent penicillin reactions, positive penicillin skin testing, or hypersensitivities to multiple β-lactams [62–64]. Fortunately, skin testing for penicillin allergy is safe during pregnancy and can be used to confirm the allergy along with its severity. This is important because a meta-analysis showed that approximately 90% of unconfirmed reports of penicillin allergy could be ruled out by using this formal skin testing method [5,64].

During pregnancy, those allergies classified as greater than low risk are treated as high-risk allergies. For GBS-positive individuals reporting low-risk penicillin allergies, cefazolin, a first-generation cephalosporin antibiotic, is considered an effective alternative to penicillins for IAP [5]. Although cephalosporins are also β-lactam antibiotics that inhibit bacterial cell wall synthesis, they have a different structure than penicillins and low cross-reactivity [64,65]. Additionally, cefazolin has broad spectrum activity and improved stability against β-lactamases, or enzymes that cleave the β-lactam ring, compared to most penicillins [66]. Evidence shows that ampicillin, cefazolin, and penicillin effectively cross the placenta, and reach minimum inhibitory concentration (MIC) levels of antibiotics in the amniotic fluid, cord blood, and neonatal blood within 1-2 hours, reaching optimal effects 4 hours after administration [3,5,36,41,67]. Penicillins and cephalosporins are also commonly used to treat asymptomatic bacteriuria and urinary tract infections during pregnancy [68]. Since cefazolin is in the same antibiotic class as penicillins, it is not recommended for individuals with high-risk penicillin allergies. Only 2% of

patients in the United States have reported a cephalosporin allergy, although there is evidence that the incidence is increasing [59].

The effective GBS treatment options for patients reporting high-risk penicillin allergies are limited to non-β-lactam alternatives such as clindamycin or vancomycin. Like the β-lactam antibiotics, clindamycin has been shown to significantly reduce vaginal GBS colony counts 2 hours following IAP and readily crosses the placenta [43,69–71]. Clindamycin is a lincosamide antibiotic that has a bacteriostatic effect but can be bactericidal at high concentrations. It is active against a wide range of bacterial species including Gram-positive cocci like GBS, and both Gram-negative and Gram-positive anaerobes [72]. In contrast to the β-lactams, clindamycin inhibits bacterial protein synthesis by binding rRNA of the 50S ribosome unit and effectively blocking ribosomal translocation [72–74]. Lincosamides are often grouped with macrolides and streptogramins based on their similar mechanism of action to comprise the Macrolide-Lincosamide-Streptogramin (MLS) class of antibiotics [75]. Historically, erythromycin, a macrolide antibiotic, was used to for IAP in patients reporting penicillin allergies; however, the number of erythromycin-resistant GBS clinical isolates has increased significantly (>50% of cases), making it a less effective treatment option [5,34,36,49].

An increase in the number of clindamycin-resistant GBS isolates has also been observed [76,77], which led the CDC to classify these pathogens as a “concerning resistant threat” in 2019 [78]. Indeed, it was estimated that clindamycin-resistant GBS contributed to 31,000 severe infections and 1,700 deaths in 2016, causing more than 40% of GBS infections [78]. As a result, vancomycin is recommended for patients who are reporting high-risk penicillin allergies and are colonized with clindamycin-resistant isolates. Vancomycin is a glycopeptide antibiotic that has bactericidal effects against Gram-positive bacteria. Like the β-lactams, vancomycin targets the bacterial cell wall, but instead of interacting with cell wall synthesis enzymes, it binds to the *N*-acetylmuramyl-pentapeptide precursor of peptidoglycan [79]. Binding to the precursor peptide inhibits its polymerization into long-chains of peptidoglycan, a process

called transglycosylation that results in an incomplete and disrupted cell wall, making the bacterial cells vulnerable to osmotic pressure [80,81]. Most Gram-negative species are intrinsically resistant to vancomycin because their outer membrane is impermeable to such large glycopeptides [65]. Since it is an expensive antibiotic and contributes to several adverse side effects including hypersensitivity reactions and nephrotoxicity, it is usually reserved to treat severe Gram-positive infections [82,83]. Regardless, vancomycin has been used for IAP and was shown to effectively reduce vaginal colony counts of GBS after 2 hours, with optimal benefit reached after 6 hours [82]. Since the concentration of vancomycin in neonates after IAP was considered subtherapeutic, weight-based dosing of vancomycin has been recommended for effective prophylaxis against GBS rather than a specific duration of time [5,36,84]. Given these drawbacks, use of vancomycin requires careful monitoring when administered during pregnancy and hence, it is often considered as a last resort treatment option [85]. More recently, clinical use of vancomycin for GBS IAP has become more common due to a rise in reports of drug allergies and resistant isolates [83], which is partly due to administration outside of CDC recommendations. One U.S. study reported that 94% of the 87 pregnant patients who received vancomycin for GBS IAP never underwent sensitivity testing to confirm serious penicillin allergies prior to use [86]. Reports of vancomycin-resistant GBS have also emerged leading some to suggest that the inappropriate use of vancomycin may cause an increase in the incidence of vancomycin resistance [87,88]. Collectively, these issues demonstrate the necessity for improved and alternative options for preventing and treating GBS disease.

ANTIBIOTIC RESISTANCE

To determine the proper dosage and concentration of antibiotics needed to effectively treat an infection, the MIC, or lowest drug concentration required to inhibit bacterial growth after 24 hours, should be established [89]. Such values are reported as clinical breakpoints for each antibiotic across bacterial species as determined by agencies including the U.S. Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), the Clinical and

Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The MIC of a given bacterial species is used to determine its susceptibility level to a certain antibiotic. If the MIC exceeds the reported threshold of susceptibility (i.e., clinical breakpoint), then the bacterial isolate is considered resistant to that antibiotic [90].

Antibiotic resistance can be intrinsic, in that the bacterium does not contain the target for a given antibiotic. For example, a Gram-negative bacterium is intrinsically resistant to vancomycin, which targets the peptidoglycan layer beneath the outer membrane that it is unable to cross [91]. Acquired resistance, on the other hand, occurs when bacteria gain a resistant phenotype through mutations or the acquisition of resistance genes from nearby bacteria via horizontal gene transfer [92]. Antibiotic resistance in GBS is most frequently acquired via horizontal gene transfer [93]. Common antibiotic resistance mechanisms employed by bacteria include reducing intracellular antibiotic concentrations via increased efflux or decreased uptake, inactivation of antibiotics via chemical degradation or enzyme modification, modifying the antibiotic itself, or alteration of the antibiotic target via mutation, elimination, or protection [58,91,92].

The overuse and improper use of antibiotics are the main contributors to the selection and spread of antibiotic resistance. GBS, along with many other bacterial pathogens, is largely resistant to tetracycline antibiotics due to its overuse [94]. Over 80% of GBS isolates carry genes that confer resistance to tetracyclines including ribosomal protection proteins (*tetM, tetO*) and efflux pumps (*tetK, tetL*) [95]. The acquisition of *tetM* and *tetO*, in particular, was suggested to impact the emergence and dissemination of more virulent strain types [94]. While tetracyclines are no longer used to clinically treat or prevent GBS in humans, resistance to the second-line agents clindamycin and erythromycin, have also emerged in GBS and increased in frequency [96]. GBS resistance to the MLS antibiotics varies by geographical region with up to 74.1% and 65.9% of cases having infections caused by isolates with resistance to erythromycin

and clindamycin, respectively [97–100]. In the United States, the increase of erythromycin and clindamycin resistance frequencies has led to the discontinued use of erythromycin for GBS IAP and the CDC to classify clindamycin-resistant isolates as a concerning threat level [96].

GBS resists killing by the MLS antibiotics through target modification, efflux pumps, or ribosomal protection proteins [95]. An isolate that is resistant to erythromycin is typically cross-resistant to clindamycin due to their similar mechanisms of action, which involves modification of the ribosome, the mutual target for these antibiotics. Methyl-transferases, which are encoded by *erm* genes, alter the ribosome resulting in cross-resistance to macrolides and lincosamides. Translation of the *erm* genes is inducible by macrolides and thus, resistance to these antibiotics is conferred in the presence of macrolides [101–105]. Similarly, ribosomal protection proteins (ABC-F proteins), encoded by *lsa* genes, confer broad-spectrum resistance to all MLS antibiotics, although this mechanism of resistance is rare in GBS [106]. Efflux pumps, on the other hand, confer resistance that is antibiotic-specific. Common MLS efflux pumps identified in GBS include the macrolide efflux pump encoded by *mefA/E* [107]. Additionally, GBS also possesses phosphotransferases and nucleotidyltransferases that directly alter macrolides and lincosamides, respectively, making them dysfunctional [95,107,108].

Gentamicin, an aminoglycoside antibiotic that inhibits protein synthesis, is sometimes used in combination with penicillins to treat severe cases of GBS EOD, it is not used for IAP. GBS isolates, along with other *Streptococcus spp.*, have low-level intrinsic resistance to aminoglycosides due to poor uptake of these large molecules [109]. High-resistance to gentamicin through the synthesis of aminoglycoside-inactivating enzymes has also been reported in GBS, but rates are relatively low (~0.3%) [109–112]. While resistance to fluroquinolones has also been observed in GBS, these antibiotics are not used to prevent GBS disease during pregnancy [95].

Fortunately, GBS remains susceptible to the penicillins, which are the antibiotics of choice to treat and prevent GBS infection [5]. Modification of penicillin-binding proteins is the

most common mechanism of β-lactam resistance in Gram-positive organisms [113]. Penicillins are still widely used in the clinic to treat and prevent GBS disease, however, reports of reduced penicillin susceptibility in GBS isolated from cases of invasive disease and vaginal colonization of pregnant patients are concerning [88,97,114–129]. Indeed, modified penicillin binding proteins have been observed in β-lactam-resistant *Streptococcus pneumoniae*. This increasing potential of penicillin resistance in GBS is a serious public health concern, particularly since resistance to penicillin-alternatives including the cephalosporins, has been reported [121,130,131], and resistance rates for β-lactam alternatives are high [96]. Only two cases of vancomycin-resistant GBS have been reported, both of which caused invasive GBS disease in non-pregnant adults. In GBS, *vanG* confers resistance to vancomycin by altering its target site in the peptidoglycan [87,132]. Observed resistance has been reported for all effective antibiotic treatment options for GBS, including the antibiotic of last resort, vancomycin. Although the incidence of isolates with reduced susceptibility to penicillins is low, it is likely that these levels will increase over time, based on trends in other similar pathogens.

LIMITATIONS OF IAP: A CALL FOR IMPROVED GBS TREATMENT AND PREVENTION

In addition to inducing the spread of antibiotic resistance, antibiotics can also negatively impact the body's microbiota, or the resident microorganisms living within a given niche. In the process of clearing out the pathogenic bacteria, antibiotics can also eliminate commensal bacterial populations that are beneficial for many physiological and biological processes. Broad-spectrum antibiotics are especially detrimental, as they are meant to eliminate a wide range of bacterial species. Because the human microbiome, or the collection of microbes and their genes and gene products in a niche, begins to develop before birth, alterations during development can result in long-term consequences such as autoimmunity, chronic diseases [133,134], obesity, and diabetes mellitus [135,136]. IAP for GBS has been shown to cause dysbiosis in the microbiomes of both the pregnant person and their neonate by targeting Gram-positive bacterial populations. These include not only GBS but commensals such as *Lactobacillus* and

Bacteroides species, which are key components of a healthy vaginal and gastrointestinal microbiome, respectively [137–140].

Apart from consequences of general antibiotic use, there are other limitations related to the IAP protocol for GBS prevention. The recommended duration of IAP to achieve maximum benefit is a minimum of 4 hours before childbirth, but patients often deliver in less than 4 hours [5,21]. This, along with the nature of IAP being administered during labor, renders this strategy ineffective for the prevention of GBS-associated preterm or stillbirths. Despite being effective at reducing rates of EOD, IAP has not contributed to a reduction in LOD [141]. Moreover, 60% of EOD cases were born to pregnant people who were negative for GBS colonization at the time of antenatal screening [142]. Additionally, screening-based methods and administration of IAP for all pregnant people is more difficult to implement in areas where resources and provision of care is limited [143]. Even though screening-based methods have been proven to be more effective in preventing GBS disease, some countries have chosen to use risk-based approaches as they are more cost-effective and may reduce the potential for antibiotic overuse [144]. Nonetheless, the implementation of IAP varies across geographic locations, as a meta-analysis discovered that only 60 of 95 countries (63%) polled had an IAP policy in place and further, 76% of the countries without a policy were classified as low- to lower-middle-income countries [143]. Together, these limitations highlight a clear need for alternative prevention strategies for GBS infection.

GBS VACCINES ON THE HORIZON

The basis for a GBS vaccine was brought about by the identification of circulating serotype-specific GBS antibodies in pregnant people along with historical evidence demonstrating the role of the capsular polysaccharide in protective immunity against GBS in mice [50,145–147]. Thus, the polysaccharide capsule, which dictates the serotype, became a promising GBS vaccine target. Because the capsule is not immunogenic on its own, protection was only achieved after conjugating capsular carbohydrates to an immunogenic “carrier” protein

[148]. The first GBS conjugate vaccines were specific to serotype III, the most prevalent among cases of invasive GBS disease, and later monovalent conjugate vaccines against serotypes Ia-VIII were created [148–151]. These new conjugated multivalent vaccines aim to elicit protection against multiple capsule types.

Regulatory and legal concerns are important barriers to GBS vaccine development as safety is a main concern regarding vaccination during pregnancy. In 2006, Patten *et al.* surveyed focus groups of pregnant people and healthcare professionals regarding their attitudes towards a GBS vaccine. While most pregnant patients and all healthcare professionals were supportive of vaccinations, in general, many of the patients were hesitant about vaccination during pregnancy based on safety concerns and the potential for adverse side effects [152]. Many vaccines that protect pregnant people and their infants from diseases including tetanus, pertussis, and influenza, have been proven safe and effective, highlighting the potential for GBS vaccination during pregnancy [153]. The implementation of a safe and effective vaccine against GBS was deemed the most cost-effective prevention strategy, as a vaccine of moderate efficacy was estimated to reduce GBS infections and deaths by more than 33% [154–156]. However, phase III safety and efficacy trials are challenging for GBS vaccines as they require large sample sizes and are costly because of the relatively low incidence of invasive neonatal GBS disease. Additionally, such trials will need to be performed in the absence of IAP strategies to accurately examine vaccine effectiveness [157]. The timing of vaccine administration is crucial, and studies have suggested that the most ideal time to vaccinate is early in the third trimester [158–160]. This timing would promote the generation of protective levels of antibody early enough to prevent GBS-associated preterm and stillbirth outcomes, without interfering with early fetal development. Alternatively, vaccination during adolescence has also been proposed, which could eliminate the need for antibodies that can cross the placenta and prevent GBS colonization [160].

Currently, the GBS vaccine of most promise and closest to licensure is the hexavalent cps conjugate vaccine, GBS6, developed by Pfizer. GBS6 elicits protection against serotypes Ia, Ib, and II-V, which provides coverage against roughly 97% of strains associated with neonatal disease worldwide [161]. After demonstrating effective antibody-mediated protection against GBS in preclinical trials with mice, GBS6 advanced to clinical development in 2017. Clinical studies are promising and have demonstrated tolerance in healthy nonpregnant adults and strong immune responses that persist for at least 6 months with antibody concentrations peaking within 2 weeks after vaccination [162]. Phase 2 placebo-controlled clinical trials for GBS6 in healthy pregnant adults are underway. Thus far, they have not resulted in adverse side-effects and effectively induce antibody responses to all six serotypes that could be transferred to infants at levels high enough to reduce the risk of invasive GBS disease [163].

A GBS vaccine will certainly enhance protection from and prevent invasive GBS disease and other severe outcomes that is not possible with current prevention strategies. Some argue, however, that a vaccine will not replace the need for antenatal-screening and IAP since the latter is effective across all GBS capsule types. Indeed, the vaccines currently under development will only provide protection against, at most, a subset of six capsule types [2]. Regardless, a GBS vaccine could provide additive protection in regions where IAP protocols are not well established along with reducing GBS-associated outcomes that are not impacted by IAP such as LOD, preterm births, and stillbirths. Because the implementation of IAP is often not feasible for regions with limited resources, great benefit could come from a GBS vaccine, a more cost-effective prevention strategy, in these regions. Moreover, the increasing prevalence of antibiotic-resistant isolates is a major public health concern worldwide and an effective GBS vaccine could eliminate the need for antibiotic prophylaxis entirely, as both susceptible and resistant GBS would be targeted. Although the global distribution of serotypes has remained stable over time [15,47], implementation of a multi-valent conjugate vaccine targeting only the most prevalent capsule types may introduce a selective pressure and result in the emergence of

more rare cps types. Such instances of emergence and vaccine escape have been observed in *Streptococcus pneumoniae* since the introduction of the heptavalent pneumococcal polysaccharide vaccine [164] as well as *Neisseria meningitidis* and the monovalent meningococcal serogroup C conjugate vaccine [165].

GBS CHARACTERIZATION & STRAIN DIVERSITY

Characterizing the polysaccharide capsule

GBS is surrounded by a polysaccharide capsule, which contributes to its virulence but also serves as a useful feature to phenotypically characterize GBS isolates. Indeed, the capsule dictates the serotype of which ten (Ia, Ib, II-IX) have been described. Generally, capsular serotyping involves the use of antibodies that target type-specific capsular polysaccharides. Several methods have been used for capsular serotyping including the Lancefield capillary precipitin method [166], latex agglutination [167,168], co-agglutination [169], double immunodiffusion [170], and enzyme immunoassays [171]. The Lancefield method, which involves capillary precipitation of polysaccharide and teichoic acid antigens expressed on the bacterial cell wall [166,172], is the most widely accepted serotyping technique for GBS. Additional studies, however, have demonstrated that latex agglutination is more sensitive (i.e., yields fewer non-typeable (NT) capsules) and less labor-intensive than other methods [167,168,173]. Still, cross-reactivity has been observed using the latex agglutination method and such cases require confirmation with the Lancefield method [168]. Overall, serotyping methods are limited in their ability to distinguish isolates that share a serotype but are genetically different, resulting in up to 18% of strains with NT capsules [173–176].

Improved molecular methods for capsular genotyping have been developed to more accurately differentiate GBS capsule types. These include DNA dot blot hybridization [172], amplicon sequencing of serotype-specific gene fragments [177,178], and PCR-based restriction fragment length polymorphism (RFLP) to detect polymorphisms within *cps* genes [173,179]. More recently, capsular genotyping using whole-genome sequence data has been developed

[180]. Like capsular serotyping, these genotyping methods can classify the GBS polysaccharide capsule into one of the ten known capsule types based on the genetic variation within the *cps* gene cluster instead of capsule expression. The GBS capsular polysaccharide (cps) is encoded by 16-18 genes, some of which are highly variable across capsule types: *cpsG-K* for types Ia-VII and IX, and *cpsR-K* for type VIII [177,181,182]. These highly variable genes encode glycosyltransferases and polymerases that are specific to a given capsule type. This variable region is flanked by conserved genes involved in the export of capsule proteins encoded by *cpsA-F* as well as genes (*cpsL, neuB, neuD, neuA, and neuC)* required for sialic acid synthesis and activation. In general, capsular genotyping methods correlate well with capsular serotyping, but have a higher sensitivity as they can detect new antigenic variants and effectively characterize capsules that were NT by serotyping [173].

Global distribution of capsule (cps) types reveal cps-Ia, -Ib, -II, -III, and -V to be the most common among strains associated with neonatal GBS disease, with 97% of cases having infections caused by these capsule types [15]. The distribution of these cps types varies by geographic region, as cps III is most prevalent across all regions except South America where cps-Ia predominates, for example. Additionally, cps types Ib and V are more prevalent in Eastern Asia and South America compared to other regions examined, respectively. Although, a recent rise in the frequency of historically rare cps-VI isolated from cases of invasive GBS disease has been observed in Canada [183]. Capsule type III predominates globally, accounting for 61.5% of invasive GBS disease cases overall and 47% and 73% of neonatal EOD and LOD cases, respectively. The next most prevalent capsule type linked to invasive disease is cps-Ia, which accounts for 19.1% of all invasive cases (22.8% EOD and 14.2% LOD) [15]. Similar distributions have been observed for pregnant patients with recto-vaginal colonization, as cps types I-V account for 98% of the colonizing GBS isolates worldwide. Capsule type III was also highly prevalent (25%) during pregnancy, but was less common in some South American and Asian countries [19].

Capsular switching, which occurs following the exchange of cps genes and results in the expression of a distinct capsule type, has been observed among GBS isolates *in vivo* [184–187]. The frequency of capsule switching in GBS is debated in the literature as GBS is not naturally competent [184,186]. Both horizontal gene transfer and homologous recombination across conserved and variable regions of the *cps* locus have been documented as methods for capsular switching, and have resulted in variation that extends beyond the *cps* loci [185,186].

While capsule switching frequently occurs via single or multiple recombination events within the *cps* loci [187–189], evidence of extensive recombination events has been shown to contribute to variation throughout the entire GBS genome in addition to the *cps* loci [94,188]. For example, a prior study uncovered thousands of SNPs in a sequence type (ST)1 serotype VI genome when it was mapped to an ST1 serotype V genome, thereby providing evidence of recombination across >50% of the genomes, despite belonging to the same ST [185]. Such events may explain the emergence of more rare *cps* types, like cps VI, and present important clinical implications as the widespread variation introduced throughout the genome may confer more virulent and resilient phenotypes. Given that cps III and VI are structurally very similar [190], cps VI likely has the same level of pathogenicity as cps III isolates, which are frequently associated with invasive GBS disease.

Multilocus sequence typing (MLST)

MLST is another molecular genotyping technique used to characterize GBS isolates that can better reflect evolutionary relationships compared to capsule typing. First described in 1998, MLST assesses the allelic variation within housekeeping genes and has been used to classify many pathogens including *Neisseria meningitidis* and *Streptococcus pneumoniae* [191,192]. In GBS, seven housekeeping gene sequences (*adhP, atr, glcK, glnA, pheS, sdhA, tkt*) are assessed to reveal an isolate's allelic profile or sequence type (ST) [193]. This classification tool has significantly expanded epidemiological and public health research as typing data can be easily uploaded and disseminated via PubMLST, an online, open-access database [194].

Phylogenetic analyses of MLST data have helped identify which GBS STs are most closely related (i.e., sharing 6-7 identical alleles) and cluster together within clonal complexes (CCs) [184,195]. MLST of GBS has revealed that isolates with the same ST can have different capsule types, while other studies have demonstrated that genotypically diverse isolates can share the same capsule type. Together, these findings provide further evidence that the capsule genes are vulnerable to horizontal gene transfer [196,197]. Similar to the capsule types, some STs are associated with specific sources regardless of the geographic origin. The majority of GBS isolates from colonized pregnant women and invasive neonates, for instance, are represented by five STs: 1, 12, 17, 19, 23 [198,199]. These STs represent the predominant genotypes in distinct clusters within MLST-based phylogenies along with other phylogenetically related STs representing the five most common CCs (1, 12, 17, 19, 23). Several epidemiological studies in different patient populations have found ST-17 and ST-19 isolates to predominate among cases of invasive neonatal disease, while STs 1, 12, and 23 were more commonly linked to colonization during pregnancy [184,198,200]. These are general trends, however, as some studies in distinct geographic locations have reported varying distributions. For example, a study of colonized pregnant patients in Nigeria detected unique STs that were not common in other locations [16]. It is likely that geographic-specific factors and exposures impact strain distributions and consequently, the diversification of existing lineages and emergence of new lineages can be observed in each location [198].

Prior studies have also demonstrated that strains representing specific CCs are often associated with specific capsule types as is the case for CC-1 and cps-V, CC-23 and cps-Ia, CC-19 and cps-III, and CC-17 and cps-III. Moreover, neonatal invasive isolates belonging to the CC-17 and CC-19 lineages more frequently have cps-III capsules, while CC-1 strains associated with vaginal colonization more commonly possess cps-V [198]. ST-17 cps III strains, in particular, have caused invasive neonatal EOD more frequently than other STs [198,200] and were more common among infants with LOD and meningitis [198]. Significant evidence of

recombination has been demonstrated in GBS and among CCs, which contributes to the genetic diversity observed across genotypes [201]. Unlike other CCs, however, the CC-17 lineage lacks evidence of recombination [198] and was found to be more closely related to GBS strains and lineages linked to bovine mastitis [199,200]. Thus, the CC-17 lineage was suggested to have independently diverged from other lineages harboring unique virulence characteristics [198,201].

Associations between genotypic lineages and clinical phenotypes

Although vaginal colonization is the main risk factor for invasive GBS disease and other adverse pregnancy outcomes, it does not always result in an infection, or severe clinical phenotype. As a multitude of outcomes can arise from GBS colonization, genotypic characterization of clinical GBS has revealed that STs and cps types vary in their virulence potential and differentially impact clinical presentation. Importantly, variation in IAP effectiveness was also demonstrated across GBS genotypes as some STs are more commonly lost following antibiotic treatment while others can persist for up to 6 weeks postpartum [202,203].

Isolates within the CC-17 lineage, which is significantly associated with severe invasive disease phenotypes, were found to possess unique virulence gene alleles and pilus-island profiles, for example [201,204,205]. Studies have demonstrated the enhanced virulence capabilities of ST-17 isolates, deeming CC-17 the hypervirulent lineage, when compared to the lineages linked to asymptomatic colonization [173,193,198,200,201,206,207]. These include an enhanced ability to colonize and invade host cells, escape antibiotic-mediated killing, and withstand the harsh environment of the phagolysosome. ST-17 isolates were also found to be more commonly associated with cases of persistent vaginal colonization even after IAP treatment, compared to other STs, demonstrating resiliency in the presence of antibiotics [202,203]. Given that GBS strains vary in their ability to cause disease, multiple studies have been conducted to understand the mechanisms of pathogenesis.

GBS PATHOGENESIS

In the context of human pregnancy, invasive GBS infection (pneumonia, sepsis, meningitis) begins with asymptomatic colonization of the vaginal tract. From the vaginal tract, GBS can ascend through the cervix into the uterine cavity where it can invade the extraplacental membranes and trigger preterm labor and/or infect the fetus *in utero*. Vaginal colonization can also result in neonatal disease in the absence of ascending infection via aspiration of infected vaginal fluid during the birthing process. In order to invade and survive in such a diverse range of host tissue and environments, GBS contains a multitude of factors that promote survival and facilitate the disease process within the human host [208,209].

As an opportunistic pathogen, those factors that enhance colonization are also important for invasive infections. GBS encodes many adhesins, which facilitate binding interactions with host cells, including cervical and/or vaginal epithelial cells and associated extracellular matrix (ECM) components. Together, these adhesins also contribute to GBS dissemination and tissue damage via crossing the blood-brain-barrier, binding brain endothelial, gastrointestinal epithelial, and lung epithelial cells, for example [208,210–213].

The possession of multiple features that facilitate adherence to host cells and ECM proteins makes GBS well adapted to colonize the human vaginal tract, the precursor to invasive infection. Surface proteins that mediate interactions with the ECM include fibrinogen-binding proteins (FbsA-C), serine-rich repeat proteins (Srr1-2), laminin-binding protein (Lmb), C5a peptidase (ScpB), fibronectin-binding protein (SfbA), and adhesin protein (BibA) [214–221]. FbsA and FbsC have been shown to specifically promote adherence and biofilm formation, respectively [216,222]. Likewise, the Srr proteins were found to be important for host cell adherence, of which Srr1 was critical for vaginal colonization and persistence [215]. GBS also expresses proteins that facilitate adhesion to vaginal epithelial cells including an immunogenic bacterial adhesin (BibA), and hypervirulent GBS adhesin (HvgA). BibA expression is well conserved across GBS lineages, although four *bibA* (formerly gbs2018A) variants have been

identified with one being exclusive to ST-17 strains [201,205,223–225]. Similarly, a specific *hvgA* (formerly gbs2018C) allele is also unique to the ST-17 lineage [210]. HvgA was suggested to be important for meningitis by facilitating adherence to intestinal epithelial cells and microvascular endothelial cells of the blood-brain barrier. Other factors that contribute to invasion of host cells include the β-hemolysin/cytolysin and the carotenoid pigment, which are both encoded by the *cyl* operon, work together to disrupt the placental membranes, lungs, and blood-brain-barrier [226,227]. Similarly, a serine protease (CpsA), hyaluronate lyase (HylB), and CAMP factor are key facilitators of GBS dissemination through host tissues, which is an important prerequisite for invasive and ascending infection [228–230].

In addition to facilitating colonization and invading host cells, some factors work to promote GBS survival in the presence of host immune responses and other stressors. For example, the expression of pili, structures extending from the surface of the bacterial cell, promote adherence to host cells and persistence through biofilm formation [231,232]. In GBS, pili are comprised of three structural proteins, a backbone protein, two ancillary proteins, and two pilus-specific class C sortase enzymes that coordinate the attachment of the subunits to each other as well as the peptidoglycan of the cell wall [204,233–235]. The backbone protein is made up of three main subunits (PilA-C). The PilA subunit, in particular, has been shown to contribute to cell adherence, while PilB is more important for tissue invasion [215,234,236–239]. Together, the pilus proteins are encoded by genes located within a pilus island (PI). Three pilus islands (PI-1, PI-2a, and PI-2b) that each encode structurally diverse pili have been identified among GBS isolates [204,233,240,241]. The PI-2a pili are important for adherence to vaginal epithelial cells and biofilm formation [242,243], whereas PI-2b plays a role in immune evasion and host cell invasion along with PI-1 [244,245]. Epidemiological studies have shown that most human GBS isolates harbor a combination of PI-1 and one of the PI-2 variants. ST-17 isolates, for example, have PI-1 and PI-2b types [204]. Similarly, lipoteichoic acid (LTA), which contributes to epithelial cell attachment, helps GBS resist antimicrobial host factors via

alanylation [246]. Other factors aid in GBS survival of reactive oxygen species including the hemolytic pigment and superoxide dismutase (SodA) [247,248]. The polysaccharide capsule is also important for immune evasion as the sialic acid residues mimic host sialic acid epitopes [249,250]. This mimicry effectively decreases immune recognition, delays neutrophil recruitment, and blocks opsonophagocytic clearance by immune cells.

GENOMIC EVOLUTION OF GBS

The GBS pangenome

Whole-genome sequencing (WGS) has allowed for a more detailed understanding of GBS evolution, leading to a more definitive picture of its molecular epidemiology. Full genome sequences of two GBS clinical isolates were first published in 2002 [251,252]. The genomic analyses identified underlying conserved regions with the presence of variable genomic islands and a significant number of tRNAs, ABC transporters, two-component regulatory systems, and mobile DNA elements throughout the GBS genome. WGS has not only allowed for better characterization of GBS isolates but has also enhanced understanding of how distinct GBS lineages have evolved over time.

In 2005, the first pangenome analysis was conducted on eight diverse GBS strains with distinct capsule types and STs, providing further insight on GBS evolution and evidence of large recombination events [253]. The “pangenome” describes the complete set of genes in all isolates of a given bacterial species and is broken down into the “core genome” and the “accessory genome”. The core genome represents the set of genes that are conserved across all strains within the species and generally include housekeeping and other essential genes, whereas the accessory genome comprises the genes that are variable across isolates like virulence genes. Thus, the core genome represents the backbone of each bacterial species while the diverse pathogenic potential and host range observed across strains are attributable to the accessory genome [253–256]. Studies have further demonstrated that GBS has an open

pangenome, a concept that was first described in GBS, meaning the size of the pangenome increases as the number of independent strains included in the analysis increases [257–263].

GBS has high genome plasticity because of its open pangenome, which allows for rapid adaptation to a range of environmental conditions and host niches. Several studies have demonstrated such genome plasticity and evolution in GBS is largely driven by large-scale recombination events mediated by mobile genetic elements (MGEs) [94,188,264,265]. Significant recombination has contributed to the extensive genomic diversity observed throughout the species, resulting in the emergence of distinct genotypic lineages and serotype profiles [94,185,201]. Whereas evidence of recombination is limited within clonal populations, the expansion of clonal populations was demonstrated to evolve via the accumulation of point mutations [205,266–268]. The extensive diversity observed within subsets of GBS isolates makes it challenging to capture the species dynamics and understand the full extent of its diversity, as hundreds of sequences are needed for a comprehensive analysis. Nonetheless, such species-wide diversity is unsurprising due to the broad host and geographical range of GBS and its ability to exist in multiple host niches as an opportunistic pathogen. In a study of over 200 GBS genomes, it was demonstrated that those isolates associated with human disease had evolved from a limited number of clones, thereby aligning with the CCs identified via MLST [94].

Multiple molecular mechanisms contribute to genome plasticity, which ultimately drives evolution, including genetic recombination, MGEs, and point mutations [269]. As a species, GBS has a broad host and geographical range, but also demonstrates strong specificity to host niches. Thus, GBS evolution has been described as a continuous generation of new lineages via recombination with simultaneous evolution of successful lineages by the accumulation of point mutations [188,205,259,265,270,271]. Indeed, evolution within specialist clonal lineages, such as those that are more successful in causing invasive GBS disease in humans, occurs

through the accumulation of point mutations while the emergence of new, divergent GBS lineages, is a result of extensive recombination and DNA transfer through MGEs.

Antibiotics as a driver for clonal expansion

Resistance to tetracycline antibiotics, mediated by *tetM*, is prominent among human GBS isolates. Tetracyclines are no longer used to treat GBS disease in humans, but the widespread distribution of tetracycline resistance observed across GBS isolates is evolutionarily relevant. Genomic analyses have revealed the presence of MGEs carrying *tetM*, suggesting that GBS isolates that cause disease in humans evolved via clonal expansion from the lineage that initially acquired the tetracycline resistance gene [94]. In addition, one of the MGEs that carries *tetM*, for example, also encodes a surface adhesin protein, which is important for colonization [266]. As MGEs are known to carry multiple antibiotic resistance genes as well as genes important for virulence, it is likely that the selective pressure of antibiotics has contributed to the evolution and persistence of more virulent lineages. This has important clinical implications as antibiotic selection of such elements has not only contributed to the rise in antibiotic resistance in GBS, but also hypervirulence. Overall, genomics has provided further clarity on how GBS has evolved to cause invasive infections and continues to evolve through clonal expansion, which can result in the emergence of more virulent and resistant isolates.

Together, larger scale pangenome analyses of diverse lineages have shed light on the genomic evolution and diversity of GBS as a species. Such analyses provide a more comprehensive understanding of the complex virulence and survival mechanisms that result in severe disease and has enhanced our predictive power of clinical outcomes based on genomic signatures.

THE IMPORTANCE OF PERSISTENT COLONIZATION

Evidence of persistent vaginal colonization has been observed following IAP in several cohort studies [203,272,273]. Persistent colonization not only threatens the effectiveness of IAP as a GBS prevention strategy, but poses a greater risk for invasive disease in infants and

subsequent pregnancies [272–274]. In fact, pregnant people testing positive for GBS colonization during pregnancy have a 50% risk of colonization in subsequent pregnancies [275]. Persistent colonization and tolerance to antibiotic stress may partly explain why a reduction in the number of LOD cases has not been observed as well [141,273]. Because GBS is not always cleared from the vaginal tract following IAP, additional studies are required to determine the mechanism(s) by which GBS escapes antibiotic-mediated killing and persistently colonizes the vaginal tract.

Prior studies have demonstrated that certain genotypic lineages are more likely to persist. Manning *et al.*, for instance, identified ST-17 and ST-19 isolates to be significantly associated with persistent colonization compared to all other STs. Comparatively, isolates belonging to ST-12 were more readily cleared by IAP. One possible explanation is the differential expression of virulence factors important for adherence to vaginal cells and colonization. The CovRS two-component system is a master regulator of virulence in GBS and has been shown to contribute to persistent colonization of the vaginal tract by regulating expression of several adherence factors. These include the C5a peptidase, BibA, HvgA, pili, Fbs proteins, and Lmb [210,244,276–280]. In ST-17s, fibrinogen binding is mediated mainly by FbsB rather than FbsA, and strains within this lineage do not express *fbsC* because of a frameshift mutation that is unique to this lineage [222,281]. Additionally, ST-17 isolates have increased expression of Srr2 than Srr1 [282,283]. These observations are in line with the described association between ST-17 and invasive GBS disease, indicating that the mechanism of persistence may be different than in other lineages.

As was demonstrated for other pathogens [284], invasion of host cells may promote long-term colonization and a way to escape antibiotic-mediated killing. In GBS, the fibronectin-binding protein SfbA, for example, facilitates invasion of the vaginal and cervical epithelium, which is a proposed niche for establishing persistent colonization [285,286]. Many of the aforementioned adhesin proteins and structures facilitate invasion of host tissues as well. In

addition, Korir *et al.* demonstrated that ST-17s induce uptake into macrophages in the presence of antibiotics and are capable of withstanding the phagosome environment longer than the less virulent ST-12 strains [207]. Similarly, the ST-17 strain showed enhanced invasion of decidualized stromal cells when compared to the other STs along with increased expression of several genes (e.g., *fbsB*) important for invasion [287].

While some pathogens may employ escape strategies to avoid antibiotic killing and persist in the vaginal tract, others may engage mechanisms to survive in the presence of the antibiotic. A key example is the formation of biofilms, which is an effective strategy used by multiple pathogens to persist and withstand environmental and immune stress. In GBS, the polysaccharide capsule and pili largely contribute to biofilm formation [242,288]. Studies have shown that GBS biofilm formation and adherence to vaginal epithelial cells are both enhanced under low pH conditions, which demonstrates that GBS is well equipped to persist within the acidity of the vaginal tract (pH 4.5) [289–291]. Studies in *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* have also demonstrated that biofilm-associated cells were more resistant to antibiotics compared to planktonic cells [292–295]. This difference is likely due to the slowed growth rate needed to achieve the survival state associated with biofilms. Indeed, slowed growth is a common strategy used by bacterial species to survive starvation conditions, which results in physiological and phenotypic changes in order to enter a state of dormancy [296,297]. This phenotypic adaptation strategy is referred to as the stringent response, which is a universal bacterial stress response to overcome amino acid limitation. The stringent response is facilitated by *relA*, which encodes an ATP:GTP 3'-pyrophophotransferase that phosphorylates GDP and GTP to (p)ppGpp [298,299]. The stringent response has been associated with reduced antibiotic susceptibility to cell wall synthesis inhibitors like β-lactams, due to the slowed growth rate. It was also demonstrated that (p)ppGpp, a product of the stringent response, can bind ribosomes to slow protein translation, which simultaneously blocks the target of protein synthesis inhibitors as well [300–303].

The stringent response has been shown to contribute to bacterial survival of antibiotics via antibiotic tolerance, resistance, and persistence, and is marked by elevated (p)ppGpp [304–313]. Brauner *et al.* has defined each of these three antibiotic survival strategies as distinct phenomena [90]. Antibiotic resistance describes the ability of bacteria to grow in the presence of high antibiotic concentrations, as determined by MIC thresholds, for any length of time. Antibiotic tolerance, however, is different in that bacteria can survive temporary exposures to antibiotics, which exhibit a slower rate of killing. Lastly, antibiotic persistence occurs when a subpopulation of bacteria can survive antibiotic exposure for a longer period of time [90] and is due to either phenotypic or genotypic tolerance to antibiotics as a result of the stringent response or genetic mutations, respectively [314]. There has been only one report of the stringent response in GBS, which demonstrated its role in enhancing resistance to killing by the host immune system and increasing expression of cytotoxin [315]. It is possible that the stringent response is utilized by some lineages of GBS to promote persistence in the vaginal tract and resistance to antibiotic killing as demonstrated in other species. Indeed, Korir *et al.* demonstrated that ST-12 isolates were able to tolerate exposure to ampicillin *in vitro* for 36 hours, compared to ST-17 isolates [207]. Furthermore, other studies have identified penicillin tolerance among clinical GBS isolates, which is the most effective antibiotic for prevention and treatment of GBS disease [316–318]. Although it is unclear as to whether such evidence of GBS isolates that survive antibiotic exposure display antibiotic tolerance or persistence strategies, these observations have important clinical implications for the effectiveness of IAP.

BACTERIAL MEMBRANE VESICLES

Membrane vesicles (MVs) are spherical particles ranging from 20-500nm in size that are surrounded by a lipid bilayer and produced by a wide range of bacterial species [319]. MVs have been identified in all Gram-negative species and are characterized as either outer membrane vesicles (OMVs), which package material from the periplasmic space, or less commonly, inner membrane vesicles (IMVs) which contain components from the cytoplasm

[320]. More recently, MV production has been observed in Gram-positive species as well. A wide array of material is packaged within MVs including lipids; glycolipids and phospholipids, nucleic acids; genomic DNA, plasmids, small RNAs, and proteins; membrane proteins, enzymes, and toxins [321–325]. The composition of MVs varies across species and is influenced by environmental factors including temperature, nutrient, antibiotic, and oxidative stress, toxins, and quorum sensing molecules [326–329].

In pathogenic species, MVs have been shown to contain toxins and virulence factors important for bacterial pathogenesis and survival [321,330]. Additionally, MVs can act as bacterial delivery systems as they have been shown to disseminate quorum signals, genetic information, and other small molecules to neighboring bacterial cells. Together, MVs serve as "bacterial soldiers" as they not only communicate and share genetic material to nearby cells, but also induce host cell damage away from the bacterial cell.

Mechanisms of MV formation

The formation of MVs is well described in Gram-negative species as either a result of membrane blebbing or explosive cell lysis [330,331]. MVs formed from blebbing are more likely to selectively package material rather than explosive cell lysis, although MVs formed through both mechanisms are physiologically relevant [332,333]. Although MVs from Gram-positive species were identified in 1973, their mechanism of formation was unclear until recently as the route of MV release through the thick layer of peptidoglycan was puzzling [334]. Studies suggest that MV formation in Gram-positive bacteria is possible due to the fluidity of cell wall synthesis, rearrangement, and degradation [335,336]. Gram-positive MVs are suggested to form at the cytoplasmic membrane via phenol-soluble modulins that lead to membrane budding through building osmotic pressure and are released through the thick cell wall via enzymes that degrade and alter peptidoglycan synthesis [330,331,337–341].

Certain proteins and pathways involved in regulating MV production have been described in Gram-negative species, but such modulation in Gram-positives remains unclear

[332,342]. Some studies hypothesize that MV biogenesis is modulated by global regulators like the two-component system, CovRS, in *Streptococcus pyogenes*, [343,344] while others suggest single genes such as *sigB* in *Listeria monocytogenes* and *virR* in *Mycobacterium tuberculosis* may be responsible [345,346]. Further investigations are warranted to understand the underlying mechanisms of MV regulation in Gram-positive species.

MV production in GBS

Currently, only three independent studies have demonstrated evidence of MV production in GBS [347–349]. Surve *et al.* was the first study to describe MVs in an ST-7 cps Ia isolate [253] and demonstrates their ability to induce inflammation and damage to the extraplacental membranes in a mouse model [347]. Of note, this chorioamnionitis-like presentation was induced in the absence of bacterial cells, demonstrating that GBS MVs can contribute to extraplacental membrane damage on their own. This damage led to either preterm birth or invasive infection of the neonate *in utero*. Armistead *et al.* investigated the role of β-hemolysin and pigment, which are cytotoxic factors that promote dissemination of GBS in the host. They demonstrated that these factors are not only found within the GBS MVs but they are also functionally active [348]. Moreover, they showed that MVs produced by hyper hemolytic GBS strains are more cytotoxic to host cells than those produced by non-hemolytic strains, further demonstrating the role of MVs in GBS pathogenicity.

Our group has previously examined the formation and composition of MVs among isolates representing three distinct GBS lineages (STs 1, 12, and 17) recovered from cases of invasive disease and vaginal colonization [349]. MV production quantity was significantly higher in ST-17 isolates compared to ST-1 isolates, and significantly different between the ST-12 invasive and colonizing isolates, demonstrating differential production across and within STs, respectively. Furthermore, the protein composition of MVs was distinct for each ST examined, with a high abundance of virulence proteins, including hyaluronidase and C5a peptidase, in the ST-17 MVs. From this work, we demonstrated that both production volume and composition

vary across and within STs, highlighting strain diversity and consequent differences in pathogenicity. Together, these findings demonstrate that diverse GBS strains are capable of producing MVs that contribute to GBS pathogenesis in an ST-dependent manner by facilitating attachment and invasion of host cells, reduction of oxidative killing, as well as eliciting pro-inflammatory responses in the host.

MV production and antibiotic stress

While many of the early investigations have examined MV biogenesis following growth in standard broth conditions, several studies have demonstrated the impact of stress on MV production. Indeed, MV production has been shown to be produced following exposure to stressors in the environment and provide a protective role for the bacterial cell [326,350–352]. Antibiotic stress, in particular, upregulated MV biogenesis in several species of Gram-positive and Gram-negative pathogens including *Acinetobacter baumannii*, *Bacillus subtilis, Enterococcus faecium, Listeria monocytogenes, Pseudomonas aeruginosa,* and *Staphylococcus aureus* [319,326,351,353–360]. Increased MV release has been observed mainly in response to cell-wall-targeting antibiotics such as β-lactams and glycopeptides [354–356,361].

In addition to the increased volume of MVs, antibiotic exposure also alters the contents packaged inside MVs. For example, studies have shown that antibiotic-induced MVs contain factors that are more cytotoxic to host cells compared to MVs produced in the absence of antibiotic stress, suggesting that antibiotic treatment induces more host damage [347,351]. In addition to increased host cytotoxicity, MVs produced in the presence of antibiotics have contributed to enhanced biofilm formation, thereby promoting survival during antibiotic stress [356,362–365]. These protective mechanisms elicited by MVs in response to antibiotic treatment highlight their potential role in promoting persistent and chronic infections by evading antibiotic-mediated killing.

MVs have been shown to directly contribute to resistance and tolerance of antibiotic stress through four main mechanisms. Antibiotic target molecules have been identified within MVs, demonstrating that the MVs can serve as decoy targets and effectively interact with antibiotics away from the bacterial cell [356,365–367]. Other studies have demonstrated that MVs can aid in the removal of unwanted material such as misfolded proteins, damaged material, and even antibiotics. Thus, MVs can facilitate bacterial survival of antibiotic killing by effectively transporting antibiotics outside of the cell [368,369]. Another mechanism by which MVs mediate antibiotic resistance is through carrying and disseminating antibiotic-degrading enzymes, of which β-lactamases have been most commonly observed [370–373]. Lastly, as genetic material, including plasmids, has been commonly found within MVs, studies have explored the possibility of MVs carrying antibiotic resistance genes. Indeed, these genes have been found within MVs, which could be transferred to neighboring bacteria and exhibit resistance to a given antibiotic when challenged [332,339,363,364,374]. This finding has important clinical implications as these observations indicate that MVs threaten the effectiveness of antibiotics to treat some bacterial infections. Because little is known about GBS MVs, it is possible that their production may also serve as a mechanism for persistent colonization after IAP, by promoting survival while evading antibiotic killing and enhancing colonization potential through biofilms, for example. More studies, however, are needed to define their role in GBS pathogenesis.

CURRENT KNOWLEDGE GAPS & DISSERTATION OVERVIEW

Since the implementation of IAP, the burden of GBS EOD in neonates has effectively decreased, but this prevention strategy has several limitations including its inability to reduce the incidence of LOD or other GBS-associated adverse pregnancy outcomes [141]. Increasing evidence of persistent GBS colonization in pregnant people, especially following IAP intervention, is a grave concern as it increases the risk of severe invasive disease and adverse outcomes, especially in cases of subsequent pregnancies. While GBS is largely susceptible to

β-lactam antibiotics, the ability of susceptible isolates to persist despite penicillin or ampicillin prophylaxis threatens the effectiveness of IAP as a prevention strategy. While antibiotic resistance is well characterized in GBS for the MLS antibiotics, the underlying mechanisms linked to antibiotic tolerance and persistent colonization are not well understood.

WGS analyses have revealed notable associations between genotypic lineages and clinical outcomes. For example, ST-17 and ST-19 isolates are significantly more common among pregnant patients with persistent colonization [202]; however, the reasoning behind this connection is unclear. Pangenome analyses of GBS have led to the discovery of how these genotypic lineages have emerged over time and have exposed the plasticity of the GBS genome, in that it can readily adapt to changing environments [253]. Nonetheless, an understanding of *in vivo* GBS evolution in the context of IAP is lacking and would provide insight for patients who remain colonized or transmit the pathogen to their susceptible babies despite IAP. Understanding how GBS persistently colonizes the vaginal tract in the presence of antibiotics is crucial for improving GBS treatment and prevention strategies.

Bacterial production of MVs have been shown to play important roles in virulence, cell communication, and response to environmental stressors, including antibiotics. MV production in GBS is understudied, as only three investigations have been published to date [347–349]. Moreover, GBS MVs have yet to be examined in the context of antibiotic stress. In other species, exposure to antibiotic stress induced excess production of MVs that contributed to bacterial survival via mediating resistance to antibiotic killing. It is likely that GBS MVs may also facilitate bacterial survival in the presence of antibiotics and could consequently contribute to persistent colonization of the vaginal tract over time.

This dissertation aims to address the following overarching question: How does GBS persistently colonize the vaginal tract and survive antibiotic exposure through IAP? I therefore sought to further understand persistent colonization after IAP by 1) examining the genomes of clinical GBS isolates before and after IAP treatment; and 2) elucidating the role of GBS MV

production during antibiotic stress. In chapter 2, we investigated the impact of IAP on genomic evolution *in vivo* by examining a subset of 97 clinical GBS isolates obtained from pregnant individuals sampled for vaginal colonization before and after IAP treatment. This objective is expanded into two sub-aims that consist of: 1) characterizing the isolates using sophisticated WGS methods coupled with assembly-based analyses; and 2) examining nucleotide-level variation with reads-based analyses to identify point mutations between paired isolates to understand the evolutionary impact of IAP on persistent colonization. We hypothesized that IAP imposes a selective pressure on GBS isolates and that persistent isolates contain genomic features that promoted persistence and survival in the vaginal tract following IAP. In chapter 3, we examined the production of GBS MVs by a persistent ST-17 isolate in the presence of β-lactam and macrolide antibiotics to elucidate their role in bacterial survival and persistence in the presence of antibiotics. This objective is divided into the following two sub-objectives: 1) examine the production of MVs from antibiotic-treated GBS; and 2) characterize the protein composition of MVs to determine how antibiotics impact their content and functionality. We hypothesized that GBS produces excess MVs in the presence of antibiotics that contain key proteins important for bacterial survival during IAP and long-term persistence in the host.

Findings from these studies will enhance understanding of the mechanisms used by GBS to survive antibiotic stress and shed light on how more resilient strains emerge *in vivo* under selective pressures like IAP. Defining the impact of IAP on GBS evolution *in vivo* is also valuable to understand how GBS evolves and adapts to other selective pressures, such as vaccines. Promising vaccine candidates for GBS disease are being developed, but evidence of adaptive evolution in the presence of IAP suggests that new, more resilient GBS strains may emerge and escape vaccine protection, as has been demonstrated in other species like *S. pneumoniae* [164].

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CHAPTER 2:

WHOLE GENOME ANALYSIS OF COLONIZING GROUP B *STREPTOCOCCUS* ISOLATES REVEALS PRESENCE OF MUTATORS THAT IMPACT VIRULENCE AND SURVIVAL FOLLOWING ANTIBIOTIC PROPHYLAXIS

ABSTRACT

Through vaginal colonization, Group B *Streptococcus* (GBS) causes severe pregnancy outcomes including neonatal disease. Although intrapartum antibiotic prophylaxis (IAP) has successfully reduced the incidence of early-onset neonatal disease, persistent GBS colonization has been observed despite IAP. We therefore sought to determine whether IAP selects for certain genomic signatures that enhance the ability of GBS to withstand antibiotics and persistently colonize the vaginal tract. Whole-genome sequencing was performed on 97 isolates recovered from the vaginal tracts of 58 individuals before (35-37 weeks prenatal) and after IAP/childbirth (6 weeks postpartum). The pangenome was evaluated by aligning the core genes and constructing a maximum likelihood phylogeny. Core-gene mutations were extracted from each pair of isolates from those colonized with GBS at both the prenatal and postpartum samplings. Biofilm assays were performed to determine the impact of specific mutations on phenotypes important for colonization. The pangenome analysis of 1,368 core genes showed that most (85%) paired isolates clustered together. Three pairs, however, were located on distinct branches despite having the same ST, while the remaining distant pairs had different STs. Such evidence of strain-level variation led us to identify 7,025 mutations across 792 distinct genes between paired isolates with the same ST. Three postpartum isolates accounted for 98% of these mutations and were classified as “mutators” due to the presence of point mutations in genes encoding DNA repair systems. Of the genes that acquired >4 total point mutations (n=366) across the mutators, 16.7% were under positive selection, including those encoding surface-associated, attachment, and regulatory proteins. Moreover, two of the mutators had a >2-fold increase in biofilm formation relative to their paired prenatal isolate. These findings provide evidence of microevolution in GBS recovered from pregnant patients after IAP and suggest that antibiotic treatment selects for mutations that promote survival *in vivo*. Furthermore, mutators may provide a reservoir of beneficial mutations that enhance fitness, which will simultaneously increase the likelihood of newborn transmission.

INTRODUCTION

As an opportunistic bacterial pathogen, *Streptococcus agalactiae*, or Group B *Streptococcus* (GBS) asymptomatically colonizes up to 35% of vaginal tracts and is the main risk factor for consequent invasive infection [1]. These invasive outcomes can arise during pregnancy, as vaginal colonization can lead to severe invasive neonatal disease, sepsis of the pregnant person, and other adverse pregnancy outcomes including preterm birth and stillbirth. GBS disease in neonates occurs in two onsets: early-onset disease (EOD) presents as sepsis and/or pneumonia within the first week after birth, and late-onset disease (LOD) presents as bacteremia and/or meningitis within the first 3 months after birth [2–5].

Screening for vaginal-rectal colonization of pregnant people is recommended between 35-37 weeks gestation. To prevent neonatal infections, individuals testing positive for GBS are given intrapartum antibiotic prophylaxis (IAP) during labor, for which penicillin is the most effective [6–10]. The IAP regimen has been successful in reducing cases of EOD in neonates but has not reduced the incidence of LOD [11–13]. It is also not effective in preventing preterm or stillbirths caused by GBS and can negatively impact the microbiome of the infant [14–16]. Because GBS colonization is intermittent in nature, this poses another challenge for effective prevention of invasive disease. Indeed, Van Dyke *et al.* showed that 60% of EOD cases were born to individuals who were negative for GBS at the time of screening [17]. In summation, there is a clear need for improved treatment and prevention methods that can reduce the multitude of GBS outcomes, more broadly and effectively.

Genotypic characterization of GBS, which includes classifying the polysaccharide capsule (cps) into serotypes and examining multilocus sequence typing (MLST) data, has identified significant associations between genotypic lineages, or sequence types (STs), and the severity of clinical outcomes [18]. Strains belonging to ST-17, for instance, were suggested to be hypervirulent since epidemiological studies identified higher frequencies of cps III ST-17 strains among neonates with invasive disease [18–21] along with unique virulence gene alleles

that enhance their ability to withstand antibiotic and oxidative stress and associate with host cells compared to strains representing other STs [22,23]. The virulence-epidemiological associations among some lineages, like ST-19 and ST-23; however, are less definitive despite their high clinical prevalence and may vary across populations [18,21]. This variability extends to the effectiveness of IAP treatment across GBS strains as some are successfully cleared while others can persist for up to 6 weeks postpartum [24]. Specifically, those GBS strains belonging to ST-17 and ST-19 were significantly associated with persistent colonization after IAP, while ST-12 strains were more commonly lost in a cohort of 212 pregnant people sampled for prenatal and postpartum GBS vaginal colonization [25,26]. The same study found IAP treatment to be associated with persistent colonization, suggesting that a large proportion of GBS strains can survive and tolerate antibiotic stress in the vaginal tract [25]. The mechanism by which GBS persistently colonizes the human vaginal tract despite antibiotic treatment, however, remains elusive.

Persistent colonization by GBS, despite antibiotic intervention, demonstrates that it can escape antibiotic-mediated killing and is not routinely cleared from the vaginal tract, which is a serious concern as it increases the risk of invasive disease, especially for subsequent pregnancies [27–29]. Moreover, this trait may contribute to LOD in babies after IAP cessation and could partly explain why the national rates of LOD have not decreased after IAP was recommended for all GBS-positive pregnant people in the U.S. [12,29]. We therefore hypothesize that IAP imposes a selective pressure on GBS residing in the vaginal tract, such that genomic features that promote colonization are selected for to enhance persistence and survivability. Since genotypic assessment of GBS by MLST is limited to the allelic variation of only seven genes, we sought to examine the relatedness of these clinical isolates using a more sophisticated method: whole genome sequencing (WGS). To investigate the evolutionary impact of IAP and its previously determined association with persistent colonization, we assessed the genomes of 97 GBS isolates collected from 58 pregnant people who were

sampled before (prenatal) and after (postpartum) childbirth and IAP. The insights gained from these genomic analyses enhance understanding of those factors that contribute to the resilience of GBS in the face of antibiotics and the host environment. Through these analyses we have gained a greater understanding of how IAP impacts the colonizing GBS population at the whole genome level, concurrently providing insight to avenues for treatment and prevention improvements.

RESULTS

Study population

Among the 58 pregnant people evaluated in the study, 41 were colonized with GBS at both prenatal and postpartum samplings indicating “persistent” colonization, while the remaining 17 individuals were colonized at only the prenatal visit indicating “loss” of colonization. Fourteen patients exhibited fever, 10 (71.4%) of whom were colonized with GBS at both prenatal and postpartum visits. Of the neonates born to individuals colonized at both visits, 12 (29.3%) had symptoms indicative of an illness. Moreover, most (91%) pregnant individuals received IAP with penicillin only (n=17), ampicillin only (n=26), cefazolin (n=2), or clindamycin only (n=6), while two of them received a combination of ampicillin and clindamycin (n=1) or penicillin and flagyl (n=1). Five subjects did not receive IAP. Eight (13.8%) patients received additional antibiotics postpartum; four of whom were colonized at both visits.

Sequencing metrics and strain characteristics

Ninety-seven GBS isolates were recovered from 58 pregnant people at the prenatal visit and 39 of the 58 (67.2%) individuals at the postpartum sampling; all isolates were included in the WGS analysis. Following sequencing, the raw reads were trimmed, and sequence quality was assessed from which all 97 genomes were deemed high-quality based on quality parameters and sequence characteristics (**Table S2.1**). All 97 genomes were assembled yielding a range of 13 to 1,731 contigs per genome with a mean of 90. Five isolates were excluded due to poor assembly quality; these included three prenatal isolates and two

postpartum isolates from five different patients, leaving 92 high-quality assembled genomes available for downstream analyses. Of these 92 genomes, 75 were recovered from individuals with persistent colonization (i.e., GBS colonization at both the prenatal and postpartum samplings). These 75 genomes represent complete prenatal-postpartum isolate pairs from 34 pregnant patients.

As was previously determined using MLST and cps typing [25], which corresponds to the molecular serotype, the 92 isolates represent 20 distinct STs that were classified into clonal complex (CC)-1 (n=23), CC-12 (n=14), CC-17 (n=16), CC-19 (n=17), CC-22 (n=1), CC-23 (n= 19), and CC-26 (n=1) (**Table S2.1**). One isolate (ST-26) was classified as a singleton and did not group with strains belonging to a predefined CC in our prior phylogenetic analysis [25]. In addition, eight distinct cps types were represented among the 92 isolates, with cps III (n=27) being the most common; four isolates were classified as non-typeable (NT).

Pangenome analysis reveals that strains distribute by clonal complex.

The pangenome analysis conducted with Roary [30] identified 5,054 unique genes, with 1,368 core genes, which are shared among ≥99% of the 92 strains (**Table S2.2)**. In addition, 213 genes were classified as soft-core genes found in 95-99% of the genomes, while 945 were classified as shell genes found in 15-95% of the genomes. Most genes (n=2,528), however, were classified as cloud genes, which were only detected in up to 15% of the 92 genomes. A maximum likelihood (ML) tree generated from the 1,368 core-gene alignment of the 92 genomes revealed five distinct sequence clusters grouping together with 52-100% bootstrap support (**Figure 2.1**). Each cluster is associated with a predefined CC (i.e., none of the strains with the same CC were split across clusters). The CC-19 strains (n=17) group together in a cluster with 100% bootstrap support, whereas CC-17 (n=16) clustered together with 78% bootstrap support. The less prominent CCs in this dataset, CC-22 and CC-26, were classified into an “Other” category along with one singleton (ST-67), which grouped together and were most closely related to the CC-17 cluster. The CC-1 (n=23) and CC-12 (n=14) strains clustered

together with low (62%) bootstrap support but formed separate clusters within this group. Comparatively, the genomes representing CC-23 (n=19) grouped closely together but comprised a mixture of outgroups and smaller clades with 2-5 genomes in each. Despite the concordance between the predefined CCs and pangenomic clusters, a neighbor-net tree constructed using the 1,368 core genes confirmed these groupings and detected significant evidence for recombination (parsimony homoplasy index (PHI) p-value = 0.00) among the 17,825 parsimony-informative sites (**Figure S2.1**).

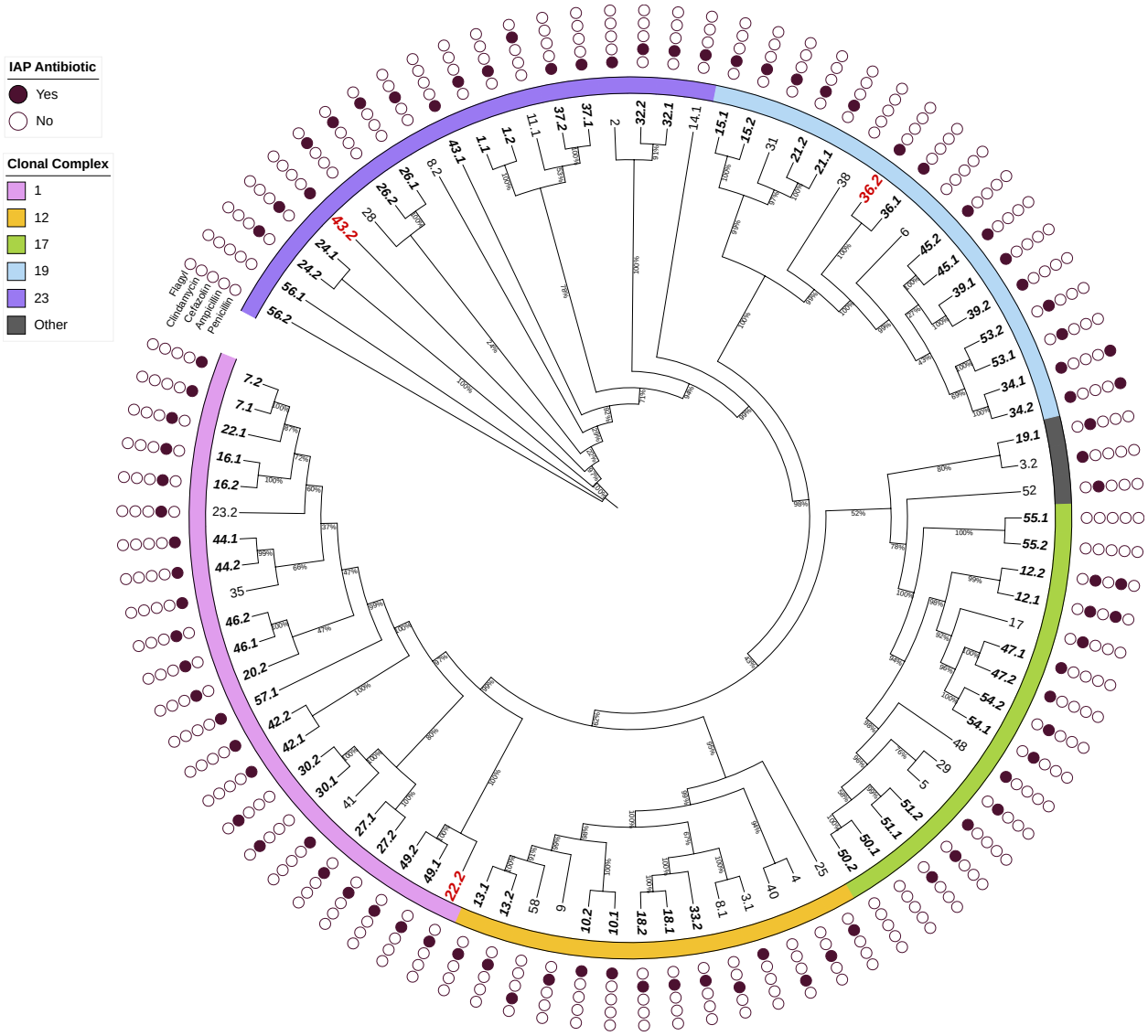

Figure 2.1: Maximum likelihood core-genome phylogeny of high-quality GBS genomes. Isolates (n=92) are labeled by pair ID such that paired isolates (persistent-same or persistent-different) contain a ".1" (prenatal) or ".2" (postpartum) to indicate the sampling timepoint with respect to childbirth and IAP treatment. The persistent-same isolates are designated with bold-italic font on their leaf labels. Mutator isolates are noted in red font. Bootstrap values are noted on the tree branches (0-100%). Isolates' clonal complex (CC) based on MLST are represented as follows: CC-1 (pink), CC-12 (yellow), CC-17 (green), CC-19 (blue), CC-23 (purple), and the less predominate CCs (CC-22, CC-26, and a singleton) grouped as "Other" (dark grey). The maroon circles display the antibiotic(s) administered to that individual for IAP treatment which may include (from inside to outside) penicillin, ampicillin, cefazolin, clindamycin, and/or flagyl.

Specific virulence or antibiotic resistance profiles are not linked to persistent colonization

To investigate the role of antibiotic resistance and virulence in these isolate's ability to colonize the vaginal tract, we performed assembly-based gene extraction analyses on all 92 genomes. Our analysis of antibiotic resistance genes (ARGs) across five resistance gene databases identified nine distinct ARGs conferring resistance to tetracyclines (*tetL, tetM, tetO, tetW, lmrP*), macrolides-lincosamides-streptogramins (MLS) (*ermA, lmrP, mreA*), fluoroquinolones (*norB*), and cationic peptides (*mprF*). Two of these ARGs, *lmrP* and *mprF*, were detected in all isolates (**Figure 2.2**).

Similarly, use of the virulence finder database (vfdb) enabled the identification of 50 distinct virulence genes across the 92 genomes and included those genes important for adherence and invasion of host cells (n=18, 36%), immune modulation (n=17, 34%), metabolism (n=1, 2%), toxin production (n=13, 26%), and dissemination (n=1, 2%) (**Figure 2.3, Table S2.3**). The greatest variation of virulence gene presence across the isolates was observed within the *cps* loci and pili-associated genes. A principal component analysis (PCA) plot, however, revealed no differences in virulence gene compositional profiles when stratified by isolate sampling timepoint (prenatal versus postpartum), type of antibiotic treatment, *cps* type, or colonization phenotype (**Figure S2.2A-D**). Although some distinct clustering was observed among virulence gene profiles when stratified by ST, significant overlap of clusters was still present (**Figure S2.2E**). Additionally, Chi-square analyses revealed no significant associations between colonization phenotype (persistent or lost) and the number of adherence (n=1,031, p = 0.995) or immune modulatory (n=1,231, p=0.519) genes present. The same was true for the other functional gene categories: exoenzyme (n=74, p=0.672), exotoxin (n=1,103, p=0.723), invasion (n=68, p=0.619), and metabolism (n=85, p=0.941). Altogether, these data suggest that possession of specific combinations of virulence genes is not linked to an isolate's ability to persistently colonize the vaginal tract after IAP.

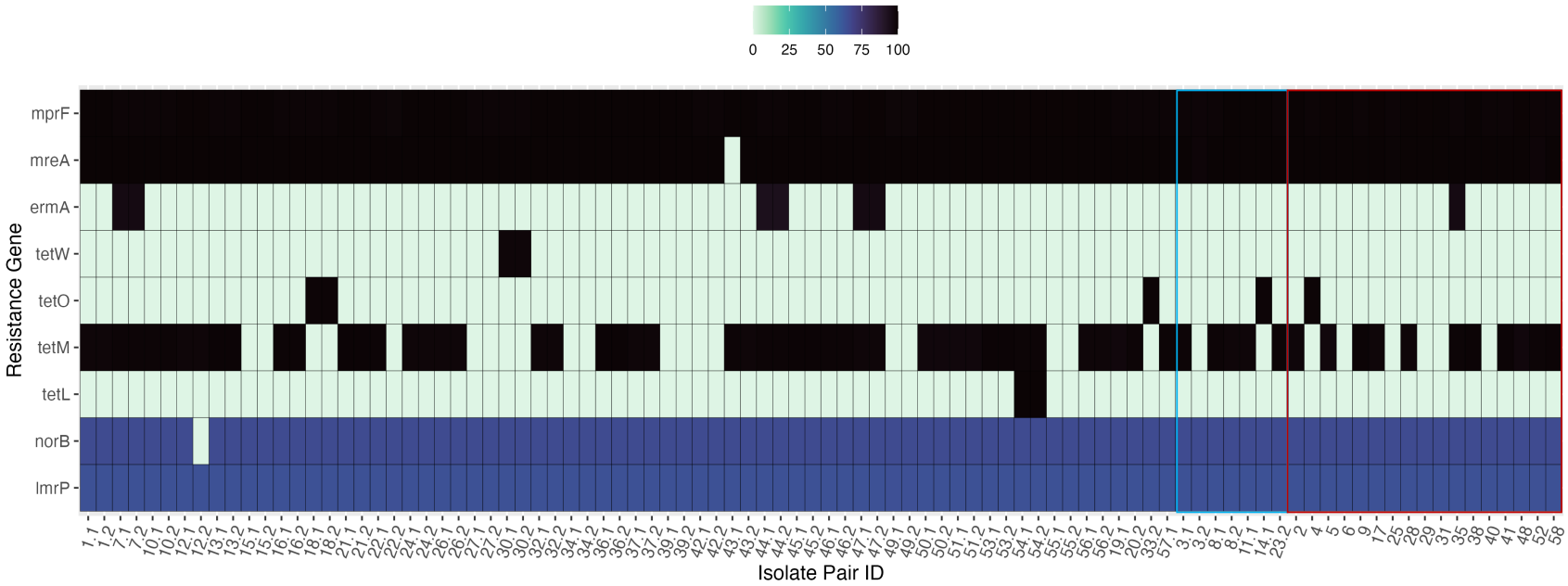

Figure 2.2: Antibiotic resistance genes (ARGs) detected in the genomic analysis by isolate. ARGs were extracted from 92 assemblies, noted along the x-axis by pair ID, including 68 persistent-same isolates (1.1-57.1), 7 persistent-different isolates (3.1-23.2, blue line), and 17 lost isolates (2-58, red line) using five different ARG databases (CARD [82], MEGARes [84], ARG-annot [83], Resfinder [86], and NCBI AMRFinderPlus [86]) with the ABRicate pipeline [https://github.com/tseemann/abricate]. Gene names are displayed on the y-axis. The color gradient displays the average percent identity of a gene across the databases, with black representing 100% identity and mint green representing <10% identity. ARGs extracted include those that confer resistance tetracyclines (*tetL, tetM, tetO, tetW, lmrP*), macrolides-lincosamides-streptogramins (MLS) (*ermA, lmrP, mreA*), fluoroquinolones (*norB*), and cationic peptides (*mprF*).

Nevertheless, it is notable that some gene-level variation was observed between paired isolates collected from the same person at the prenatal and postpartum visits in both the ARG and virulence gene analyses. For both the two paired isolates belonging to distinct STs (pair IDs 3 and 8), the prenatal isolate had different virulence gene profiles than their respective postpartum isolates, indicating colonization with two genetically distinct isolates (**Figure 2.3**). By contrast, three discrepancies were identified in the presence of specific ARGs when paired isolates of the same ST were compared (**Figure 2.2**). For two of the three discrepancies, sequence validation revealed that these genes were not completely absent, but instead, they did not meet the gene calling criteria for inclusion. For example, the *mreA* gene was detected in less than two databases in the prenatal isolate (pair ID 43), while *norB* fell below the 80% coverage threshold in the postpartum isolate (pair ID 12). The last discrepancy was for *tetM*, which was present in the prenatal isolate but absent in the postpartum isolate of pair ID 22. The presence of *tetM* was notably variable throughout the entire dataset. Similar results were observed for the presence of specific virulence genes, as 14 discrepancies were also detected between six isolate pairs with the same ST (**Figure 2.3**). Upon validation, however, most of these discrepancies involved genes that fell below the identity and coverage thresholds, indicating that they are present but distinct and require further evaluation.

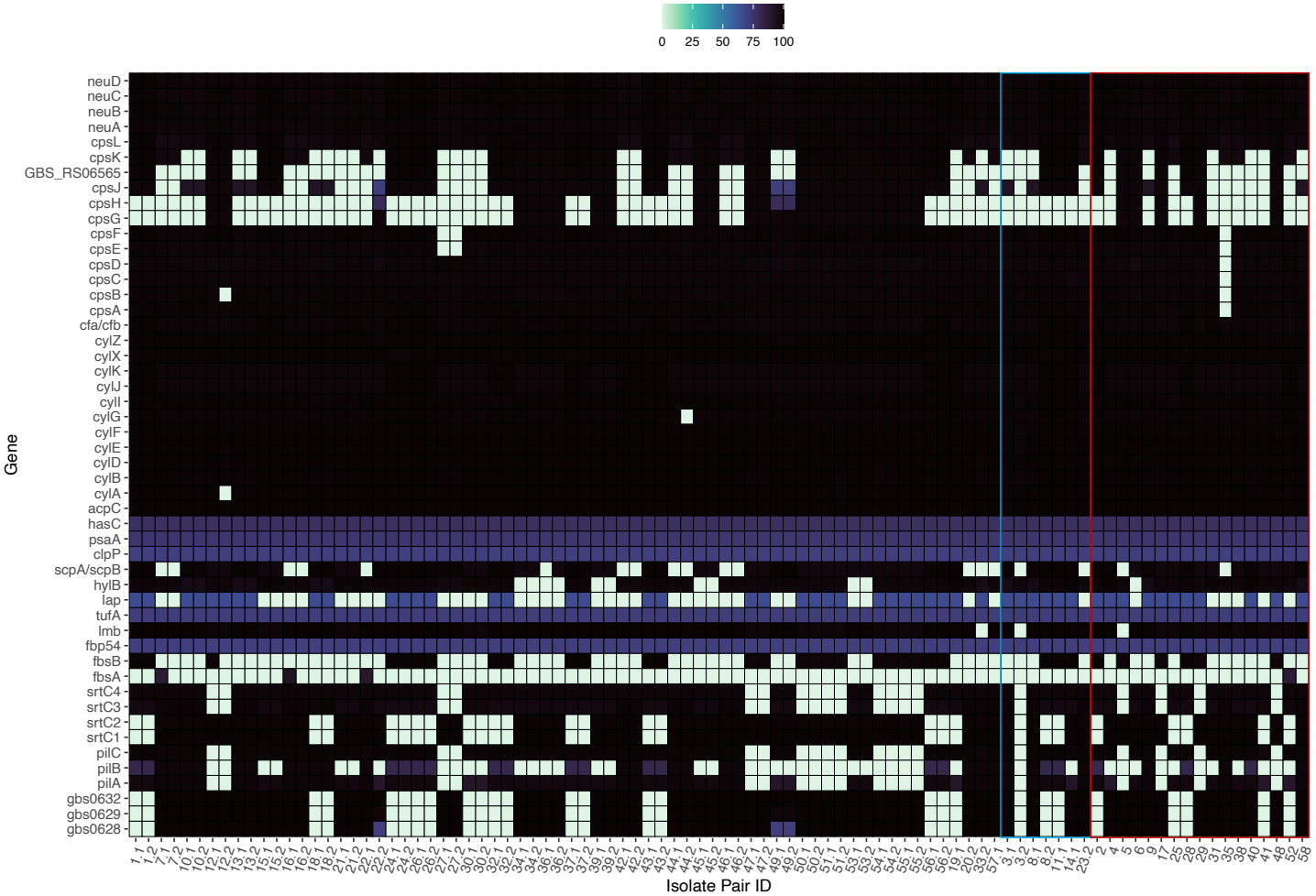

Figure 2.3: Virulence genes detected the genomic analysis by isolate. Virulence genes were extracted from 92 assembled genomes, noted along the x-axis by pair ID, including 68 persistent-same isolates (1.1-57.1), 7 persistent-different isolates (3.1-23.2, blue line), and 17 lost isolates (2-58, red line) using the ABRicate pipeline [https://github.com/tseemann/abricate] with the virulence-finder-database (VFDB) [87]. Gene names are displayed on the y-axis. The color gradient displays the average percent identity of a gene across the databases, with black representing 100% identity and mint green representing <10% identity. Genes extracted include those that encode proteins associated with host cell adherence and invasion, dissemination, exotoxin production, immune modulation, metabolism, and stress response.

Phylogenetic distance was identified among a subset of paired isolates

Since the postpartum isolates did not share a specific virulence or resistance gene profile relative to the prenatal isolates, we next compared placement in the ML core-gene phylogeny for each pair of isolates from 34 patients with persistent colonization. Most (n=29; 85%) of the paired genomes grouped together with >91% bootstrap support (**Figure 2.1**). Among the five paired genomes that did not group together, two pairs had postpartum isolates (IDs 3.2 and 8.2) representing different STs than the prenatal isolates (IDs 3.1 and 8.1), placing them on different branches of the tree. Interestingly, the prenatal CC-12 isolates from each of these pairs grouped together with 100% bootstrap support. Although the remaining three pairs had prenatal and postpartum isolates belonging to the same ST, the paired genomes did not cluster together in the ML phylogeny. One of these pairs (ID 22, CC-1) was separated into different subclades on opposite ends of the larger clade, whereas the other two pairs (IDs 43, 56) are singletons within the CC-23 cluster. Despite having the same ST within their respective pairs, the phylogenetic distance between these isolates highlights variation in the core gene sequences. Consequently, we hypothesized that differences at the nucleotide level may explain these disparities within the paired isolates of the same ST.

Mutations among persistent isolate pairs highlight microevolution following IAP

To detect nucleotide-level variation, we conducted a reads-based analysis of the core genes from the 32 paired isolates with the same ST; each prenatal isolate was used as the reference genome for its respective postpartum isolate. In all, 7,025 mutations were detected between 24 (75.0%) of the 32 pairs, the majority of which occurred within coding regions (n=6,065; 86.3%). Another 958 (13.6%) mutations occurred in uncharacterized regions and the remaining two were detected in rRNAs (**Table S2.4**).

Most of the mutations (n=5,982) were classified as single nucleotide polymorphisms (SNPs), though 712 complex mutations, which represent a combination of a SNP and multiple nucleotide polymorphism (MNP), were also detected as were 172 insertions, 124 deletions, and

35 MNPs (**Figure S2.3A**). While most SNPs were classified as synonymous mutations (n=3,469; 57.9%), 1,771 (29.6%) were classified as missense SNPs along with a small proportion (n=26; 0.4%) of nonsense SNPs (**Figure 2.4)**. The complex mutations represented a relatively equal frequency of synonymous (n=311; 43.7%) and missense (n=299; 41.9%) mutations, while the MNPs were mostly missense mutations (n=25; 71.4%). Among the insertions and deletions, frameshift mutations (n=107; 36.1%) were most prevalent followed by conservative outcomes (n=6), defined as a deletion or insertion of one or more entire codons, and disruptive outcomes (n=7), defined as a codon change plus a codon deletion or insertion. Another deletion also resulted in the loss of a stop codon, while two others resulted in the loss of a start codon. For the insertions, however, more conservative (n=7) than disruptive (n=4) mutations were detected and importantly, two of the insertions resulted in a nonsense mutation and four others resulted in the loss of a stop codon. This high degree of nucleotide variation between the prenatal and postpartum isolates is striking given that the isolates within each pair belong to the same ST and most (n=29) are near each other in the ML phylogeny (**Figure 2.1**).

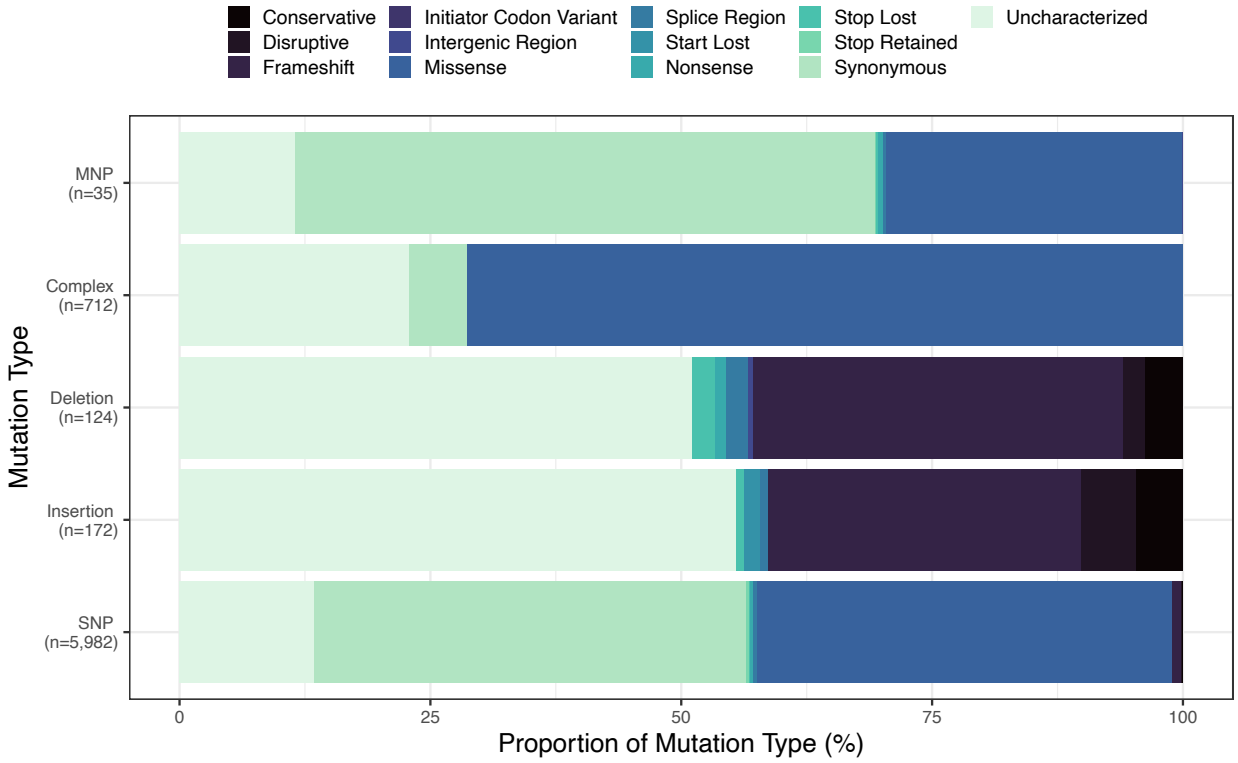

Figure 2.4: Core-genome mutations detected in the persistent-same paired genomes. Five types of mutations (y-axis) were detected using Snippy v4.6.0 (https://github.com/tseemann/snippy) between persistent-same pairs (n=32) including multiple nucleotide polymorphisms (MNP), complex mutations, deletions, insertions, and single nucleotide polymorphisms (SNPs), with the raw mutation count in parentheses for each type. Mutation outcomes, distinguished by color, are plotted as a proportion of each mutation type (x-axis). The categories are not mutually exclusive as some mutations were classified as multiple types (i.e., an insertion classified as disruptive and as a frameshift will be included in both mutation outcome categories.

Skewed distribution of mutations across persistent isolates reveals mutator strains

An assessment of the mutation frequencies across the isolate pairs revealed a skewed distribution, with three outlier pairs containing 204, 262, and 6,454 mutations (**Figure S2.3B**). Based on the total assembly lengths of the postpartum genomes for each respective pair of outliers, the mutation ratios (i.e., the number of mutations per number of base pairs) are 9.37×10^{-5}, 1.32×10^{-4}, and 0.003 for isolates GB00158 (pair ID=36), GB00281 (pair ID=43), and GB00026 (pair ID=22), respectively (**Table S2.4**). These rates were 53-, 75-, and 1,747-fold

higher, respectively, than the average mutation rate ($1.77x10^{-6}$ mutations/bp) across the remaining 29 isolate pairs.

Because mutators arise at a low frequency in the population due to mutations within genes encoding DNA replication and proofreading machinery, mismatch repair (MMR) pathways, and the 8-oxo-dG (GO) system [31–34], we examined these genes more comprehensively. Of the three outliers, pair 22 has the highest number of nonsynonymous mutations, which are located in multiple DNA repair systems, including MMR (n=14) and GO (n=8) pathways, and across other genes involved in DNA replication (n=18), DNA repair (n=7), and DNA recombination (n=9). Pair ID 36 has a deletion in the gene encoding a DNA polymerase IV, a missense SNP in *mutY* of the GO system, and a 33 bp insertion within an MMR locus. Moreover, pair ID 43 has a missense SNP in a gene encoding a DNA topoisomerase and a 1 bp deletion downstream of the MMR loci. The increased mutation rates along with the mutations in these key regions suggest that these isolates are mutators that arose in the three postpartum isolates after IAP treatment and childbirth.

Distinct classes of MMR loci are common but not associated with hypermutability

To define specific genomic alterations in the mutator strains, we examined the six MMR pathway genes (7,466 bp region) encoding DNA mismatch repair proteins MutS (*mutS*) and MutL (GBS_230), a cold shock protein, an MFS transporter/LmrP homolog (GBS_436), the Holliday junction branch migration protein RuvA (*ruvA*), and DNA-3-methyladenine glycosylase I (GBS_235) across the persistent paired isolates sharing the same ST (n=64, 32 pairs). A multiple sequence MUSCLE alignment of the MMR loci and construction of a neighbor joining (NJ) (**Figure 2.5**) phylogeny revealed 4 main unique groups of MMR nucleotide identities, which were designated as “classes” and were assigned arbitrary MMR class numbers 1-4. Although, construction of a neighbor-net tree based on 103 parsimonious informative sites within the 7,466 bp MMR region did not reveal significant evidence of recombination (p-value=0.0899), the four distinct MMR classes are evident (**Figure S2.4**). Each of the four MMR classes were further

divided into subclasses (n=13) based on 100% nucleotide identity across the MMR region. Of the four classes, class 4 is the largest group (n=28, 43.7%) with four subclasses (4A-4D), followed by class 2 (n=21, 32.8%) which had the largest number of subclasses (n=5, 2A-2E). The third largest class, class 1 (n=12, 18.7%), consists of three subclasses while the smallest class, class 3 (n=3, 4.7%), contains only one subclass. Subclass 4A contained the largest number of isolates (n=14, 21.8%) compared to the other 12 subclasses. A nucleotide BLAST analysis revealed that subclass 2A was most common among previously published GBS genomes with 43 genomes having matches with 100% nucleotide identity (**Table S2.5)**. Furthermore, classes 1, 2, and 4 each contained two, four, and two subclasses that were completely unique (i.e. did not return any 100% nucleotide identity matches with published GBS genomes).

All two of the mutator isolates (GB00158, GB00281) are placed in the same MMR class (class 4) while the supermutator (GB00026) is grouped into class 3. Of note, each mutator isolate has a distinct MMR subclass, representing 3A (GB00026), 4B (GB00158), 4A (GB00281). These data suggest that a single MMR genotype is not associated with hypermutability and random point mutations within these DNA repair genes are critical for adaptation. Further examination of these point mutations may also highlight other genomic regions that are under evolutionary pressure due to antibiotic exposure. Indeed, MMR classes seem to align with clusters in the core-genome phylogeny suggesting that certain MMR classes may be associated with CCs (**Figure S2.5**). It is notable, however, that two individuals (pair IDs 36 and 51) had postpartum isolates with different MMR subclasses when compared to their prenatal isolates despite their close proximity in the core-genome ML phylogeny.

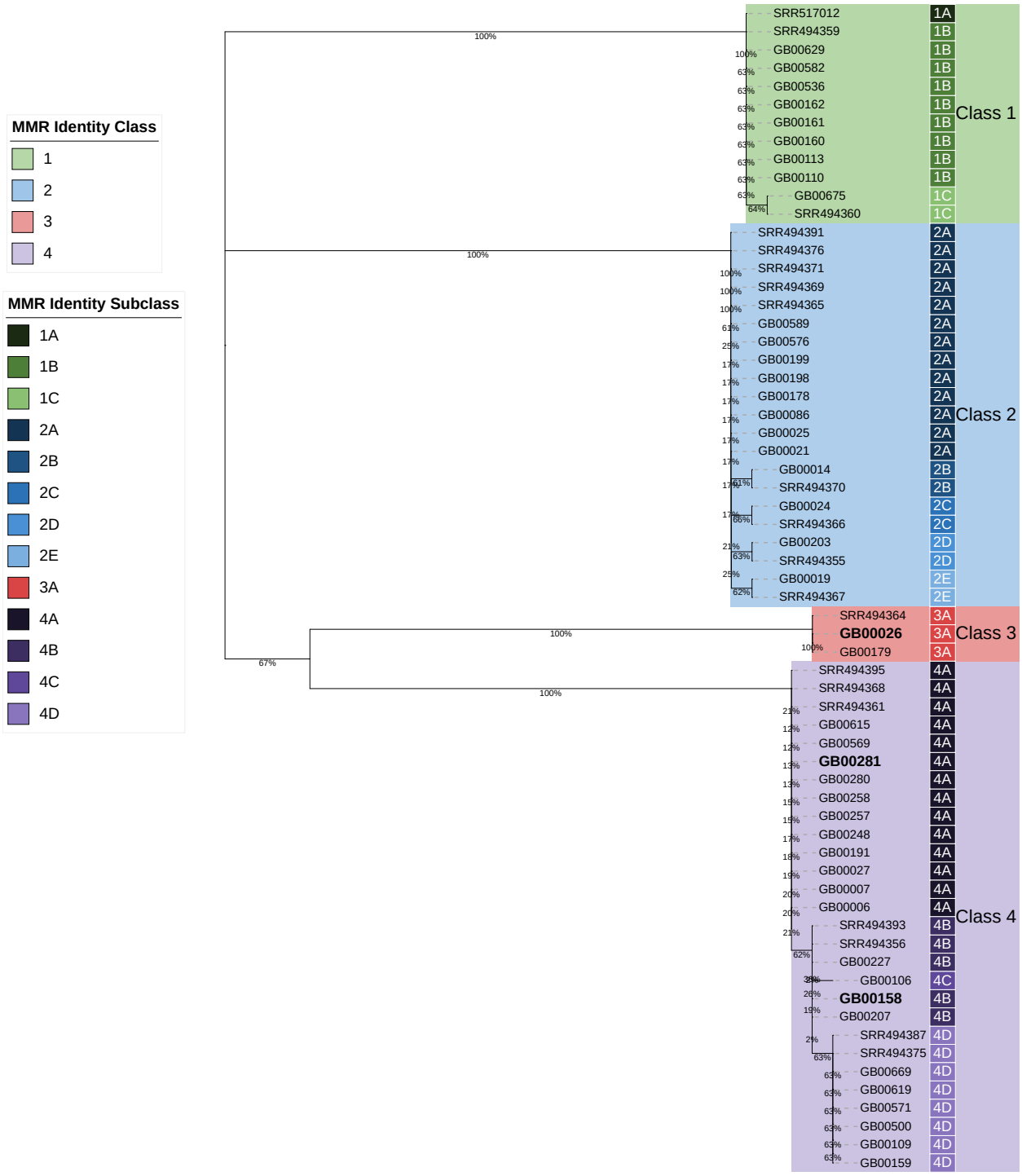

Figure 2.5: Neighbor joining phylogeny of persistent-same isolates based on MMR region alignment. Nucleotide alignment of the MMR region (7,466bp) was performed across 64 persistent -same isolates from which a neighbor joining phylogeny was constructed. Four MMR identity classes (green, blue, red, and purple) were identified based on clustering relationships. Within each class, MMR identity subclasses (1A-4D) were identified based on 100% nucleotide identity across the MMR region. Bootstrap values are displayed on tree branches (0-100%). Mutator isolates are noted with larger and bolded text.

IAP poses a selective pressure that differentially impacts genotypic lineages

To further examine point mutations that are more likely to disrupt functionality of the resulting protein, we assessed the types of SNPs and their respective frequencies across the isolates with both the mutator and non-mutator phenotypes. The distribution of SNPs across the 20 isolate pairs shows that CCs 1, 23, and 19 have the greatest overall number of SNPs, but this is largely driven by the presence of the three mutators isolates in each group (**Table 2.1**). When the mutators are excluded, however, isolates within CCs 1, 12, and 23 acquired the most SNPs between timepoints, averaging 3, 7, and 4 SNPs per non-mutator isolate, respectively. Isolates within CCs 17 and 19, on the other hand, acquired much fewer SNPs overall, averaging between 1-2 SNPs per non-mutator isolate. Furthermore, all CCs, except CC-17, had more nonsynonymous than synonymous SNPs across the non-mutator isolates, suggesting an overall pattern of positive/diversifying selection indicating a trend of adaptive evolution. The CC-17 group, however, has more synonymous than nonsynonymous SNPs, indicating an overall negative/purifying selection outcome. Two of the three mutators acquired more nonsynonymous than synonymous SNPs suggesting diversifying/adaptive evolution, while the other mutator with nearly 5,000 SNPs has more synonymous than nonsynonymous SNPs indicating an overall purifying selection theme. Overall, these data suggest that some genotypic lineages are more vulnerable to the evolutionary pressure imposed by IAP treatment, even without considering the SNP outcomes of the mutator isolates.

Table 2.1: Number of characterized SNPs identified between persistent isolate pairs.

Clonal Complex	Pair ID	Nonsynonymous	Synonymous	Isolate Totals
CC1	22	1598	3343	4941
	7	4	3	7
	49	1	1	2
	27	1	1	2
	42	1	0	1
	CC Total (NM)	**7**	**5**	**12**
	CC Total (ALL)	**1605**	**3348**	**4953**
CC12	10	7	2	9
	18	4	2	6
	CC Total	**11**	**4**	**15**
CC17	47	1	0	1
	51	0	1	1
	55	0	1	1
	CC Total	**1**	**2**	**3**
CC19	36	71	43	114
	34	3	0	3
	21	2	1	3
	15	1	0	1
	CC Total (NM)	**6**	**1**	**7**
	CC Total (ALL)	**77**	**44**	**121**
CC23	43	109	69	178
	26	8	2	10
	32	1	3	4
	37	3	1	4
	1	1	1	2
	56	1	0	1
	CC Total (NM)	**14**	**7**	**21**
	CC Total (ALL)	**123**	**76**	**199**
Grand Total (NM)	**17 pairs**	**39**	**19**	**58**
Grand Total (ALL)	**20 pairs**	**1817**	**3474**	**5291**

Mutators have high frequencies of missense SNPs and genes under positive selection after IAP

As mutators have been shown to serve as a reservoir of beneficial mutations for the surrounding bacterial population, we sought to further examine the SNPs identified in the three mutator isolates by investigating both the frequency and functional impact of characterized SNPs, i.e., those within coding sequences (CDS) only, throughout the genome. The three mutators acquired 5,233 characterized SNPs across a total of 1,167 genes (avg = 2 SNPs per

gene) (**Figure 2.6A, Table S2.4**) compared to 58 SNPs across a sum of 56 genes in the non-mutator isolates (avg = 1 SNP per gene) (**Figure 2.6B**). The overall distribution of SNPs across genes in the mutators shows that most of these genes (n=683) acquired <5 SNPs; however, >20 SNPs were acquired in 26 genes involved in DNA replication and repair, antimicrobial resistance, phage proteins, host cell attachment and invasion, and virulence. Among the 366 genes that had acquired >4 total SNPs in the three mutator isolates, most (77.6%, n=284) had evidence of negative selection. Sixty-one genes (16.7%) were under positive selection, while the remainder (5.74%, n=21) had evidence for neutral selection. Filtering for genes that acquired 5 or more nonsynonymous SNPs, resulted in 662 SNPs across 91 genes, most (98.3%, n=651) of the nonsynonymous SNPs were classified as missense (**Figure 2.6C**).

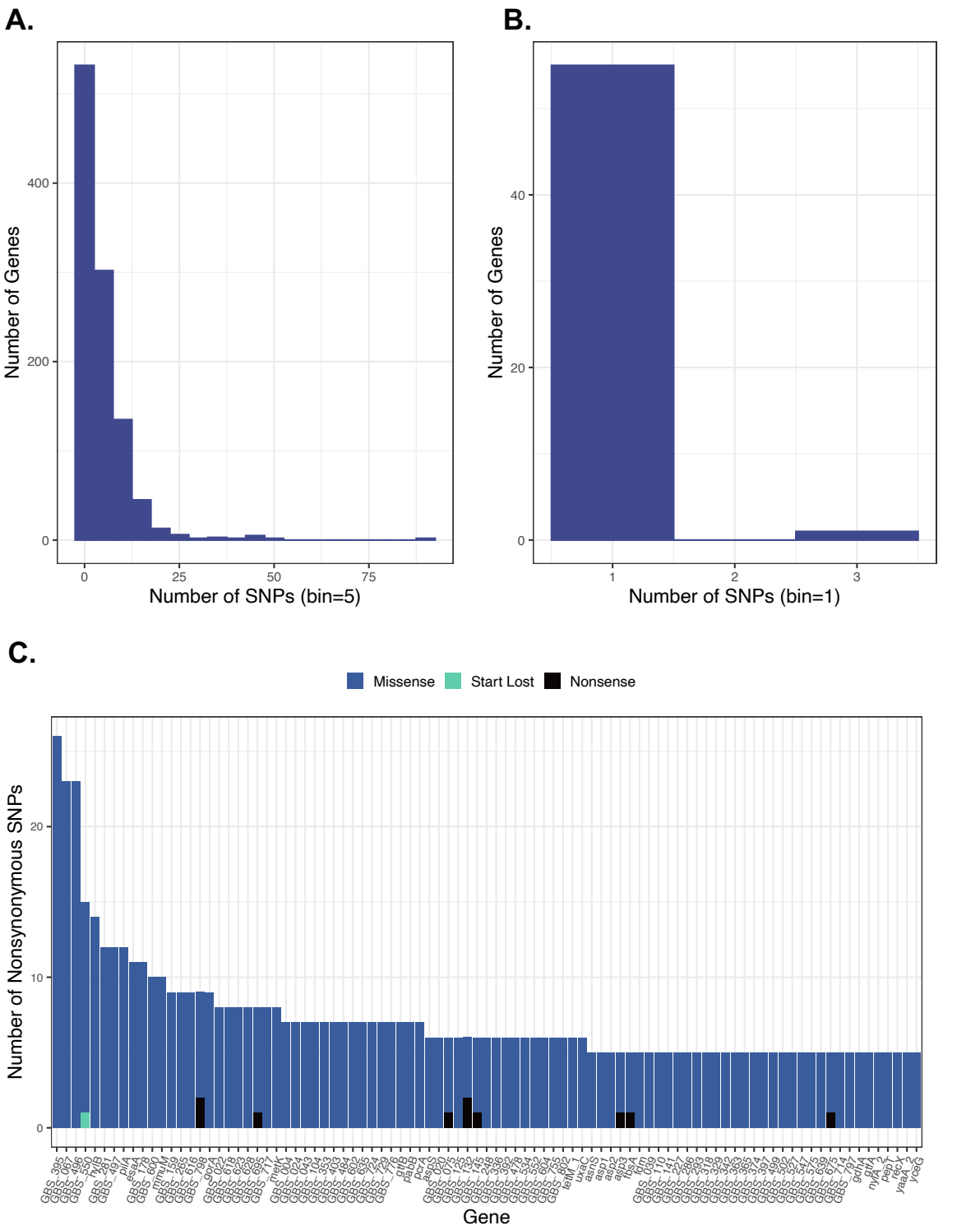

Figure 2.6: Distribution of SNPs detected in genes among the persistent-same isolate pairs recovered following IAP. Histograms displaying the distribution of the number of characterized SNPs (y-axis) by the number of genes (x-axis) is shown for the A) mutator and B) non-mutator isolates. C) Among the three mutator isolates, the types of nonsynonymous SNPs (missense = blue, start lost = teal, nonsense = black) are indicated by gene (x-axis) for those genes with >4 nonsynonymous SNPs.

Among the 61 genes with evidence of positive selection, eight had dN/dS values greater than 4: *asnS* (asparagine tRNA ligase), GBS_318 (GNAT family acetyltransferase), GBS_534 (protein-ADP-ribose hydrolase), GBS_639 (thiol reductase thioredoxin), GBS_675 (U32 family peptidase), GBS_724 (hypothetical protein), GBS_776 (hypothetical protein), and *yaaA_2* (peroxide stress protein YaaA). These genes are important for oxidative stress (*yaaA_2,* GBS_639), bacterial competition (GBS_534) [35], protein translation (*asnS*), virulence (GBS_675) [36], and aminoglycoside resistance (GBS_318) [37], while two genes (GBS_724, GBS_776) encode hypothetical proteins. Two of these eight genes (*asnS* and GBS_724) had very strong evidence of positive selection with a dN/dS of infinity as they acquired only nonsynonymous mutations (n=5 and 7, respectively). Other notable genes with evidence of positive selection include another tRNA ligase (*aspS*), *recX* (recombination regulator RecX), and *esaA* (type VII secretion protein EsaA). As many key virulence and stress response genes are undergoing adaptive evolution across these mutator strains, it implies that such processes may be important for persistent colonization despite IAP.

Similarly, of the 651 missense SNPs identified in the mutator isolates, several occurred in the same genes, ranging from 4 to 26 total missense SNPs in a gene. For example, GBS_067, GBS_395, GBS_496, which encode an accessory Sec-dependent serine-rich glycoprotein adhesin, a leucine-rich repeat domain-containing protein, and a phage tail protein, respectively, all acquired >20 missense SNPs in the mutator isolates. Key virulence genes, *esaA* (type VII secretion protein EsaA), *hylB* (hyaluronate lyase), *pilA* (PI-2a pilus adhesin PilA) that are important for colonization, acquired >10 missense SNPs. Four other genes acquired >10 missense SNPs, GBS_178 (class C sortase), GBS_281 (GBS Bsp-like repeat-containing protein), GBS_497 (phage tail tape measure protein), GBS_550 (pullulanase). Ten nonsense mutations were also acquired collectively across the three mutator isolates, which occurred in eight distinct genes: *asp3* (accessory Sec system protein Asp3), fbsA (fibrinogen-binding adhesin FbsA), GBS_075 (alanine racemase), GBS_132 (BCCT family transporter), GBS_145

(bifunctional metallophosphatase/5'-nucleotidase), GBS_675 (tyrosine-type recombinase/integrase), GBS_695 (Xaa-Pro peptidase family protein), GBS_798 (hypothetical protein). In addition to acquiring >10 missense SNPs, GBS_550 (pullulanase), acquired a mutation that resulted in the loss of a start codon. As nonsynonymous SNPs alter the eventual encoded protein and may consequently impact the resulting functionality of the protein and its associated pathways, it is likely that these genes or particular genomic signatures may enhance the isolate's ability to colonize host cells and/or survive antibiotic stress.

Postpartum isolates more commonly exhibit enhanced biofilm formation

One of the strategies isolates employ to enhance survival and host colonization is biofilm formation. Indeed, we identified nonsynonymous mutations in biofilm-related genes in the postpartum persistent isolates such as p*ilA*, as well as *gtfB* and GBS_067, which are both part of the accessory Sec system (**Figure 2.6C**). To investigate changes in phenotypic functionality, we performed *in vitro* biofilm assays on the persistent isolate pairs to assess whether the postpartum isolates exhibit enhanced biofilm capabilities relative to their prenatal isolate. Indeed, over half (54.8%, n=17) of the 31 persistent isolate pairs available for analysis had postpartum isolates with increased biofilm formation (fold-change >1) compared to its respective prenatal isolate (**Figure 2.7, Figure S2.6**). Five of these observed increases were significantly higher and occurred in isolates with a range of 0 to over 5,600 SNPs relative to the prenatal isolates combined. Among those pairs with increased biofilm production in the postpartum isolates, two represented the mutators. Only the mutator isolate from pair 22, however, had a significant increase in biofilm formation with the greatest fold-change across all pairs tested (fold-change = 8.14). Notably, two postpartum isolates from pairs 26 and 27 exhibited a significant decrease in biofilm formation; these isolates had acquired 11 and 2 mutations, respectively.

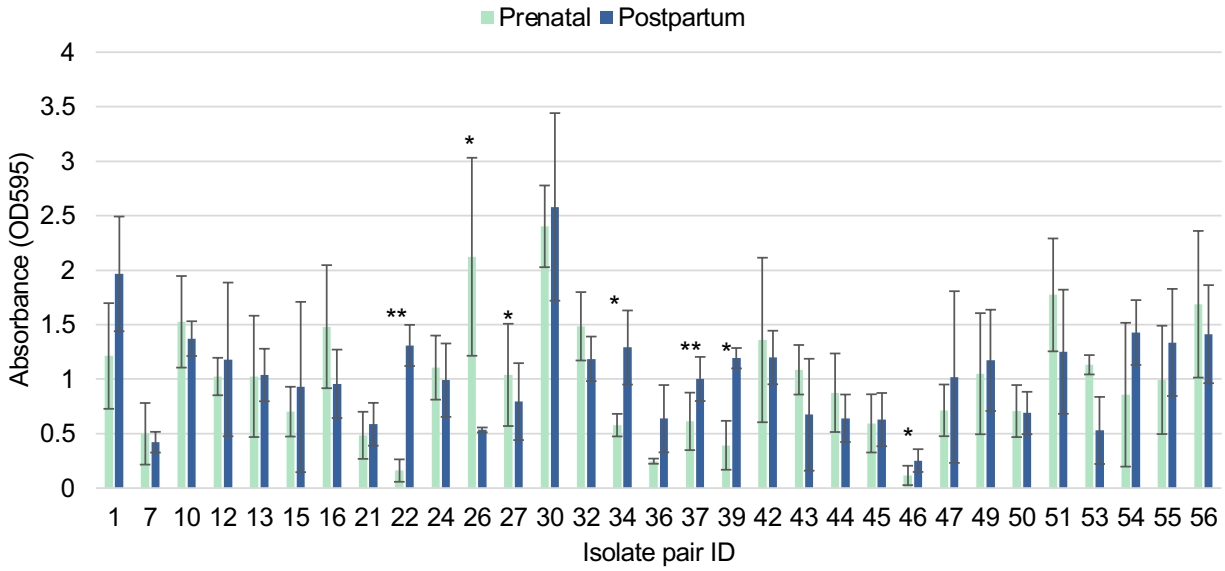

Figure 2.7: Assessment of biofilm formation between persistent-same isolate pairs. Biofilm formation was assessed across persistent-same isolate pairs (n=62 isolates, 31 pairs) via absorbance (OD595) values (y-axis), averaged across biological replicates (n=3). Prenatal (mint green) and postpartum (dark blue) isolates are grouped by pair ID (x-axis). One-tailed, paired T-tests were calculated to assess significant differences in biofilm formation between isolate pairs (*=p <0.05, **=p<0.01). Each measurement was normalized to a negative media control. Error bars display standard deviation.

DISCUSSION

GBS colonization during pregnancy is a concerning threat for consequent invasive disease and thus, antibiotics are recommended during childbirth to kill the bacterium and decrease the likelihood of EOD in the newborn. Because we observed in our previous work that 59.4% of colonized pregnant people were also colonized at their postpartum visit following IAP treatment [25], this persistent colonization presents an even greater risk of transmission to and infection of infants, or LOD. It further demonstrates that GBS can withstand antibiotic stress or escape antibiotic-mediated killing altogether. Conducting WGS on the paired isolates from before and after IAP herein has enhanced our understanding of GBS adaptation in the face of antibiotics and has helped elucidate mechanisms behind persistent colonization.

Although MLST has proven to be useful for examining evolutionary relationships between GBS strains, it can fail to detect strain-level variation since only seven housekeeping

genes are examined. Use of WGS, however, has allowed us to conduct a pangenome analysis based on 1,368 core genes and identify genomic differences between closely related isolates. Indeed, the ML phylogeny grouped isolates into five distinct clusters, with most persistent colonizers clustering by prenatal-postpartum pair (**Figure 2.1**). Three pairs (IDs 22, 43, 56), however, did not cluster together despite having the same ST and acquired by 6,454, 262, and 4 total mutations, respectively, providing evidence of within-ST variation (**Table S2.4**). Moreover, differences were identified in 72% (n=21) of the isolate pairs that clustered together (n=29), highlighting the enhanced sensitivity of WGS. Intriguingly, more SNPs were detected in the non-mutator pairs belonging to CC-23 compared to isolates pairs representing CCs 17 and 19, and the average mutation rate across non-mutator isolates belonging to CCs 1, 12, and 23 (n=11, 3.66×10^{-6}) was significantly higher (p=0.02) than the average mutation rate of the CC17 and CC19 non-mutator isolates (n=6, 1.37×10^{-6}). These observations, along with the extended strain variation observed within the CC-23 lineage supports the suggested classification of these lineages as generalists that are more vulnerable to genomic alterations and capable of colonizing a broad host range [18,25,38]. In contrast, the CC-17 and -19 lineages have been have been linked to higher frequencies of neonatal disease [20,21] and are likely more specialized. While two of the three mutator isolates belong to the CC-1 and CC-23 lineages, the third mutator belongs to the CC-19 lineage. Therefore, it is not clear, however, if such differences are due to mutability or variation in the mechanisms used to survive antibiotic stress.

Although acquisition of ARGs can promote bacterial survival in the presence of antibiotics, we failed to detect newly acquired resistance genes in the postpartum isolates relative to the prenatal isolates from the same person. Hence, resistance to the antibiotics used for IAP was ruled out as a mechanism of persistent colonization. Although most individuals (88.6%) received β-lactam antibiotics for IAP, for example, no β-lactam resistance genes were detected. Of those patients who received clindamycin (11.3%), their postpartum isolates were neither phenotypically resistant [26] or harbored the clindamycin resistance gene, *ermA*.

Interestingly, two different individuals (IDs 35, 44) were colonized with isolates containing *ermA*, yet these were found to be susceptible to clindamycin [26]. This finding suggests that while genotypically present, *ermA* may be altered resulting in attenuated expression/function in some cases. Although *lmrP*, which encodes a multidrug exporter in *Lactococcus lactis* conferring resistance to MLS antibiotic [39], was detected in all isolates, the lack of phenotypic resistance to these antibiotics suggests an alternate function of this gene in GBS. While *lmrP* is part of the MMR operon in *Streptococcus* species, its role remains elusive [40,41]. Similar to the ARG analysis, the presence of specific virulence genes was also not associated with persistent colonization, as none of the postpartum isolates had distinct virulence genes when compared to their respective prenatal isolates in the persistent-same pairs. Rather, acquisition of mutations within existing genes was more commonly detected in the postpartum isolates.

Studies in other bacterial species have identified increased mutation frequencies after antibiotic exposures, suggesting that antibiotic stress may drive genetic diversity and consequently contribute to a rise in antibiotic resistant and more virulent isolates by damaging DNA replication machinery, inducing stress response pathways, and increasing bacterial competence [42,43]. Our analysis of evolutionary selection shows that over 16% of genes that acquired mutations after antibiotic exposure are undergoing positive selection. This finding suggests that IAP is exerting a selective pressure on GBS genomes, fostering more resilient strains. Indeed, studies have documented a rise in GBS cps VI strains, which have been classified as a rare cps type among cases of both vaginal colonization and infant disease [1,44], along with an increase in erythromycin- and clindamycin-resistant isolates [45,46]. In our study, three isolates (3%) were classified as cps VI and all were persistent colonizers, including the postpartum isolate (GB00026) that acquired the largest number of mutations. The respective prenatal isolate (GB00025) shared the same ST, but was classified as cps V, providing evidence of a capsule switch within this pair from cps V to cps VI. The change in capsule types

within this pair (ID 22) explains, in part, the large amount of variation detected between these isolates. Capsule switching in GBS frequently occurs via single or multiple recombination events within the *cps* locus [47–49], but evidence of extensive recombination events have been shown to bring about variation throughout the entire GBS genome in addition to the *cps* locus [47,50]. For example, a prior study that uncovered thousands of SNPs in ST-1 serotype VI genomes when they were mapped to a ST-1 serotype V genome, and provided evidence of recombination across >50% of the genomes, despite being the same ST [51]. Such events may explain the emergence of more rare *cps* types, like cps VI, which presents important clinical implications as the widespread variation introduced throughout the genome as a result of these rare capsule switches likely enhances virulence and resistance to current GBS treatment and prevention methods.

The observation that three isolates had considerably higher mutation rates led us to explore the mutator phenomenon. Indeed, previous studies have identified isolates with high mutation rates across several bacterial species that have been characterized as “mutators” [33,52,53]. Mutators typically arise at low frequencies in bacterial populations due to DNA polymerase errors, dysfunctional proofreading mechanisms, and/or failure of mismatch/error correcting machinery [52]. One such mismatch correcting system is the MMR pathway, which is an important barrier for recombination events [54]; all three mutator isolates had mutations in the MMR-encoding region. Dysfunction of this system is a common mechanism of how mutators arise, increasing the likelihood of horizontal gene transfer and consequent spread of antibiotic resistance [55]. For instance, mutations and disruptions in *mutS* were detected and have been linked to hypermutators in other bacterial species [56–58], including *Streptococcus pyogenes* in which prophage integration between the *mut* genes has resulted in a growth-phase-dependent mutator state [40,41]. In clinical *Pseudomonas aeruginosa* isolates, disruption of such DNA repair pathways to give rise to mutators resulted in attenuated virulence but favored long-term persistence in the host, similar to the protective strategy of biofilms [56,59]. It is also possible

that variation in the MMR-encoding region impacts colonization phenotypes, as alignment of this region across the persistent isolates revealed 4 distinct MMR classes and 13 distinct MMR subclasses. Interestingly, each mutator had a different MMR subclass, although only patient 22 acquired mutations (n=51) within the MMR coding genes in the postpartum isolate. A 33 bp insertion in the *mutS* and *mutL* intergenic region and a 1 bp deletion resulting in a frameshift mutation directly downstream of the MMR loci, were also discovered in the postpartum isolates for patients 36 and 43, respectively.

The presence of GBS mutators may serve as a potential source of enhanced survival and colonization for the host's GBS population, and even for other bacterial species occupying the vaginal tract. Indeed, studies have revealed a link between hypermutation and persistent infections, suggesting that persistent isolates may be a source for mutators [60]. Since this is the first time mutators have been described in GBS, our findings present an important clinical implication for GBS disease and the current IAP treatment regimen. Indeed, it was suggested that the presence of mutators threatens the potential for increased antibiotic resistance frequencies [61], further limiting IAP treatment options. Moreover, these mutators, may serve as a reservoir for beneficial alleles that confer enhanced virulence and survival outcomes for other isolates in the population. This trait is especially important in the event of subsequent pregnancies for persistently colonized people.

Similar to the MMR pathway, disruptions in the GO system, a base excision repair pathway, have also been shown to result in mutators through accumulation of oxidative stress product, 7,8-dihydro-8-oxo-2'-deoxyguanine (8-oxo-dG) in several bacterial species, including *Streptococcus mutans* [31,32,62]. In addition to the endo and exonucleases associated with base excision repair, the three main enzymes that are responsible for removing this common form of oxidative DNA damage include a formamidopyrimidine-DNA glysoylase, an adenine glycosylase, and an 8-oxo-dG hydrolase, which have been classified as *mutM, mutY,* and *muT* in *Escherichia coli*, respectively [63]. In our analysis, two of the three mutators (pair IDs 22 and

36) acquired nonsynonymous mutations in *mutY,* and pair ID 22 also acquired nonsynonymous mutations in genes encoding uracil DNA glycosylases (GBS_683, GBS_684) which are likely to be "*mutM"* and in genes encoding 8-oxo-dGTP disphosphatases (GBS_014, GBS_015) which we hypothesize to be "*mutT*". We also identified mutations in other genes important for oxidative stress, including *yaaA* (dNdS = 5) and GBS_639 (dN/dS=5) encoding the peroxide stress protein YaaA and thiol reductase thioredoxin, respectively, which were under strong positive selection. Previous work examining interactions between GBS and macrophage-like cells in the presence of antibiotics in conjunction with our core-genome mutation analysis supports the idea that some strains may be employing escape-based strategies to avoid antibiotic-mediated killing. Notably, we previously demonstrated that the persistent colonizing ST-17 strain (SRR517012, postpartum ID 51.2) had an enhanced ability to survive phagosomal stress than an ST-12 strain (SRR494379, prenatal ID 4) that was readily killed following IAP [22]. While mutations that disrupt genes in the GO system would not be beneficial in terms of handling oxidative damage, they result in a larger reservoir of mutations, including those that may enhance survival and virulence.

On the other hand, some isolates may be employing more antibiotic-tolerance based strategies to withstand the presence of antibiotics for extended periods of time. These tolerance strategies may include induction of a slow-growing, latent stage while in the presence of the stressor, known as the stringent response. This phenomenon is supported by our findings of missense SNPs occurring in several genes important for protein synthesis, and more globally, the stringent response. Enhanced colonization and protection against antibiotic killing may also be attributable to biofilm formation. The presence of pili-associated genes (*pilA-C, srtC1-*4) across isolates in this dataset was the most variable (**Figure 2.3**) and we identified >5 SNPs in *pilA* (n=23, dN/dS = 1.09) and *srtC4* (n=8, dN/dS = 1). We also observed nonsynonymous SNPs in Sec-system related genes, *GBS_067* and *asp3*, which have also been shown to play a role in biofilm formation [64]. Diversifying evolution within such genes suggests that biofilm formation

may be an important strategy for GBS persistent colonization and antibiotic tolerance in the vaginal tract. In fact, we observed a trend of increased biofilm formation in over half of the postpartum isolates from the persistently colonized pairs with the same ST (n=17/31), but only five were significantly different than the prenatal isolate. This finding suggests that biofilm formation may be a persistence strategy for some, but not all strains. Indeed, previous work has identified that biofilm production among GBS isolates is influenced by variation in the pili genes and certain pilus-island types are associated with stronger biofilm production than others [65]. Notably, biofilm formation was assessed under standard broth conditions in this study. Further investigations of biofilms in more clinically relevant conditions such as lower pH or in the presence of antibiotic stress are needed to confirm biofilm formation as a strategy for persistent colonization.

Isolates obtained from the same subject over time, before and after antibiotic treatment, presents a unique and valuable opportunity to assess genomic evolution *in vivo*. Examination of the nucleotide-level variation has exposed key genes that may play a role in persistent colonization and survival during antibiotic exposure. The findings have important clinical relevance as they have enhanced our understanding of the toolkit of mechanisms GBS employs to circumvent current IAP treatment and stress. The identification of mutators threatens the existing effectiveness of IAP treatment as they increase the incidence of horizontal gene transfer and homologous recombination, contributing to the spread of antibiotic resistance.

GBS cps conjugate vaccines based on the most common cps types have been the most successful in eliciting specific antibody production, presenting the greatest potential for preventing severe GBS disease of the neonate and pregnant person [66–68]. One of the major concerns with this vaccine is the ability of capsule switching in GBS, particularly to rare cps types that are not included in the conjugate vaccine. Moreover, this vaccination method may introduce a selective pressure that results in isolates switching capsules to escape vaccine coverage, as has been observed in *Streptococcus pneumoniae* since the introduction of the

heptavalent pneumococcal polysaccharide vaccine [69] and *Neisseria meningitidis* and the monovalent meningococcal serogroup C conjugate vaccine [70]. Mutator strains also bring concerning implications of vaccine effectiveness, as they provide a large resource of beneficial mutations and increased recombination through which the rare alleles and genotypes could emerge. These changes could result in capsule switching, as we have observed in one of the mutator isolates (pair 22) that switched to the rare cps VI, and evasion of immune responses within a host and population. Altogether, this work helps to bring awareness of mutators as an important public health concern, highlighting antibiotics as a selective pressure and informs potential avenues for improving treatment methods, not only for GBS, but for many other pathogens as well.

METHODS

Bacterial isolate selection & characterization

A subset of 97 GBS isolates that were recovered from part of a larger cohort study of 212 pregnant people were included in this analysis [25,26]. The isolates were recovered from vaginal-rectal swabs taken from the same individual at two sampling visits: 1) during late 3rd trimester (35-37 weeks gestation), and 2) 6-weeks postpartum [26], and were previously characterized by MLST, serotyping, and cps typing [25]. Patients colonized with GBS at both prenatal and postpartum samplings were considered to have persistent colonization (n=80 isolates, 41 patients), while patients who only had GBS at the prenatal sampling were considered to have lost the pathogen by the postpartum sampling (n=17 isolates & patients).

Whole-genome sequencing (WGS)

A set of 40 of the 97 isolates was sequenced previously and the raw reads were downloaded from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (**Table S2.1**). The remaining 57 isolates were sequenced for this study, in which DNA was extracted from overnight cultures of GBS grown in Todd-Hewitt broth (THB) at 37°C + 5% CO_2 using the E.Z.N.A. (Omega Bio-tek Inc., Norcross, GA, USA) or Wizard HMW

(Promega, Madison, WI, USA) DNA extraction kits according to manufacturer's instructions. Construction of DNA libraries was performed by the Michigan Department of Health & Human Services (MDHHS) using the Nextera XT library prep kit (Illumina, San Diego, CA, USA) followed by sequencing on the MiSeq (Illumina) with 2x250 bp paired end reads.

Raw reads for all 97 genomes were trimmed with Trimmomatic v0.39 [71] using "gentle trimming" parameters to trim adapters and remove sequences with an average quality score of <15 or less than 36 nucleotides in length. The paired-end reads, or single-end reads for SRR517012 which was sequenced using an unpaired method, were assessed for quality using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) with high-quality genomes defined as those that passed ≥10%. High quality trimmed reads were assembled *de novo* using SPAdes v3.13.1 (kmers 21, 33, 55, 77, 99, 127) with mismatch error correction [72]. Assembly quality was assessed using QUAST v5.0.2 [73] and MultiQC v1.15 [74] with default parameters, where assemblies were considered high-quality if N50 and L50 scores were greater than 15,000 and less than 50, respectively. Assembled genomes were annotated using Prokka v1.14.6 with the –proteins option that allows for the use of a custom-made database [75]. This database was curated from 25 closed reference *S. agalactiae* genomes downloaded from GenBank to ensure that gene naming was consistent and specific for *S. agalactiae*. The ST designation was confirmed for each strain based on the whole-genome assemblies using PubMLST [76]. The previously determined ST designation was used for 3 strains [25] that could not be classified using WGS data due to poor assembly quality in one of the seven MLST loci (**Table S2.1**).

Pangenome and phylogenetic analysis

For pangenome analysis of all isolates, Roary v3.11.2 was used to create a multiFASTA alignment of core genes from all 92 high-quality assemblies using the -i (blastp of 95%) and -e (PRANK aligner 170427) parameters [30]. Analysis of the MMR region (n=6 genes, 7,466 bp region) was performed by manual extraction of the 6-gene region in Geneious v2022.2.2 [77]

from assembled persistent isolate genomes (n=64) followed by a MUSCLE nucleotide alignment and construction of a neighbor-joining phylogeny in MEGA v.11 [78]. Maximum likelihood phylogenies were generated with RAxML v8.2.12 (parameters -m GTRGAMMA, 500 bootstrap replicates) [79], and phylogeny visualizations and annotations were performed using the Interactive tree of life (iToL) [80]. Neighbor-net trees were generated in SplitsTree v4.19.0 [81], while the pairwise homoplasy index (PHI) was used to test for evidence of recombination with a window size of 100 with k as 1, where a p-value of <0.05 is considered to be significant.

Whole gene and nucleotide-level mutation extractions

Antibiotic resistance genes (ARGs) and virulence genes were extracted from the 92 high-quality assemblies with ABRicate (https://github.com/tseemann/abricate) using the following five ARG databases: CARD [82], ARG-annot [83], MEGARes [84], Resfinder [85], NCBI AMRFinderPlus [86] and the virulence-finder-database (VFDB) [87], respectively. Presence of a gene was confirmed if it surpassed the ≥10% identity and ≥80% coverage parameters and was identified in two or more databases in the case of ARGs. Antibiotic resistance phenotypes were determined previously by disk diffusion [26] and were used for comparison to the ARG data.

Core-genome mutations were extracted from all high-quality paired isolates with the same ST (n=64, 32 pairs). For each pair, the assembled prenatal genome was used as the reference, while the trimmed reads from the postpartum isolate were mapped against the reference. Each genome was interrogated for core-genome mutations including single nucleotide polymorphisms (SNPs), insertions, deletions, multiple nucleotide polymorphisms (MNPs), and complex mutations using Snippy v4.6.0 (https://github.com/tseemann/snippy).

For any discrepancies of gene presence/absence within an isolate pair with the same ST, i.e., isolates considered nearly identical, results were further confirmed using Geneious v2022.2.2 [77] by confirming ≥15X read coverage, and mapping up and downstream regions

with respect to a given gene to assess the location and completeness of the gene along a contig. Similarly, core-genome mutations were validated using a ≥10X read coverage cutoff.

Biofilm Assays

Biofilm assays were performed in triplicate on the paired isolates with the same ST that were still available (n=62 isolates, 31 pairs), with values normalized to blank media controls as previously described [65] with the modification of stagnant growth for 24hrs. Results were analyzed for magnitude of fold-change between isolate pairs with respect to the postpartum isolate, as well as any differences in biofilm formation within each isolate pair.

Data analysis

All raw output from WGS analyses were managed in Microsoft Excel. R v4.1.2 [88] was used in RStudio v2022.07.1+554 [89] for importing data (readr v2.1.4) [90], data wrangling (devtools v2.4.5, dplyr v1.1.3, forcats v1.0.0, plyr v1.8.8, tidyr v1.3.0, tidyverse v2.0.0) [91–96], and visualization (ggplot2 v3.4.3, viridis v0.6.4) [97,98]. R package factoextra v1.0.7 [99] was used to perform and visualize PCA with 95% confidence ellipses for virulence genes using the prcomp() and fviz_pca() functions. Chi-square tests were performed in Microsoft Excel or Epi Info™ v7 (Centers for Disease Control and Prevention, Atlanta, GA, USA); statistically significant associations were classified at $p \leq 0.05$. For unnamed genes that acquired mutations, arbitrary “gene ids” using the GBS_00X notation were assigned for easier reference. For all SNP-only analyses, SNPs were filtered to exclude those that occurred in rRNA and/or uncharacterized, non-coding regions. Nonsynonymous SNPs included those characterized by Snippy as “initiator codon variant”, “missense”, “start lost”, “stop lost”, and “stop gained” (nonsense). Synonymous SNPs included those characterized by Snippy as “stop retained” and “synonymous variant”. Measures of evolutionary selection were calculated based on the nonsynonymous to synonymous SNP ratio (dN/dS) across the genomes, as well as across each gene of interest to discern areas under positive (dN/dS >1), neutral (dN/dS = 1), or negative (dN/dS <1) selection. Calculations for evolutionary selection across genes were limited to genes

that acquired a total of 5 or more SNPs. Student t-tests were used to analyze differences in biofilm formation between isolate pairs and mutation rates across CCs; a p-value <0.05 was deemed significant.

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APPENDIX

Table S2.1: Strain and sequencing characteristics for 97 Group B *Streptococcus* genomes.

Isolate ID	GB ID	Pair ID	Clonal Complex (CC)	Sequence Type (ST)	Serotype	Capsule Type (cps)	IAP treatment	Sampling Visit	Colonization	Total Sequences	Average Sequence Length (bp)	N50(bp)	N75(bp)	L50	L75	Contigs (n)	Total assembly length (bp)	Assembly Quality Result
SRR494372	GB00002	11.1	23	23	1a	1a	clindamycin	prenatal	persistent-different	1235134	80	187561	76737	3	8	23	1970213	pass
SRR494366	GB00003	18.1	12	12	1b	1b	ampicillin	prenatal	persistent-same	370821	79	160085	69278	5	9	24	2127863	pass
GB00006	GB00006	24.1	23	23	3	1a	ampicillin	prenatal	persistent-same	115805	210	201992	87734	3	7	19	1972815	pass
GB00007	GB00007	24.2	23	23	1a	1a	ampicillin	postpartum	persistent-same	58858	207	85219	63650	6	13	31	1972407	pass
SRR494367	GB00012	44.1	1	1	3	5	penicillin	prenatal	persistent-same	316966	80	170081	90559	4	9	25	2104781	pass
SRR494370	GB00013	46.1	1	1	NT	5	ampicillin	prenatal	persistent-same	576841	80	204732	90958	3	8	25	2055424	pass
GB00014	GB00014	46.2	1	1	NT	5	ampicillin	postpartum	persistent-same	145489	209	156046	91573	4	9	21	2058104	pass
GB00016	GB00016	57.1	1	1	NT	5	penicillin	prenatal	persistent-same	397877	221	525076	91057	2	7	20	2057693	pass
SRR494368	GB00018	26.1	23	444	1a	1a	clindamycin	prenatal	persistent-same	685176	80	132862	62754	4	10	26	1968604	pass
GB00019	GB00019	44.2	1	1	5	5	penicillin	postpartum	persistent-same	71318	208	158614	68270	5	11	31	2107793	pass
SRR494371	GB00020	16.1	1	1	5	5	ampicillin	prenatal	persistent-same	474744	80	142933	69321	5	10	24	2054846	pass
GB00021	GB00021	16.2	1	1	NT	5	ampicillin	postpartum	persistent-same	69527	205	95708	74887	6	12	32	2057681	pass
GB00024	GB00024	18.2	12	12	NT	1b	ampicillin	postpartum	persistent-same	385290	207	244069	157791	3	6	23	2132983	pass
GB00212	GB00212	20.1	1	1	5	5	ampicillin	prenatal	persistent-same	351528	218	1887	972	323	801	1731	2550108	fail
GB00017	GB00017	57.2	1	1	5	5	penicillin	postpartum	persistent-same	33517	208	8700	4812	71	148	380	2026210	fail
GB00025	GB00025	22.1	1	1	NT	5	ampicillin	prenatal	persistent-same	70310	208	114793	67779	7	12	30	2056515	pass
GB00026	GB00026	22.2	1	1	NT	6	ampicillin	postpartum	persistent-same	348091	217	576826	138143	2	4	13	2084002	pass
GB00027	GB00027	26.2	23	444	NT	1a	clindamycin	postpartum	persistent-same	536752	206	254066	85474	3	7	20	1973163	pass
SRR494369	GB00082	42.1	1	2	4	4	penicillin	prenatal	persistent-same	327757	80	127021	95920	6	10	21	2036998	pass
SRR494364	GB00083	49.1	1	1	4	6	ampicillin	prenatal	persistent-same	374201	80	498832	92376	2	6	23	2052790	pass
SRR494365	GB00084	27.1	1	1	8	8	ampicillin	prenatal	persistent-same	2291747	80	290636	125012	3	6	22	2037353	pass
GB00086	GB00086	27.2	1	1	8	8	ampicillin	postpartum	persistent-same	454340	214	290736	133498	3	6	21	2040914	pass
SRR494357	GB00092	6	19	19	3	3	none	prenatal	lost	459511	80	79061	50989	10	18	51	2142871	pass
SRR494358	GB00097	17	17	17	3	3	ampicillin	prenatal	lost	606753	79	92978	63140	6	12	38	2007543	pass
GB00106	GB00106	36.1	19	19	3	3	penicillin	prenatal	persistent-same	468219	214	103421	63673	7	14	39	2093083	pass
GB00109	GB00109	45.1	19	19	3	3	penicillin	prenatal	persistent-same	542947	218	164506	67901	6	11	56	2154930	pass
GB00110	GB00110	47.1	17	17	3	3	penicillin	prenatal	persistent-same	553578	216	153573	64204	5	11	34	2047053	pass
SRR494359	GB00111	50.1	17	32	3	3	none	prenatal	persistent-same	811687	80	98815	63057	7	13	41	2066559	pass
GB00680	GB00680	33.1	12	12	1b	1b	ampicillin	prenatal	persistent-same	377678	213	11204	2990	11	215	987	3976850	fail
SRR517012	GB00112	51.2	17	17	3	3	ampicillin	postpartum	persistent-same	225367	121	44118	24127	16	31	78	2041213	pass
GB00113	GB00113	54.1	17	17	3	3	ampicillin	prenatal	persistent-same	457096	218	125350	81537	6	11	33	2094843	pass
SRR494360	GB00115	55.1	17	17	3	3	none	prenatal	persistent-same	354888	80	94196	48571	7	14	35	1969533	pass
GB00158	GB00158	36.2	19	19	3	3	penicillin	postpartum	persistent-same	447236	216	94596	64075	8	15	147	2176359	pass
GB00159	GB00159	45.2	19	19	3	3	penicillin	postpartum	persistent-same	442501	216	164506	78672	6	10	36	2137582	pass
GB00160	GB00160	47.2	17	17	3	3	penicillin	postpartum	persistent-same	301820	210	153573	64204	5	11	33	2046936	pass
GB00161	GB00161	50.2	17	32	3	3	none	postpartum	persistent-same	528307	217	137064	64163	6	11	34	2071253	pass
GB00162	GB00162	54.2	17	17	3	3	ampicillin	postpartum	persistent-same	417921	215	125008	81537	6	11	32	2094636	pass
SRR494363	GB00174	19.1	22	22	2	2	ampicillin	prenatal	persistent-same	481764	79	42301	24032	15	29	79	1987582	pass
GB00178	GB00178	42.2	1	2	4	4	penicillin	postpartum	persistent-same	505218	219	166274	91058	4	8	21	2041609	pass
GB00179	GB00179	49.2	1	1	6	6	ampicillin	postpartum	persistent-same	417302	216	671902	133732	2	3	63	2087904	pass
SRR494361	GB00190	56.1	23	23	1a	1a	none	prenatal	persistent-same	491136	80	179705	85731	3	7	21	1968770	pass
GB00191	GB00191	56.2	23	23	1a	1a	none	postpartum	persistent-same	320620	217	383638	87737	2	5	16	1973272	pass
GB00198	GB00198	7.1	1	1	5	5	penicillin	prenatal	persistent-same	388235	217	204120	90484	4	9	27	2108070	pass
GB00199	GB00199	7.2	1	1	5	5	penicillin	postpartum	persistent-same	525930	218	151387	78157	5	10	30	2101600	pass
GB00175	GB00175	19.2	22	22	2	2	ampicillin	postpartum	persistent-same	458220	219	9283	2032	70	350	1266	3923441	fail
SRR494355	GB00202	10.1	12	10	1b	1b	penicillin	prenatal	persistent-same	518966	80	146811	79291	5	10	31	2019352	pass
GB00241	GB00241	23.1	23	23	5	5	ampicillin	prenatal	persistent-different	420738	210	5134	1516	116	372	1160	2647901	fail
GB00203	GB00203	10.2	12	10	1b	1b	penicillin	postpartum	persistent-same	324089	217	243404	87491	3	6	23	2022225	pass
SRR494356	GB00206	15.1	19	19	1a	1a	ampicillin	prenatal	persistent-same	467495	80	101904	48172	8	15	41	2141765	pass
GB00207	GB00207	15.2	19	19	1a	1a	ampicillin	postpartum	persistent-same	308375	220	115051	67884	7	13	41	2147982	pass
GB00213	GB00213	20.2	1	1	5	5	ampicillin	postpartum	persistent-same	181526	218	525160	91587	2	5	16	2052666	pass
GB00217	GB00217	8.2	23	23	1a	1a	cefazolin	postpartum	persistent-different	209151	214	179930	83744	3	8	41	1986319	pass
SRR494396	GB00219	9	12	8	1b	1b	ampicillin	prenatal	lost	410322	79	232624	90210	4	8	24	2053957	pass
SRR494393	GB00226	21.1	19	28	2	2	ampicillin	prenatal	persistent-same	1218378	80	149462	67544	5	10	30	2101551	pass
GB00227	GB00227	21.2	19	28	2	2	ampicillin	postpartum	persistent-same	522716	217	149659	91518	5	9	28	2105636	pass
GB00242	GB00242	23.2	1	1	5	5	ampicillin	postpartum	persistent-different	276719	186	68203	38170	12	22	74	2097436	pass
SRR494394	GB00245	28	23	23	1a	1a	clindamycin	prenatal	lost	894685	80	145323	75298	3	9	22	1924453	pass
SRR494395	GB00247	32.1	23	24	1a	1a	ampicillin	prenatal	persistent-same	839259	80	240882	85119	3	7	21	1995894	pass
GB00248	GB00248	32.2	23	24	1a	1a	ampicillin	postpartum	persistent-same	444599	217	307852	87737	3	5	14	1999566	pass
GB00257	GB00257	37.1	23	23	1a	1a	penicillin	prenatal	persistent-same	417556	213	382621	87737	2	5	16	2018454	pass
GB00258	GB00258	37.2	23	23	1a	1a	penicillin	postpartum	persistent-same	420623	218	321354	85219	3	6	19	2019956	pass
SRR494388	GB00264	40	12	10	2	2	clindamycin	prenatal	lost	454187	80	99719	63876	5	12	33	2039255	pass
SRR494389	GB00279	41	1	2	2	2	penicillin	prenatal	lost	555722	80	156150	85445	6	10	32	2096520	pass
GB00280	GB00280	43.1	23	23	1a	1a	penicillin	prenatal	persistent-same	186224	222	401343	93434	2	5	17	2025213	pass
GB00281	GB00281	43.2	23	23	1a	1a	penicillin	postpartum	persistent-same	472214	218	383649	87737	2	5	17	1973487	pass
GB00285	GB00285	48	17	17	2	2	penicillin	prenatal	lost	357243	209	126507	59874	6	13	44	2001885	pass
SRR494390	GB00300	25	12	130	NT	8	ampicillin	prenatal	lost	488139	80	114645	64071	6	12	45	2103484	pass
GB00500	GB00500	53.2	19	19	3	3	ampicillin	postpartum	persistent-same	421391	217	167630	78664	5	10	34	2117785	pass
SRR494391	GB00535	13.1	12	8	1b	NT	ampicillin	prenatal	persistent-same	6352998	79	224573	92027	4	7	23	2019490	pass
GB00536	GB00536	12.1	17	17	3	3	ampicillin, clindamycin	prenatal	persistent-same	279976	219	125352	64607	6	11	33	1968919	pass
SRR494387	GB00543	39.1	19	36	3	3	penicillin	prenatal	persistent-same	9059749	79	115966	62892	5	12	41	2095188	pass
SRR525043	GB00548	14.1	23	88	1a	1a	ampicillin	prenatal	persistent-different	6165028	80	244464	124942	3	6	17	2034409	pass
SRR494386	GB00555	3.1	12	12	1b	NT	penicillin	prenatal	persistent-different	3953479	79	180600	95805	4	8	26	2077430	pass
SRR494384	GB00557	29	17	17	3	3	ampicillin	prenatal	lost	882310	75	94185	59776	7	13	41	2022243	pass
SRR494385	GB00561	38	19	19	5	5	none	prenatal	lost	5507265	79	79045	54419	8	16	51	2151369	pass
GB00569	GB00569	1.1	23	23	1a	1a	ampicillin	prenatal	persistent-same	100560	218	383748	87734	2	6	20	2036554	pass
GB00571	GB00571	34.1	19	19	3	3	penicillin, flagyl	prenatal	persistent-same	179794	201	111218	78839	8	13	39	2081990	pass
GB00576	GB00576	13.2	12	8	1b	1b	ampicillin	postpartum	persistent-same	414181	211	232836	181669	4	6	22	2020209	pass
GB00582	GB00582	12.2	17	17	3	3	ampicillin, clindamycin	postpartum	persistent-same	43459	211	22449	9692	25	57	186	1970008	pass
SRR494383	GB00588	31	19	447	2	2	ampicillin	prenatal	lost	6102297	79	201737	91345	4	8	24	2056785	pass
GB00589	GB00589	30.2	1	2	2	2	clindamycin	postpartum	persistent-same	288252	219	106761	70041	5	11	33	2011220	pass
SRR494382	GB00601	2	23	24	NT	1a	penicillin	prenatal	lost	6984179	79	187585	85384	3	7	21	2035161	pass
GB00609	GB00609	33.2	12	12	NT	1b	ampicillin	postpartum	persistent-same	595688	218	179436	90454	4	8	26	2173595	pass
SRR494381	GB00614	35	1	448	NT	NT	penicillin	prenatal	lost	7952358	79	107892	62547	8	14	44	2141678	pass
GB00615	GB00615	1.2	23	23	1a	1a	ampicillin	postpartum	persistent-same	267807	220	383748	92892	2	6	18	2037306	pass
GB00619	GB00619	34.2	19	19	3	3	penicillin, flagyl	postpartum	persistent-same	498528	219	119652	78716	5	10	35	2082551	pass
GB00629	GB00629	51.1	17	17	3	3	ampicillin	prenatal	persistent-same	531933	216	126496	64566	6	11	33	2042123	pass
GB00637	GB00637	8.1	12	12	NT	3	cefazolin	prenatal	persistent-different	990785	202	175450	95919	5	9	27	2080696	pass
SRR494380	GB00640	52	26	26	5	5	ampicillin	prenatal	lost	973507	75	488219	198771	2	4	14	2079645	pass
GB00649	GB00649	3.2	---	67	NT	NT	penicillin	postpartum	persistent-different	937426	199	44260	23026	15	32	104	2219550	pass
SRR494378	GB00651	58	12	8	1b	1b	clindamycin	prenatal	lost	732877	75	128193	55913	5	12	36	2061421	pass
SRR494379	GB00653	4	12	12	2	2	cefazolin	prenatal	lost	942600	75	88725	46206	8	16	49	2158227	pass
SRR494377	GB00654	5	17	17	3	3	penicillin	prenatal	lost	891465	75	126378	63179	5	11	30	2028868	pass
SRR494375	GB00663	53.1	19	19	3	3	ampicillin	prenatal	persistent-same	980145	75	105574	63922	7	13	41	2111016	pass
GB00669	GB00669	39.2	19	36	3	3	penicillin	postpartum	persistent-same	506582	212	179798	64167	4	10	64	2117663	pass
GB00675	GB00675	55.2	17	17	1a	3	none	postpartum	persistent-same	411415	216	127935	59877	6	13	35	1973886	pass
SRR494376	GB00679	30.1	1	2	2	2	clindamycin	prenatal	persistent-same	984734	75	106513	51544	5	12	38	2004962	pass
Average Values	---	---	---	---	---	---	---	---	---	**985543**	**158**	**181885**	**75119**	**11**	**30**	**90**	**2107213**	---

Table S2.2: Pangenome characteristics of the 97 GBS isolate dataset.

Genome Component	Definition	Number of Genomes	Number of Genes
Core	99-100%	91-92	1,368 (27.06%)
Soft core	95-99%	87-90	213 (4.21%)
Shell	15-95%	13-86	945 (18.69%)
Cloud	0-15%	0-12	2,528 (50.01%)
Total	0-100%	0-92	5,054 (100%)

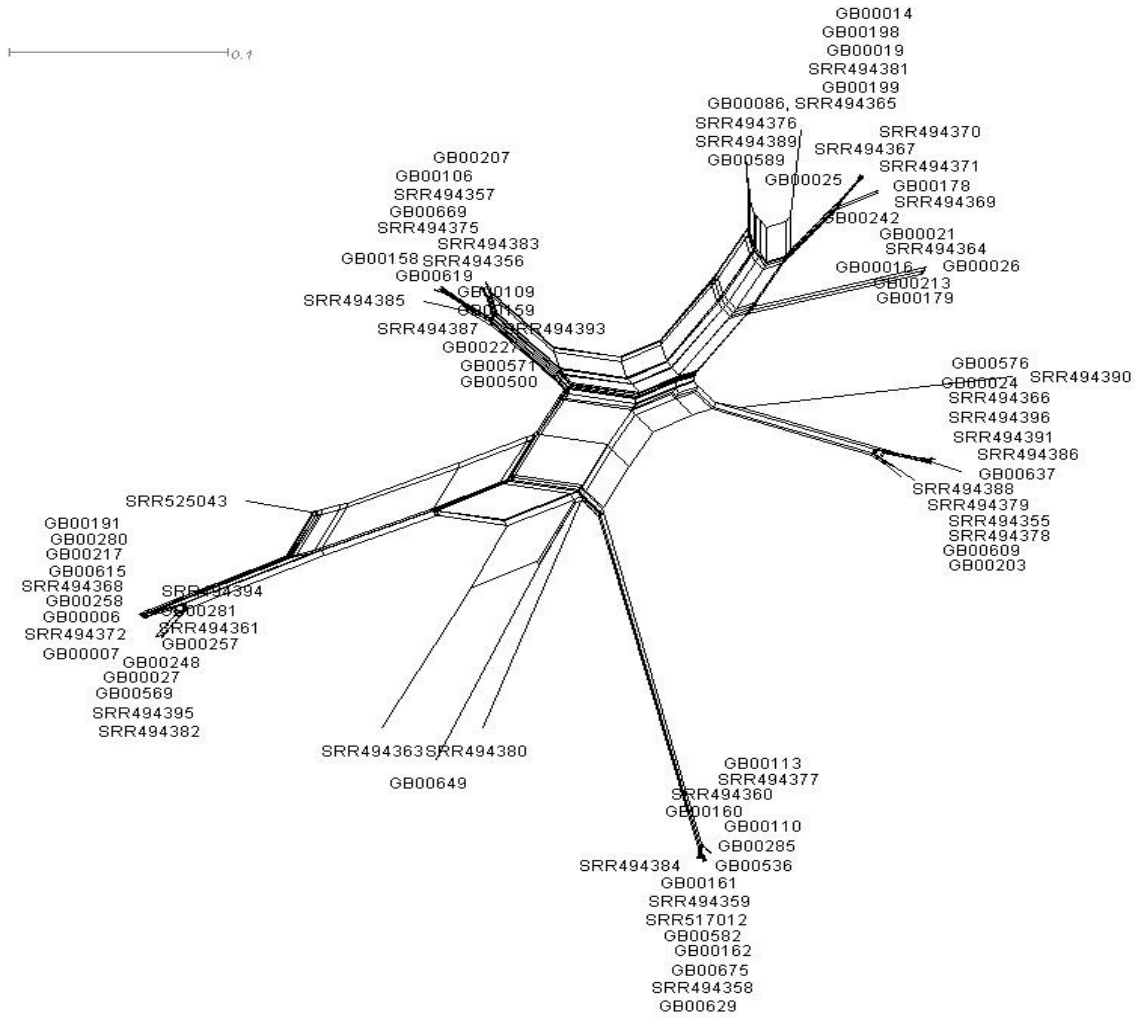

Figure S2.1: Neighbor net tree based on core-gene alignment of high-quality genomes. A multiFASTA alignment of core genes was created using Roary v3.11.2 across all high quality genomes assemblies (n=92). A neighbor net tree was constructed using SplitsTree v.4.19.0 based on 17,825 informative sites and a window-size of 100 with k as 1. Phi test revealed significant evidence for recombination ($p<0.001$).

Table S2.3: Virulence genes identified across 92 high-quality genomes.

Gene	Functional Category	Gene Product	Accession	Identity range (min-max)	Number of isolates (all=92)
fbsA	Adherence	fibrinogen binding adhesin FbsA	WP_000482192	86.86-92.13	4
cpsH	Immune modulation	capsule biosynthesis protein CpsH/hypothetical protein (GBS_RS06575)	WP_001233888	78.9-99.56	30
cpsG	Immune modulation	capsule protein CpsG/glycosyltransferase (GBS_RS06580)	WP_000578446	98.73-98.94	30
fbsB	Adherence	fibrinogen binding surface protein FbsB	WP_000743858	98.58-100	34
pilB	Adherence	PI-2a pilus major subunit PilB	WP_000723540	81.56-99.16	45
GBS_RS06565	Immune modulation	capsule protein/glycosyltransferase family 2 protein	WP_000591736	99.26-99.68	48
lap	Adherence	adhesion-mediating acetaldehyde-CoA/alcohol dehydrogenase (Listeria monocytogenes)	NP_465159	65.86-65.98	51
cpsJ	Immune modulation	capsule protein CpsJ/glycosyltransferase family 2 protein (GBS_RS06570)	WP_000660895	70.79-99.17	62
cpsK	Immune modulation	capsule protein CpsK/glycosyltransferase family 52 (GBS_RS06560)	WP_000181212	97.49-100	64
gbs0628	Adherence	PI-1 backbone protein/SpaH EbpB family LPXTG-anchored major pilin (GBS_RS03565)	WP_000777402	70.34-100	65
gbs0629	Adherence	PI-1 ancillary protein 2/SpaA isopeptide-forming pilin-related protein (GBS_RS03570)	WP_000815035	98.16-100	65
srtC2	Adherence	class C sortase SrtC2/pilus associated protein	WP_000746885	99.77-100	65
gbs0632	Adherence	PI-1 ancillary protein 1/SpaA isopeptide-forming pilin-related protein (GBS_RS03585)	WP_001868236	99.85-100	65
srtC1	Adherence	class C sortase SrtC1/pilus associated protein	WP_000529916	99.89-100	65
pilA	Adherence	PI-2a pilus adhesin PilA	WP_001233990	90.1-99.3	73
srtC3	Adherence	class C sortase SrtC3/pilus associated protein	WP_000850672	96.81-99.77	73
pilC	Adherence	PI-2a pilus subunit PilC	WP_000723812	98.38-99.78	73
srtC4	Adherence	PI-2a pilus assembly sortase SrtC4	WP_000508992	98.76-99.32	73
scpA/scpB	Invasion	C5a peptidase	WP_001227855	97.83-100	73
hylB	Exoenzyme/Dissemination	hyaluronate lyase	WP_000403400	96-100	81
lmb	Adherence/Invasion	laminin binding protein	WP_000715197	99.02-100	89
cpsE	Immune modulation	capsule biosynthesis protein CpsE (GBS_RS06590)	WP_000659582	98.85-99.57	89
cpsF	Immune modulation	capsule biosynthesis protein CpsF (GBS_RS06585)	WP_000686634	99.11-100	89
cpsB	Immune modulation	capsule protein CpsB/tyrosine-protein phosphatase (GBS_RS06605)	WP_000565385	99.04-99.86	90
cylG	Exotoxin	CylG protein/3-oxoacyl-ACP reductase FabG	WP_000861302	99.72-100	91
cylA	Exotoxin	CylA protein/ABC transporter ATP-binding protein	WP_000403526	99.78-100	91
cpsD	Immune modulation	capsule protien CpsD/tyrosine-protein kinase (GBS_RS06595)	WP_000197412	97-99.71	91
cpsC	Immune modulation	capsule biosynthesis protein CpsC (GBS_RS06600)	WP_001033074	98.12-99.57	91
cpsA	Immune modulation	capsule protein CpsA/LCP family protein (GBS_RS06610)	WP_000064997	99.38-99.79	91
fbp54	Adherence	fibronectin binding (S. pyogenes)	WP_010922232	70.27-70.89	92
tufA	Adherence	Surface expressed elongation factor (Francisella tularensis)	WP_003028672	71.16-71.33	92
cylK	Exotoxin	CylK protein	WP_001068957	98.44-100	92
cylJ	Exotoxin	CylJ protein/glycosyltransferase	WP_000033003	98.76-100	92
cylF	Exotoxin	CylF protein/aminomethyl transferase	WP_001092618	99.06-100	92
cylI	Exotoxin	CylI protein/beta-ketoacyl-(acyl-carrier-protein) synthase	WP_000118217	99.09-100	92
cylE	Exotoxin	CylE protein/haemolysin	WP_000650746	99.15-100	92
cylB	Exotoxin	CylB protein/ABC transporter permease	WP_000462410	99.32-100	92
cylX	Exotoxin	CylX protein	WP_000533775	99.35-100	92
acpC	Exotoxin	acyl carrier protein	WP_000611493	99.67-100	92
cylD	Exotoxin	CylD protein	WP_000859501	99.76-100	92
cylZ	Exotoxin	CylZ protein/3-hydroxyacyl-ACP dehydratase	WP_000164166	99.79-100	92
hasC	Immune modulation	hyaluronic acid capsule protein/UTP-glucose-1-phosphate uridylyltransferase HasC (S. pyogenes)	WP_010922799	77.12-77.46	92
cpsL	Immune modulation	capsule protein CpsL/oligosaccharide flippase protein (GBS_RS06555)	WP_001093064	96.79-100	92
neuC	Immune modulation	capsule protein NeuC/UDP-N-acetylglucosamine 2-epimerase	WP_000717643	98.61-100	92
neuA	Immune modulation	capsule protein NeuA/N-acylneuraminate cytidylyltransferase	WP_000802346	98.87-100	92
neuD	Immune modulation	capsule protein NeuD/acetyltransferase (GBS_RS06540)	WP_000727597	99.05-100	92
neuB	Immune modulation	capsule protein NeuB/N-acetylneuraminate synthase	WP_000262522	99.22-100	92
psaA	Metabolism	manganese ABC transporter substrate-binding protein	WP_000733059	73.75-74.11	92
clpP	Stress/Adherence/Invasion	Stress protein/phagosome escape, adhesion and invasion (Listeria)	NP_465991	70.42-70.76	92
cfa/cfb	Exotoxin	CAMP factor	WP_001101136	73.75-100	111 (19 duplications)

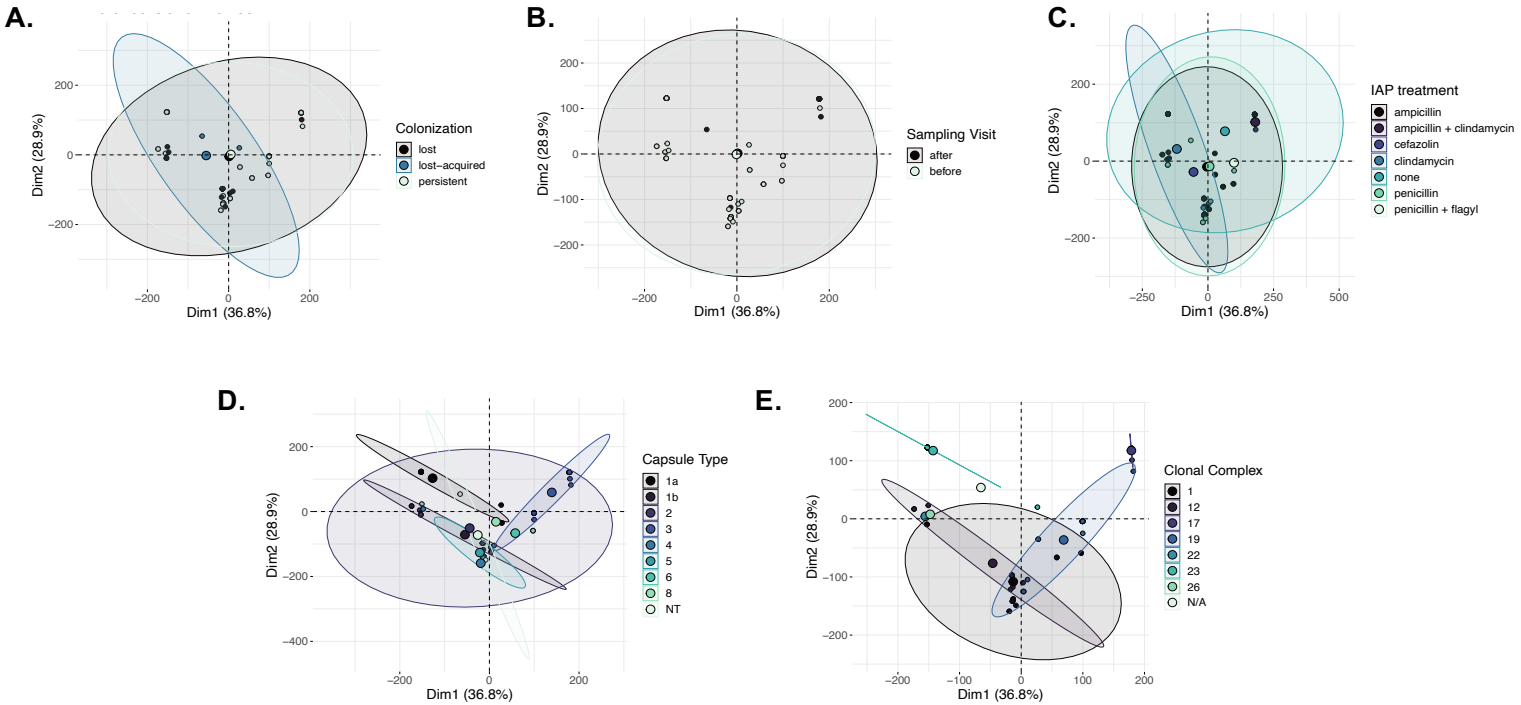

Figure S2.2: Principal component analysis (PCA) of isolates' virulence gene profiles. Percent identity values of virulence genes detected across 92 whole genomes using ABRicate and the virulence finder database (vfdb) were used to perform a PCA to assess diversity of virulence gene profiles. The single PCA was stratified by colonization phenotype (A), sampling timepoint relative to IAP/delivery (B), IAP treatment (C), capsule type (D), or clonal complex (E). For all panels, the first principal component is displayed on the x-axis and the second principal component on the y-axis, representing 36.8% and 28.9% of the variation in the sample set, respectively. Each point on the plot represents an isolate's profile of virulence genes (i.e. the set of virulence genes that are present or absent in a given isolate). Ellipses represent 95% confidence interval of relatedness and the larger points for each category is representative of the cluster's median in which the ellipse is drawn. For panels that have categories with too few points to calculate a confidence ellipse (C-E), these categorical clusters are represented with their median value without an ellipse.

Table S2.4: Core genome mutation analysis between persistent isolate pairs.

Isolate ID	Pair ID	Raw SNPs	Characterized SNPs*	Deletions	Insertions	Complex Mutations	MNPs	Total Mutations (Raw)	Total Assembly Length (bp)	Mutation Rate**	Genes with SNPs*	Average SNPs* per gene
GB00615	1.2	2	2	1	0	0	0	3	2037306	1.47253E-06	2	1
GB00199	7.2	10	7	1	1	0	0	12	2101600	5.70994E-06	7	1
GB00203	10.2	9	9	3	1	0	0	13	2022225	6.42856E-06	9	1
GB00582	12.2	0	0	0	0	0	0	0	1970008	0	---	---
GB00576	13.2	0	0	0	0	0	0	0	2020209	0	---	---
GB00207	15.2	1	1	0	0	0	0	1	2147982	4.65553E-07	1	1
GB00021	16.2	0	0	2	0	0	0	2	2057681	9.71968E-07	---	---
GB00024	18.2	7	6	1	1	1	0	10	2132983	4.68827E-06	4	1.5
GB00227	21.2	5	3	0	1	0	0	6	2105636	2.8495E-06	3	1
GB00026	22.2	5571	4941	76	89	683	35	6454	2084002	0.003096926	918	5.373
GB00007	24.2	0	0	0	0	0	0	0	1972407	0	---	---
GB00027	26.2	12	10	3	1	0	0	16	1973163	8.10881E-06	10	1
GB00086	27.2	2	2	0	0	0	0	2	2040914	9.79953E-07	2	1
GB00589	30.2	1	0	0	0	0	0	1	2011220	4.97211E-07	---	---
GB00248	32.2	7	4	0	3	0	0	10	1999566	5.00109E-06	4	1
GB00619	34.2	4	3	0	1	0	0	5	2082551	2.4009E-06	3	1
GB00158	36.2	129	114	16	59	0	0	204	2176359	9.37345E-05	110	1.036
GB00258	37.2	4	4	1	1	0	0	6	2019956	2.97036E-06	4	1
GB00669	39.2	0	0	0	0	0	0	0	2117663	0	---	---
GB00178	42.2	2	1	0	0	1	0	3	2041609	1.46943E-06	1	1
GB00281	43.2	205	178	18	12	27	0	262	1973487	0.00013276	139	1.281
GB00019	44.2	0	0	0	0	0	0	0	2107793	0	---	---
GB00159	45.2	0	0	0	0	0	0	0	2137582	0	---	---
GB00014	46.2	0	0	0	0	0	0	0	2058104	0	---	---
GB00160	47.2	1	1	0	0	0	0	1	2046936	4.88535E-07	1	1
GB00179	49.2	3	2	0	0	0	0	3	2087904	1.43685E-06	2	1
GB00161	50.2	1	0	1	0	0	0	2	2071253	9.65599E-07	---	---
SRR517012	51.2	1	1	0	0	0	0	1	2041213	4.89905E-07	1	1
GB00500	53.2	1	0	0	0	0	0	1	2117785	4.72191E-07	---	---
GB00162	54.2	0	0	0	0	0	0	0	2094636	0	---	---
GB00675	55.2	1	1	0	2	0	0	3	1973886	1.51984E-06	1	1
GB00191	56.2	3	1	1	0	0	0	4	1973272	2.02709E-06	1	1

*SNPs within coding sequences, excluding those within rRNA and uncharacterized regions

**Mutation rate = “Total Mutations”/ “Total Assembly Length”

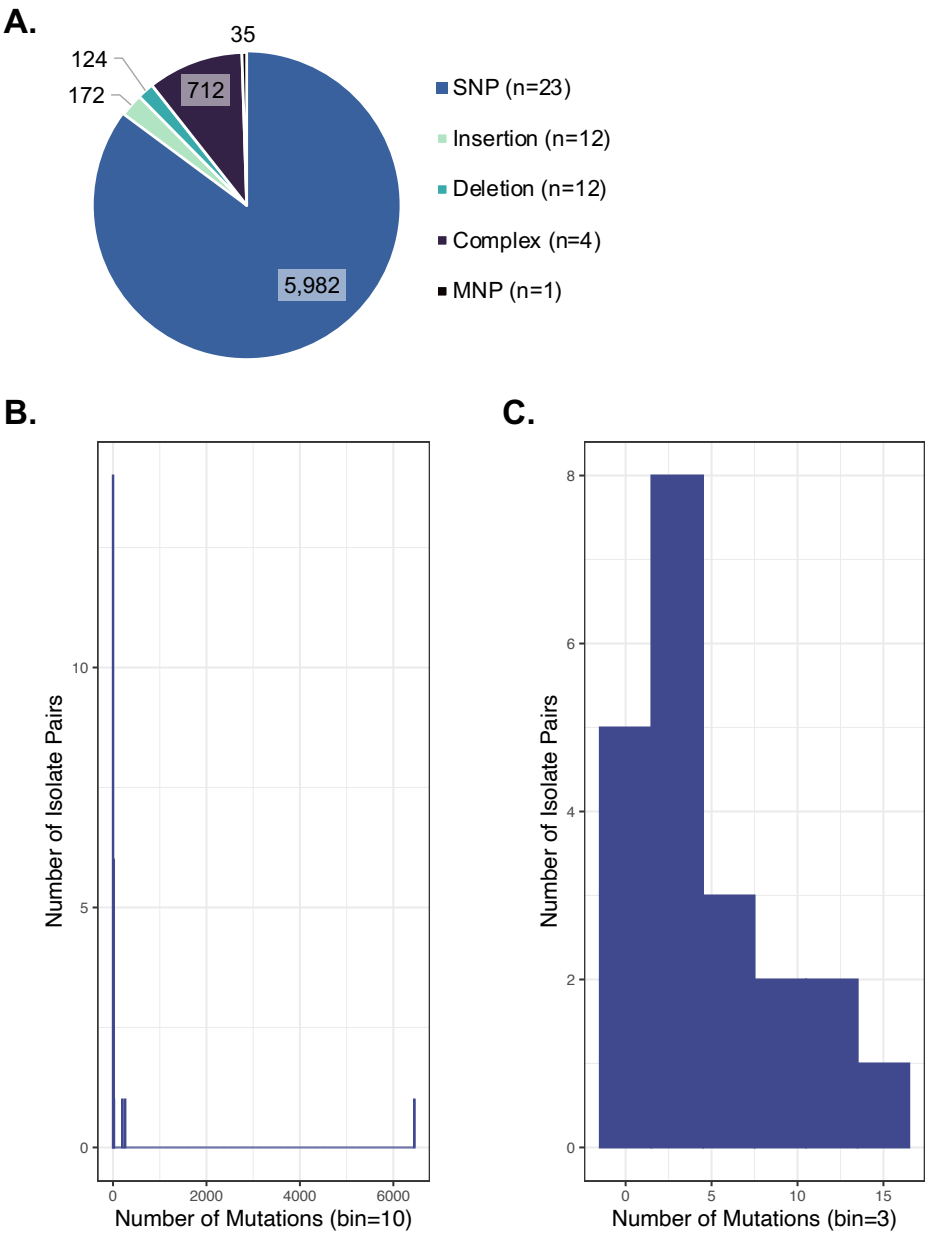

Figure S2.3: Core-genome mutations detected in the persistent-same paired genomes. A) A total of 7,025 mutations were detected using Snippy v4.6.0 (https://github.com/tseemann/snippy) between persistent-same pairs (n=32) including 35 multiple nucleotide polymorphisms (MNP, black), 712 complex mutations (dark purple), 124 deletions (teal), 172 insertions (mint green), and 5,982 single nucleotide polymorphisms (SNPs, blue). The number of isolate pairs that obtained each mutation type is shown in the legend in parentheses for each mutation type. For example, 5,982 SNPs were identified in 23 pairs and 124 insertions were found in 12 pairs. Histograms display the distribution of the number of mutations (x-axis) by number of isolate pairs (y-axis) for B) all persistent-same pairs or C) persistent-same pairs with outliers (n=3) removed. Data is shown for persistent-same isolate pairs that acquired at least 1 mutation (n=24 pairs), isolate pairs that acquired 0 mutations are not represented.

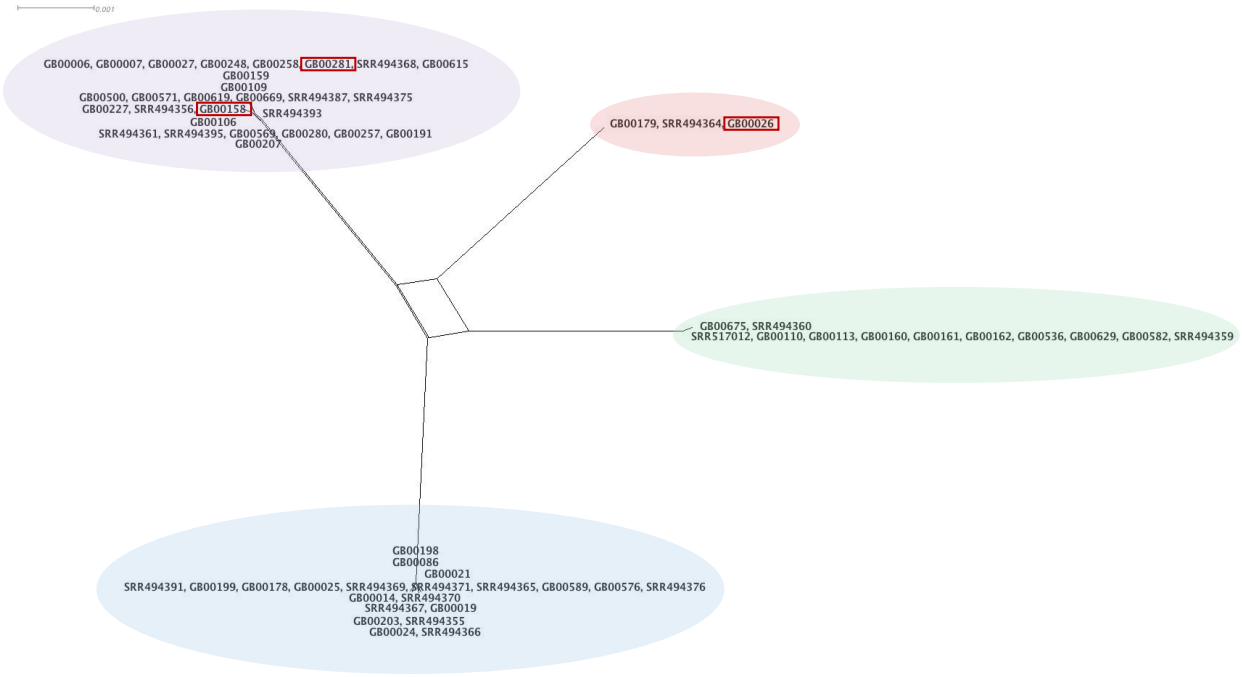

Figure S2.4: Neighbor net tree based across persistent-same isolates based on MMR region alignment. Nucleotide alignment of the MMR region (7,466bp) was performed in MEGA v.11 across 64 persistent -same isolates. A neighbor net tree was constructed using SplitsTree v.4.19.0 based on 103 informative sites which revealed four groups of isolates separated based on recombination events, indicated by parallelograms, that correspond with the four MMR classes identified (class 1 = green, class 2 = blue, class 3 = red, class 4 = purple). Phi test revealed no significant evidence for recombination (p=0.0899). Isolates identified as mutators are boxed in red.

Table S2.5: MMR alignment class types and frequency of identical BLAST matches.

Isolate ID	MMR class	MMR subclass	BLAST Identity Matches
GB00006	4	4A	7
GB00007	4	4A	7
GB00027	4	4A	7
GB00191	4	4A	7
GB00248	4	4A	7
GB00257	4	4A	7
GB00258	4	4A	7
GB00280	4	4A	7
GB00281	4	4A	7
GB00569	4	4A	7
GB00615	4	4A	7
SRR494361	4	4A	7
SRR494368	4	4A	7
SRR494395	4	4A	7
GB00158	4	4B	8
GB00207	4	4B	8
GB00227	4	4B	8
SRR494356	4	4B	8
SRR494393	4	4B	8
GB00021	2	2A	43
GB00025	2	2A	43
GB00086	2	2A	43
GB00178	2	2A	43
GB00198	2	2A	43
GB00199	2	2A	43
GB00576	2	2A	43
GB00589	2	2A	43
SRR494365	2	2A	43
SRR494369	2	2A	43
SRR494371	2	2A	43
SRR494376	2	2A	43
SRR494391	2	2A	43
GB00109	4	4D	0
GB00159	4	4D	0
GB00500	4	4D	0
GB00571	4	4D	0
GB00619	4	4D	0
GB00669	4	4D	0
SRR494375	4	4D	0
SRR494387	4	4D	0
GB00014	2	2B	0
SRR494370	2	2B	0
GB00019	2	2E	0
SRR494367	2	2E	0
GB00024	2	2C	0
SRR494366	2	2C	0
GB00026	3	3A	3
GB00179	3	3A	3
SRR494364	3	3A	3
GB00675	1	1C	0
SRR494360	1	1C	0
GB00110	1	1B	13
GB00113	1	1B	13
GB00160	1	1B	13
GB00161	1	1B	13
GB00162	1	1B	13
GB00536	1	1B	13
GB00582	1	1B	13
GB00629	1	1B	13
SRR494359	1	1B	13
GB00203	2	2D	0
SRR494355	2	2D	0
SRR517012	1	1A	0
GB00106	4	4C	0

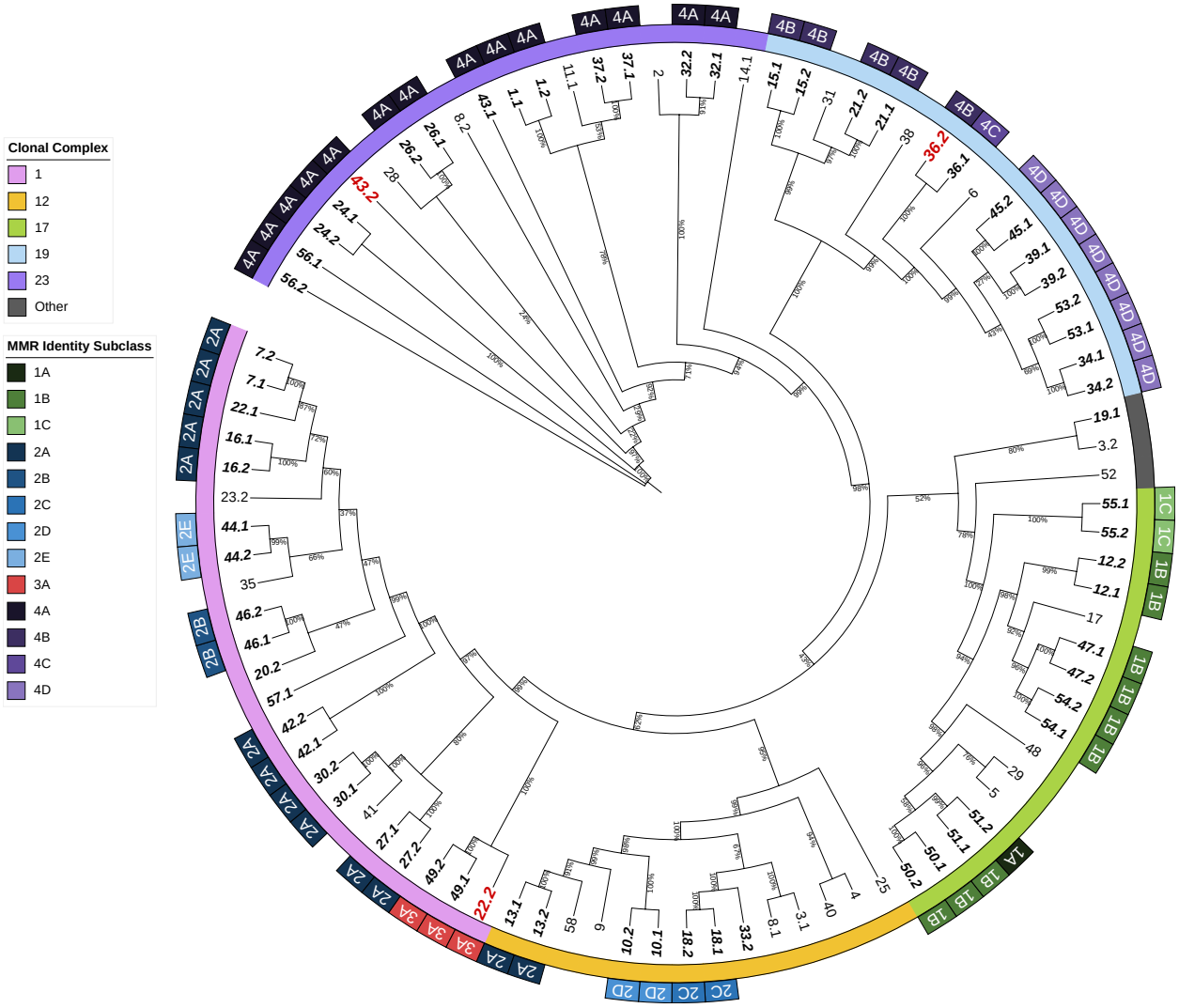

Figure S2.5: MMR class overlay onto core-gene maximum likelihood phylogeny. Isolates (n=92) are labeled by pair ID such that paired isolates (persistent-same or persistent-different) contain a ".1" (prenatal) or ".2" (postpartum) to indicate the sampling timepoint with respect to childbirth and IAP treatment. The persistent-same isolates are designated with bold-italic pairIDs and mutator isolates are in red font. Bootstrap values are noted on the tree branches (0-100%). Isolates' clonal complex (CC) based on MLST are displayed along the inner color strip: CC-1 (pink), CC-12 (yellow), CC-17 (green), CC-19 (blue), CC-23 (purple), and the less predominate CCs (CC-22, CC-26, and a singleton) grouped as "Other" (dark grey). MMR identity subclasses (1A-4D) are displayed along the outer color strip with respective subclass labels for each persistent-same isolate.

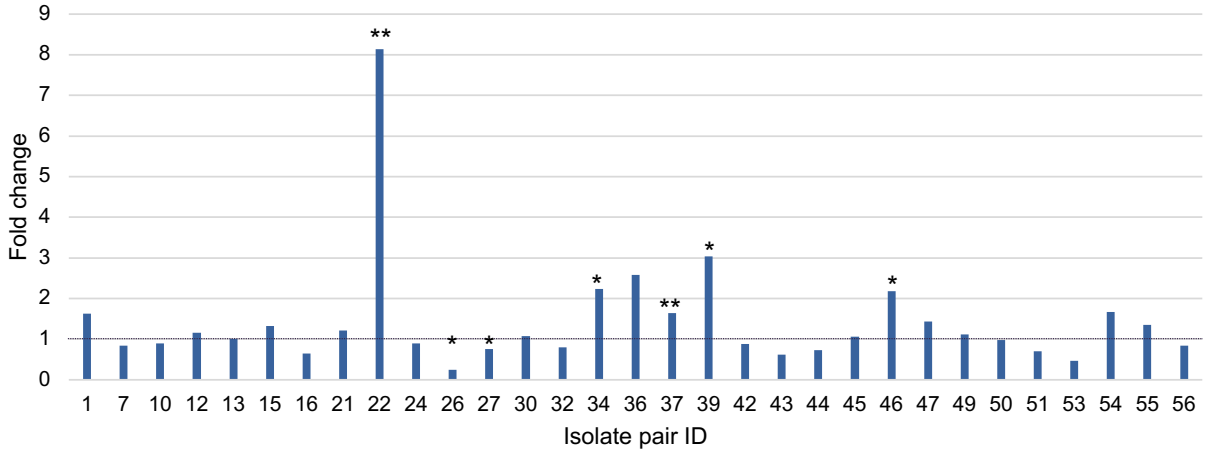

Figure S2.6: Difference in biofilm formation between persistent-same isolate pairs. Biofilm formation was assessed across persistent-same isolate pairs (n=62, 31 pairs) via OD595 absorbance values. Fold change (y-axis) in biofilm formation was calculated for each isolate pair (x-axis) as follows: average absorbance value (n=3 replicates) for the postpartum isolate was divided by that of its respective prenatal isolate. A fold change >1 indicates an increased formation of biofilm in the postpartum isolate with respect to its prenatal isolate, while a fold change <1 indicates decreased biofilm formation within the pair. One-tailed, paired t-tests were calculated to assess significant differences in biofilm formation between isolate pairs (*=P<0.05, **=p<0.01).

CHAPTER 3:
EXAMINING THE IMPACT OF ANTIBIOTICS ON MEMBRANE VESICLE BIOGENESIS IN A HYPERVIRULENT, PERSISTENT GROUP B *STREPTOCOCCUS* ISOLATE

ABSTRACT

Group B *Streptococcus* (GBS) is an important bacterial pathogen during pregnancy, colonizing up to 35% of pregnant people recto-vaginally. If transferred to the fetus during pregnancy, GBS can cause adverse pregnancy outcomes including preterm and stillbirths, as well as severe disease in the neonate following childbirth. These babies often present with sepsis and/or meningitis and therefore, intravenous antibiotics are recommended for GBS-positive pregnant people during labor. Despite this, we have discovered that some GBS isolates are able to rebound and persistently colonize the vaginal tract after antibiotic treatment. However, the mechanism(s) linked to persistence and antibiotic tolerance are poorly understood in GBS. One tolerance strategy employed by bacteria is the production of membrane vesicles (MVs), which have been demonstrated to aid in bacterial survival. To understand the role of GBS MVs in the context of antibiotics and persistence, we exposed a clinical GBS isolate recovered from a pregnant patient with persistent colonization to antibiotics and examined MV production. Through MV isolation and subsequent quantification, microscopy, and proteomics analyses, our data show that antibiotic treatment significantly increases the quantity of MVs produced relative to the untreated control regardless of the antibiotic class (ampicillin; $p=4.2x10^{-6}$, erythromycin; $p=0.01$) and yields MVs with different protein composition compared to the untreated control, with 21 and 19 proteins uniquely identified in the ampicillin- and erythromycin-treated groups, respectively. We observed increased abundances of antibiotic-specific targets in the respective ampicillin and erythromycin MVs, suggesting a mechanism to resist antibiotic-mediated killing. Together, these data suggest that excess quantities of MVs produced in the presence of antibiotics may enhance GBS survival in such conditions. Improving our understanding of GBS MVs and their role in persistent infections will aid in the development of more targeted and effective treatments for GBS disease during pregnancy.

INTRODUCTION

Group B *Streptococcus* (GBS) is an opportunistic bacterial pathogen that can cause disease in pregnant people and their neonates. During pregnancy, vaginal colonization by GBS is transient and typically asymptomatic but can cause severe invasive neonatal disease if transferred to the fetus *in utero*, during birth, or after birth during breastfeeding. The incidence of GBS vaginal colonization varies by geographical region occurring in roughly 11-35% of individuals, which contributes to an estimated 319,000 annual cases of neonatal disease [1,2]. Neonatal GBS disease can present as early onset disease (EOD) within the first week after birth resulting in pneumonia and/or sepsis, or late onset disease (LOD), which occurs within the first week to 3 months after birth, presenting as bacteremia and/or meningitis. Additionally, GBS colonization can lead to other adverse pregnancy outcomes including preterm births, stillbirths, chorioamnionitis, and sepsis of the pregnant person [2–5].

Current prevention strategies for GBS disease include screening for rectovaginal colonization at 35-37 weeks' gestation and administering intrapartum antibiotic prophylaxis (IAP) during labor for GBS-positive individuals. Since its implementation, IAP has successfully reduced cases of EOD but has not been effective in reducing the incidence of other outcomes including LOD, preterm births or stillbirths [6–9]. Moreover, increasing evidence of clindamycin and erythromycin-resistant isolates as well as cases of persistent vaginal colonization up to 6 weeks postpartum, threatens the effectiveness of IAP [10,11].

Classification of GBS through multilocus sequence typing (MLST) has led to the discovery of a hypervirulent genotypic lineage, or sequence type (ST): ST-17 [12,13]. Prior studies have identified ST-17 to be more commonly associated with cases of severe invasive disease compared to other STs and have demonstrated its enhanced ability to colonize and invade host cells, escape antibiotic-mediated killing, and withstand the harsh environment of the phagolysosome [14–16]. ST-17 isolates were also found to be more commonly associated with cases of persistent vaginal colonization even after IAP treatment and increased frequencies of

vertical transmission to infants after birth, compared to other STs, demonstrating its resiliency in the presence of antibiotics [11,17,18].

Membrane vesicles (MVs), produced by both Gram negative and positive bacteria, are membrane-enclosed entities that contain a multitude of components including toxins, virulence factors, nucleic acids, lipoproteins, and enzymes [19,20]. We and others have recently described the production of MVs in GBS [21–23] while demonstrating that they contain key virulence components including those important for attachment and invasion of host cells, reducing oxidative killing, and eliciting pro-inflammatory host responses. We further demonstrated differential production and composition of MVs across GBS genotypes, including the hypervirulent ST-17 lineage, which had significantly high abundances of key virulence proteins C5a peptidase and hyaluronidase [23]. Because this study showed differences among GBS MVs produced under standard broth conditions, we sought to investigate the production and composition of GBS MVs in the presence of antibiotics, an important stressor that GBS commonly encounters via IAP. Several studies have demonstrated that antibiotics can induce the production of MVs [24–32] which have been shown to contribute to biofilm formation and survival of antibiotic stress in other species [27,33–36].

Hence, we sought to examine whether GBS produces MVs with similar functionalities, specifically in the context of antibiotic treatment and persistent colonization. We hypothesize that GBS, like many other bacterial species, produces excess MVs in the presence of antibiotics that contain key proteins critical for survival and persistence. To test this hypothesis, we isolated MVs from an ST-17 GBS strain recovered from the vaginal tract of a patient 6 weeks postpartum following IAP [11,17]. Notably, this ST-17 GBS strain was also isolated from the same patient since the third trimester prenatal screening visit at 35-37 weeks' gestation despite IAP, indicating an ability to escape antibiotic-mediated killing and persistently colonize the host. To elucidate the effect of antibiotic stress on GBS MVs, we quantified MV production and characterized protein composition following exposure to two antibiotic classes, a β-lactam and

macrolide, that are commonly used for IAP. Findings from this work enhance our knowledge of MV biogenesis in a more clinically relevant context that may help improve GBS prevention and treatment methods.

METHODS

Bacterial Growth Conditions and Antibiotic Treatments

Experiments were conducted with GBS strain GB00112 [37], which was isolated from the recto-vaginal swab of a patient at the 6-week postpartum visit after receiving ampicillin for IAP [11]. This isolate was previously characterized as an ST-17, cps III strain, which has been linked to hypervirulence [12,13] and persistent colonization after IAP [17].

For all experiments, GB00112 was cultured in Todd-Hewitt Broth (THB) or on Todd-Hewitt Agar (THA) (BD Diagnostics, Franklin Lakes, New Jersey, USA) and incubated overnight at 37°C with 5% CO_2. Assessment of viable colony forming units (CFUs) was performed by serially diluting samples in 1x Phosphate Buffered Saline (PBS) and plating with the Eddy Jet spiral plater (IUL Instruments, Barcelona, Spain) in logarithmic mode. CFUs were enumerated from plate images in Microsoft® PowerPoint v16.66.1 using the spiral plater counting grid overlay. Absorbance measurements for bacterial growth were taken at an optical density (OD) of 600nm using a spectrophotometer.

Bacterial growth was also measured following treatment with ampicillin or erythromycin, which represent β-lactam and macrolide-lincosamide-streptogramin (MLS) classes of antibiotics, respectively. These antibiotics were selected because of their clinical relevance as they are among the more common classes of antibiotics used for IAP treatment of GBS and their distinct mechanisms of action. The β-lactams target the cell wall and have a bactericidal effect, while MLS antibiotics target protein synthesis and are bacteriostatic.

Membrane Vesicle Isolation and Purification

Isolation of MVs was performed as described previously [23] with the following modifications for antibiotic treatment. Overnight cultures of GB00112 were back-diluted 1:50 and grown to early/mid-log phase (OD_{600} 0.35-0.45). Following centrifugation at 2,000 x g for 10min, the cells were resuspended in 100mL of fresh THB with ampicillin (2.5µg/mL), erythromycin (10µg/mL), or no antibiotics (untreated control) and incubated for 4 hours, which represents the minimum suggested treatment duration for IAP. Aliquots were taken at multiple time points for CFU enumeration, which were performed in triplicate.

Following treatments, the cultures were centrifuged at 2,000 x g for 20min at 10°C to pellet bacterial cells. The supernatant was subjected to further centrifugation at 8,500 x g for 30min at 10°C to pellet out the remaining cellular debris and was filtered (0.22µM) and concentrated using 10kDa Amicon Ultra-15 centrifugal filters with a 10kDa cutoff (Millipore Sigma, Burlington, MA, USA). Concentrated supernatants were kept on ice at 4°C overnight and ultracentrifuged at 150,000 x g for 2 hours at 4°C. Pellets were resuspended in 1x PBS and ultracentrifuged again under the same conditions to wash the pellet. The resulting pellet was resuspended in a final volume of ~100µL of 1x PBS and stored at -80°C until use.

For MV isolation for proteomics analysis, Problock Gold Bacterial Protease Inhibitor Cocktail (GoldBio, St. Louis, MO, USA) was added to the concentrated supernatants to prevent protein degradation overnight. One round of ultracentrifugation was performed, followed by purification of the resuspended samples using qEV1 size exclusion columns (IZON Science, Christchurch, New Zealand) according to the manufacturer's instructions. The resulting fractions were further concentrated using Amicon Ultra-4 (10kDa cutoff) centrifugal filters (Millipore Sigma) to a final volume of ~100-200µL. More protease inhibitor (GoldBio) was added to the concentrated MV fractions, which were also stored at -80°C until further use.

Electron Microscopy of Bacterial Cells and MVs

Scanning Electron Microscopy (SEM) was performed to visualize bacterial cells and extracellular material across treatment groups. Bacterial samples were collected after the 4hr antibiotic treatment period and prepared as described [23], though the resting and washing times were performed for 10min and samples were coated with osmium of ~5nm thickness. Samples were imaged using a JEOL 7500F scanning electron microscope (JEOL Ltd., Tokyo, Japan). Bacterial cells were also visualized via thin-section Transmission Electron Microscopy (TEM) to observe cell integrity across treatment groups. Briefly, samples were collected after the 4hr treatment period, pelleted, and fixed with 2.5% glutaraldehyde solution in PBS. After primary fixation, samples were washed with 0.1M phosphate buffer and postfixed with 1% osmium tetroxide in 0.1M phosphate buffer, dehydrated in a gradient series of acetone and infiltrated and embedded in Spurr. A Power Tome Ultramicrotome (RMC, Boeckeler Instruments. Tucson, AZ) was used to obtain 70nm thin sections which were post stained with uranyl acetate and lead citrate.

TEM was also performed on the MV samples to visualize and confirm the presence of MVs after isolation. Isolated MVs (5uL) from each treatment were fixed with 2.5% glutaraldehyde in PBS and prepped as described previously [23]. Both MV and thin-sectioned bacterial samples were imaged using a JEOL 1400Flash Transmission Electron Microscope (Japan Electron Optics Laboratory, Japan) at an accelerating voltage of 100kV.

MV Quantification

Isolated MVs were quantified via nanoparticle tracking analysis using a NanoSight NS300 (Malvern Panalytical, Westborough, MA, USA) to assess differences in MV production across treatment groups (n=6 replicates) as described [23]. Samples were diluted in PBS (1:1,00-1:10,000) for each replicate and the data were averaged across five technical replicates of nanoparticle tracking videos per biological replicate for each treatment group. Raw output was exported as a .csv file and analyzed in R v4.1.2 [38] using the tidyNano package [39]. MV

counts were normalized to the total volume of the culture used for preparation as well as the final volume used for resuspension. Values that fell outside of the interquartile range x 1.5 were deemed outliers.

MV Proteomics

To assess the protein composition of isolated MVs, purified MV fractions (n=4 biological replicates per treatment) were subjected to proteomics liquid chromatography with tandem mass spectrometry (LC-MS/MS). The Pierce Bicinchoninic Acid (BCA) Assay (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify protein concentrations of MV fractions. The microplate procedure was used with 10µL sample and standard volumes according to manufacturer's instructions and supplemented with 2% SDS in water to reduce excess background signal from lipids. A total of 0.5µg MV protein for each sample was loaded into 4-20% Tris-Glycine SDS-PAGE gels (BioRad, Hercules, CA, USA) and concentrated into single bands that were fixed, stained, and excised as described previously [23,40].

For proteolytic digestion, gel bands were digested in-gel as described with minor modifications [41]. Briefly, gel bands were dehydrated using 100% acetonitrile and incubated with 10mM dithiothreitol in 100mM ammonium bicarbonate (pH~8) at 56^{o}C for 45min, dehydrated again and incubated in the dark with 50mM chloroacetamide in 100mM ammonium bicarbonate for 20min. Gel bands were then washed with ammonium bicarbonate and dehydrated again. Sequencing grade modified trypsin was prepared to 0.005ug/µL in 50mM ammonium bicarbonate and ~100µL was added so that the gel was completely submerged. Following incubation overnight at 37^{o}C, peptides were extracted from the gel by water bath sonication in a solution of 60% Acetonitrile (ACN) /1% Trifluoroacetic acid (TFA) and vacuum dried to ~2µL.

For LC-MS/MS, an injection of 5µL was automatically made using a Thermo EASYnLC 1000 (Thermo Fisher Scientific) onto a Thermo Acclaim PepMap RSLC 0.1mm x 20mm C18 trapping column (Thermo Fisher Scientific) and washed for ~5min with buffer A. Bound peptides

were then eluted over 35min onto a Thermo Acclaim PepMap RSLC 0.075mm x 250mm resolving column (Thermo Fisher Scientific) with a gradient of 5%B to 40%B in 24min, ramping to 90%B at 25min and held at 90%B for the duration of the run (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 80% Acetonitrile/0.1% Formic Acid/19.9% Water) at a constant flow rate of 300nl/min. Column temperature was maintained at a constant temperature of 50^{o}C using an integrated column oven (PRSO-V1, Sonation GmbH, Biberach, Germany). Eluted peptides were sprayed into a ThermoScientific Q-Exactive mass spectrometer (Thermo Fisher Scientific) using a FlexSpray spray ion source. Survey scans were taken in the Orbi trap (35000 resolution, determined at m/z 200) and the top 15 in each scan was subjected to automatic higher energy collision induced dissociation (HCD) with fragment spectra acquired at 17500 resolution.

The resulting MS/MS spectra were converted to peak lists using Mascot Distiller, v2.8.2 (Matrix Science Inc., Boston, MA, USA) and searched against a protein database containing all sequences available for *S. agalactiae*. These sequences were downloaded from Uniprot [42] and appended with common laboratory contaminants (www.thegpm.org, cRAP project) using the Mascot searching algorithm v 2.8.0.1 [43]. The Mascot output was analyzed using Scaffold, v5.1.2 (Proteome Software Inc., Portland, OR, USA) to probabilistically validate protein identifications. Assignments validated using the Scaffold 1%FDR confidence filter were considered true. Mascot parameters for all databases were as follows; allow up to two missed tryptic sites, fixed modification of Carbamidomethyl Cysteine, variable modification of Oxidation of Methionine, peptide tolerance of +/- 10ppm, MS/MS tolerance of 0.02 Da, and FDR calculated using randomized database search.

Data Analysis

All raw data was wrangled, visualized, and statistically analyzed using R v4.1.2 [38] in RStudio. Specifically, data wrangling was performed using devtools v2.4.5 [44], dplyr v1.1.3 [45], purr v1.0.2 [46], readr v2.1.4 [47], tidyr v1.3.0 [48], tidyverse [49]. Data visualization was performed using EnhancedVolcano v1.13.2 [50], ggplot2 [51], ggbeeswarm v0.7.2 [52],

VennDiagram v1.7.3 [53], and viridis v0.6.4 [54] packages. The tidyNano package [39] was specifically used to tidy and analyze nanoparticle tracking analysis data used to quantify MVs as described.

Pairwise Kruskal Wallis tests were performed with the asbio v1.9-6 package [55] to test for statistical differences between treatment groups for growth (OD_{600}), viable CFUs (CFU/mL), and MV protein abundance (spectral counts) using the asbio R package. One-way Anova and Tukey HSD statistical tests were performed to assess differences across treatment groups for the total number of MVs across using the rstatix v0.7.2 package [56]. The vegan v2.6-4 [57] package was used to perform Principal Coordinate Analysis (PCoA) using Euclidean distance metrics on MV protein spectral counts. PermANOVA statistical analysis with 999 permutations was performed on the resulting PCoA distance matrix using vegan::adonis2 and the pairwiseAdonis v0.4.1 [58] package. For all statistical tests, p-values of <0.05 were considered statistically significant.

RESULTS

Viable cells are present after antibiotic treatments despite a reduction in growth

As GB00112 was previously determined to be susceptible to both ampicillin and erythromycin [11], we sought to evaluate bacterial growth after a 4-hour exposure to antibiotic concentrations that are 10 times greater than the reported minimum inhibitory concentration (MIC). The absorbance (OD_{600}) (**Figure 3.1A**) and number of viable colony forming units (CFUs) (**Figure 3.1B**) were measured every hour for a total of 8 and 6 hours, respectively. After 1 hour, both the ampicillin- and erythromycin-treated cultures had significant reductions in CFUs relative to the untreated control. This reduction was less pronounced in the absorbance readings, as the ampicillin treatment significantly reduced growth 1 hour post-treatment, whereas the erythromycin treatment did not result in a significant growth reduction until 3 hours post-treatment.

Despite the reduction in growth, a qualitative assessment of the GBS cultures via SEM identified viable streptococci even after the 4-hour antibiotic treatment. Moreover, evidence of extracellular particles was observed, along with the bacteria cells, in cultures from all three treatment groups (**Figure S3.1**). The ampicillin-treated culture appeared to have a greater presence of extracellular particles compared to the control and erythromycin-treated cultures. It is likely that these extracellular particles visualized with SEM may be membrane vesicles (MVs) based on their association with the bacterial chains and relative size, though additional confirmation was needed.

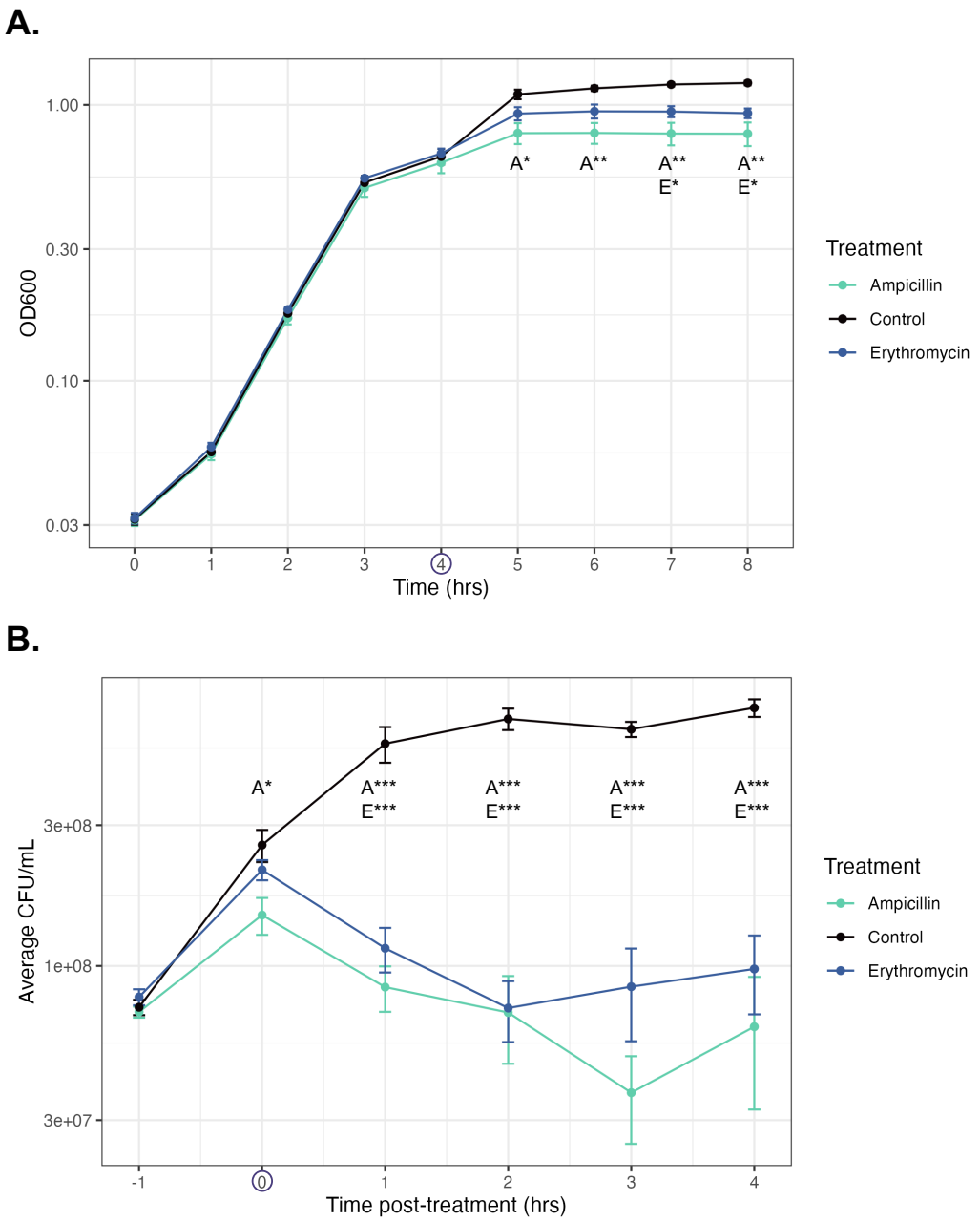

Figure 3.4: Antibiotics impact GBS growth over time. Average A) absorbance (OD_{600}) and B) viable CFUs/ml following treatment with ampicillin (turquoise) or erythromycin (blue) relative to the untreated cells (control; black). Error bars reflect standard error for each timepoint (n=4 biological replicates). Antibiotics were added to cultures after four hours of growth (purple circle on x-axis). A) OD_{600} measurements (y-axis) were taken every hour for 8 hours (x-axis). B) Samples were also diluted and plated for CFUs starting 1 hour before the treatment period (-1); the x-axis displays time relative to treatment with "0" representing the addition of antibiotics. ANOVA and Tukey HSD statistical tests were performed across treatment groups at each timepoint to test for differences relative to the control ("A"=ampicillin, "E"=erythromycin). P-values <0.05, <0.01 and <0.001 were considered significant and are noted by *, **, and ***, respectively.

Production of GBS MVs is significantly increased in response to antibiotic treatments

To validate, MVs were isolated from the bacterial supernatant from the entire culture after the 4-hour treatment period and visualized with TEM. The presence of MVs were confirmed in cultures from all three treatment groups as shown by the presence of dark, electron-dense circles enclosed by a bright, surrounding membrane approximately 100-150nm in size (**Figure 3.2**). Next, we sought to investigate the effect of antibiotic treatment on the quantity of MVs produced using nanoparticle tracking analysis. The size distribution of MVs across treatment groups ranged from 11.5nm to 999.5nm, with modes of 122.5nm, 118.5nm, and 127.5nm, for the ampicillin, control, and erythromycin groups, respectively (**Figure 3.3A**). The quantity of MVs, however, differed across groups (**Figure 3.3B**). When compared to the untreated control, which had an average of $5.6x10^6$ MVs, the antibiotic-treated cultures both had more MVs. An average of $3.7x10^7$ MVs was detected in the ampicillin-treated cultures, whereas $1.95x10^7$ MVs were detected in the erythromycin-treated cultures. Relative to the untreated control group, both the ampicillin- (Tukey's HSD $p=4.2x10^{-6}$) and erythromycin-treated (Tukey's HSD $p=0.01$) cultures had significantly greater quantities of MVs. Additionally, an increased number of MVs was detected in the ampicillin-treated cultures relative to the erythromycin-treated cultures ($p=0.002$).

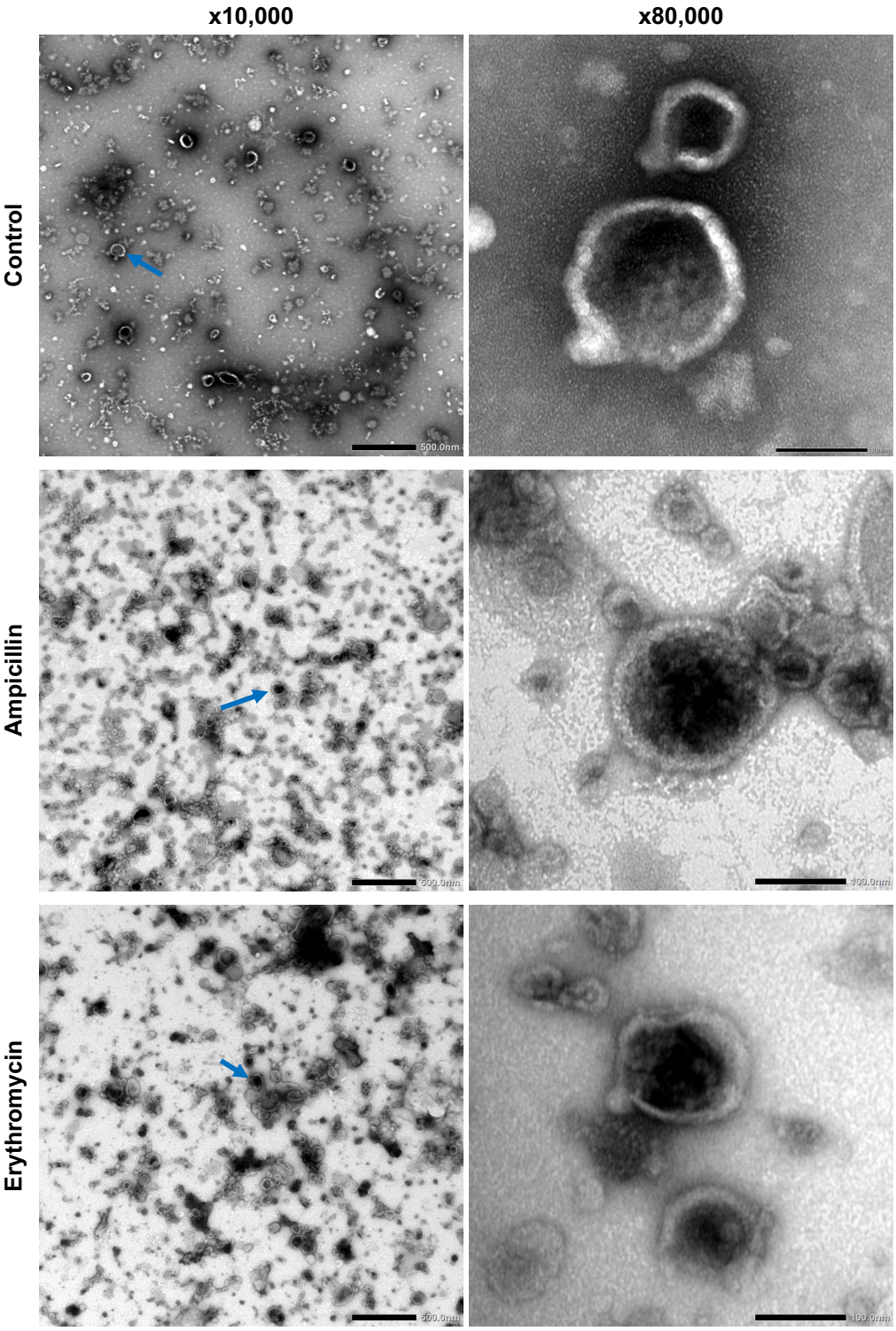

Figure 3.5: Transmission electron microscopy (TEM) images of membrane vesicles (MVs). isolated from GBS cultures without antibiotics (control, top) or following a 4hr treatment with ampicillin (middle) or erythromycin (bottom). Scale bars represent 500nm and 100nm for 10,000x (left) and 80,000x (right) magnifications, respectively. MVs are identified by their spherical shape with a dark, electron-dense center surrounded by a bright membrane. Blue arrows represent an MV in each treatment group.

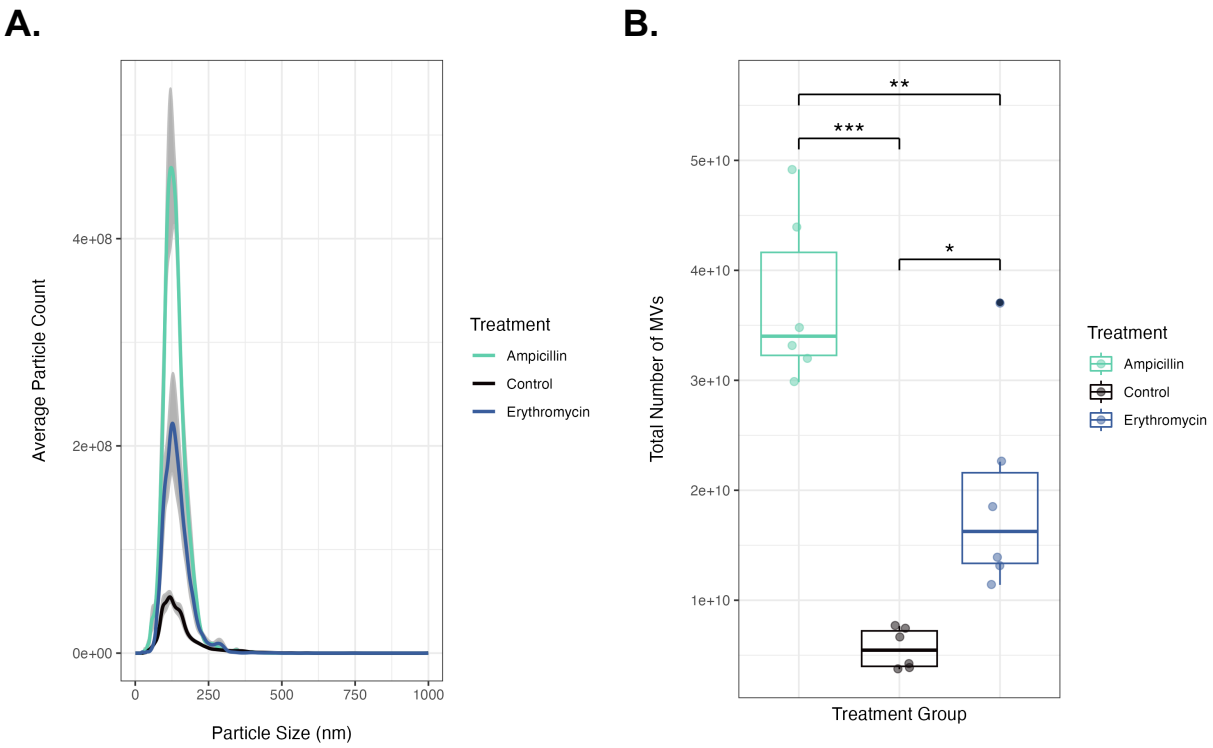

Figure 3.6: Quantification of MVs isolated from GBS cultures with and without antibiotics. A) Distributions of size (x-axis) in nanometers by average count of MVs (n=6 replicates) from each treatment group. Cultures treated with ampicillin (turquoise) or erythromycin (blue) are shown as well as those receiving no antibiotics (control, black). Grey shading reflects the standard error across replicates. B) Boxplots display the summary of the total number of MVs isolated (y-axis) for each treatment group (x-axis). Each circle represents the total number of MVs for each biological replicate (n=6); outlier replicates are displayed as opaque black dots. One-way ANOVA and Tukey HSD tests were used to detect significant differences in the number of MVs produced across treatment groups. P-values <0.05 (*), <0.01 (**), and <0.001 (***) were considered significant.

Evidence of distressed membrane provides insight on mechanism of excess MV production

Because a significant increase in MV production was observed with both antibiotic treatments, thin-section TEM was used to visualize the effect of each antibiotic on the integrity of the bacterial cell. Of note, the TEM images show dark and electron-dense cells, which have been classified as viable cells and were observed in all treatment groups. In general, slightly more non-viable cells, which appear lighter or “empty”, were observed in the antibiotic-treated cells compared to the untreated control cells. In the latter, most of the bacterial cells were

surrounded by an intact and distinct membrane. The ampicillin-treated cells, however, had a less distinct cell membrane with a distressed appearance (**Figure 3.4**). Areas of potential MV formation were also observed in the ampicillin-treated cells, appearing as blebs on the cell surface; these were absent in the untreated cells. Although the erythromycin-treated cells had a more distinct and intact membrane than the ampicillin-treated cells, they still differed in appearance relative to the untreated cells. Interestingly, the cells exposed to erythromycin also appear to have excessive membrane gaps, which may be indicative of partial cell division.

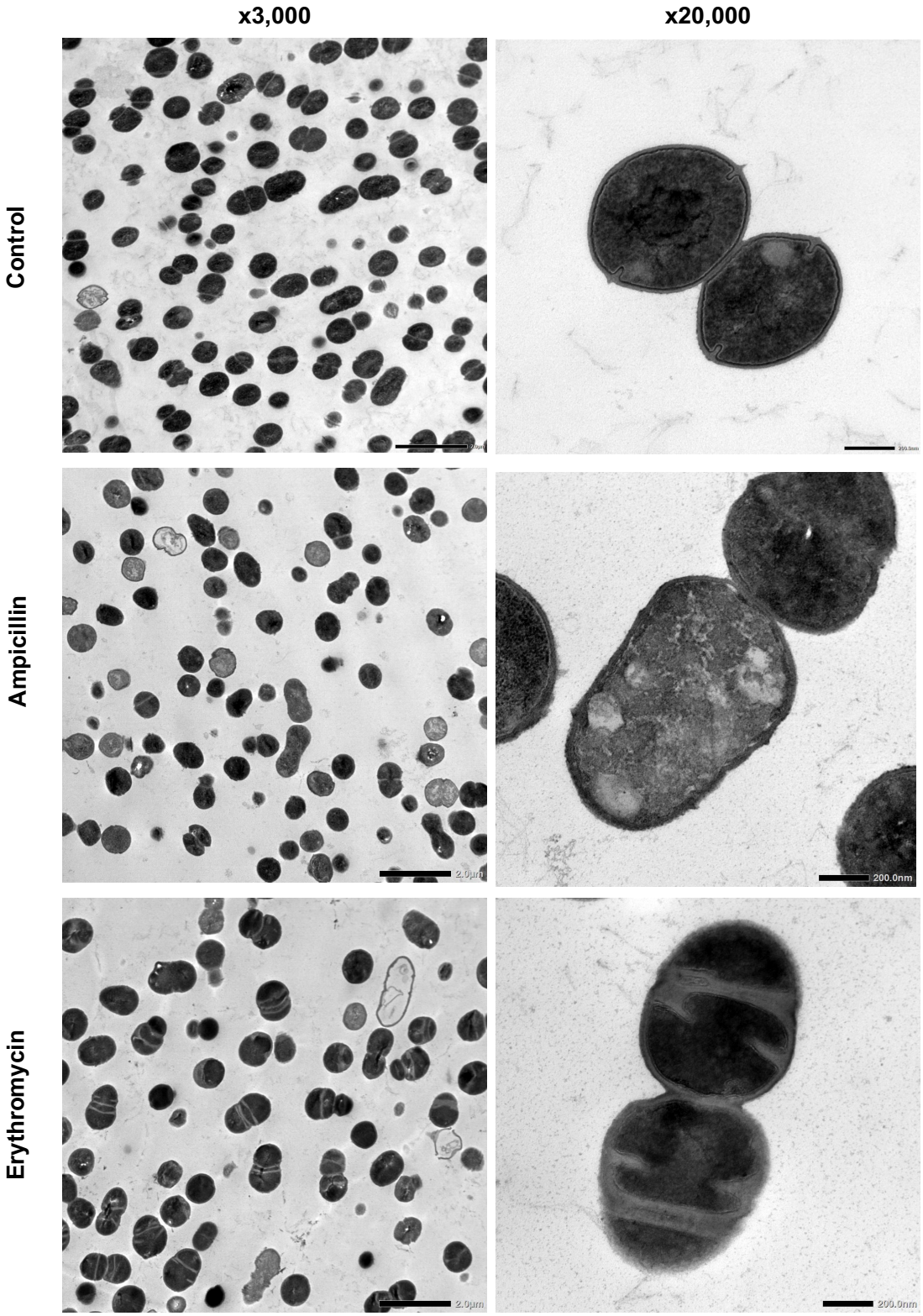

Figure 3.7: Thin section transmission electron microscopy (TEM) images of GBS cells with and without antibiotic treatment. GBS was treated with erythromycin (bottom), ampicillin (middle), or no antibiotic (control, top). Scale bars represent 2.0µm and 200nm for 3,000x (left) and 20,000x (right) magnifications, respectively.

MVs have distinct protein composition across treatment groups

To further understand the effect of antibiotics on GBS MVs, we investigated the protein composition within MVs isolated from each treatment group via LC-MS/MS. Proteomics analysis identified a total of 417 distinct proteins across all three treatment groups, with the controls having the largest number of proteins (n=330), followed by the erythromycin- (n=319) and ampicillin-treated (n=276) groups (**Figure 3.5A**). While nearly half of the proteins detected (n=201, 48.2%) were shared among all treatment groups, 47 MV proteins were shared exclusively between the antibiotic-treated samples but were absent in the untreated controls. Importantly, some MV proteins were unique to the cultures treated with ampicillin (n=21) and erythromycin (n=19), highlighting β-lactam- and MLS-specific effects, respectively. Principal Coordinate Analysis (PCoA) using Euclidean distance revealed distinct clustering of MV proteomes by treatment group (PERMANOVA p=0.001); (**Figure 3.5B**). Significant differences in protein composition were also observed for the following pairwise comparisons: ampicillin vs. control (p=0.031), erythromycin vs. control (p=0.033), and ampicillin vs. erythromycin (p=0.029).

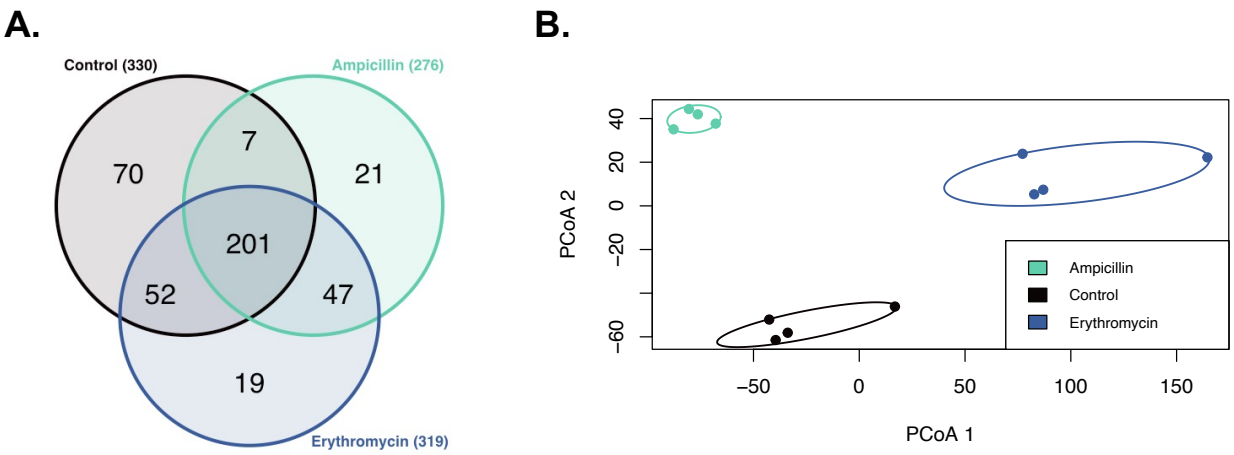

Figure 3.8: MVs have distinct protein composition across treatment groups. A) A Venn Diagram was constructed with the VennDiagram R package to depict the distribution of 417 distinct proteins identified across MVs from ampicillin- (turquoise) and erythromycin- treated (blue) cells as well as the untreated controls (black). The number of proteins identified in MVs from each treatment group is shown in parentheses. A total of 201 proteins was shared across treatment groups, while 21, 19, and 70 proteins were uniquely identified in the ampicillin, erythromycin, and control groups, respectively. B) Principal Coordinate Analysis (PCoA) was performed with Euclidean distance, using the vegan R package, to evaluate dissimilarities in MV protein content across ampicillin (turquoise), control (black), and erythromycin (blue) treatment groups. Individual points represent biological replicates (n=4), while ellipses represent 95% confidence intervals of clustering. Points that are closer together are more similar. PERMANOVA tests with 999 permutations were performed to detect significant differences in MV protein composition between treatment groups. Significant dissimilarity (p=0.001) was observed across all sample and significant differences were detected in the ampicillin vs. control (p=0.031), ampicillin vs. erythromycin (p=0.029), and control vs. erythromycin (p=0.033) comparisons.

Among the 47 proteins shared between MVs from both antibiotic groups (**Table S3.1**), several are well-known virulence proteins including CylA, which is needed for hemolysin production, hyaluronate lyase, an important factor for immune modulation, and DltS, an environmental sensor protein. Most of the proteins that were shared between the antibiotic groups include transport proteins (n=10), cell division proteins (n=4), ribosomal proteins (n=4), transferases (n=5), membrane proteins (n=3), and polysaccharide biosynthesis proteins (n=2). Other shared proteins included a potassium uptake protein, a phosphate hydrolysis protein, cell cycle protein (GpsB), cyclic-di-AMP phosphodiesterase, DegV family protein, deadenylate cyclase, a histidine kinase, DUF2207 domain protein, the MurM protein, penicillin binding

protein 4, GntP family permease, GntR transcriptional regulator, XerS recombinase, YbbR-like protein, and a YkuD domain protein.

The 21 proteins that were unique to MVs recovered from the ampicillin-treated cultures included key virulence and stress proteins such as an antitoxin protein, a Gls24 protein, and a universal stress protein (**Table S3.2**). Others were classified as ABC transporters (n=4), a ribosomal protein, metal transporters (n=2), cell division protein SepF, synthases (n=2), kinases (n=2), a methylenetetrahydrofolate reductase, phosphocarrier protein, polysaccharide biosynthesis protein, R3H domain protein, serine/threonine phosphatase, and a UPF0297 protein. Comparatively, the erythromycin-treated cultures also had unique MV proteins including virulence and stress-associated proteins such as the toxic anion resistance protein, alkaline shock protein, and the signal recognition particle receptor (FtsY). Other proteins unique to erythromycin-treated MVs were ribosomal proteins (n=3), ABC transporters (n=2), chaperonin GroEL, an amino acid antiporter, DUF2154 domain protein, transferases (n=3), glutamine synthetase, L-lactate dehydrogenase, NAD kinase, phosphoenolpyruvate carboxylase, and a sugar uptake protein.

Shared MV proteins have differential expression across treatment groups

To examine the abundance of proteins packaged in MVs, we compared the average spectral counts of proteins shared between ampicillin-treated and control MVs (n=208), erythromycin-treated and control MVs (n=253), and both antibiotics (n=248). Pairwise comparisons of mean spectral counts between treatment groups (i.e., ampicillin vs. control, erythromycin vs. control, and ampicillin vs. erythromycin) to identify significant differences in protein abundance, defined as a >1.5 $\log_2$-fold-change (L_2FC) and p-value <0.05. This analysis revealed 51 significant differences across 50 distinct proteins – one (rplF), of which was significantly upregulated in the erythromycin MVs compared to both ampicillin and control MVs (**Figure 3.6A-C, Table S3.3**). Most of the significantly different comparisons (43%, n=22) were between the ampicillin and control MVs, accounting for 10.6% of the total proteins shared

between these groups. More proteins (n=15) were significantly upregulated (i.e., higher abundances) in the MVs following ampicillin treatment, with L_2FCs ranging from 3.70-1.51 (**Figure 3.6A**). These included key virulence proteins that were detected in all three treatment groups, such as the serine protease, capsule (CpsD), stress response protein Gls24, an endolytic murein transglycosylase, and penicillin binding proteins; Pbp2A, Pbp2B, and Pbp2X. Other proteins including transport, cell division, and regulatory proteins were also more abundant in the ampicillin MVs relative to control MVs.

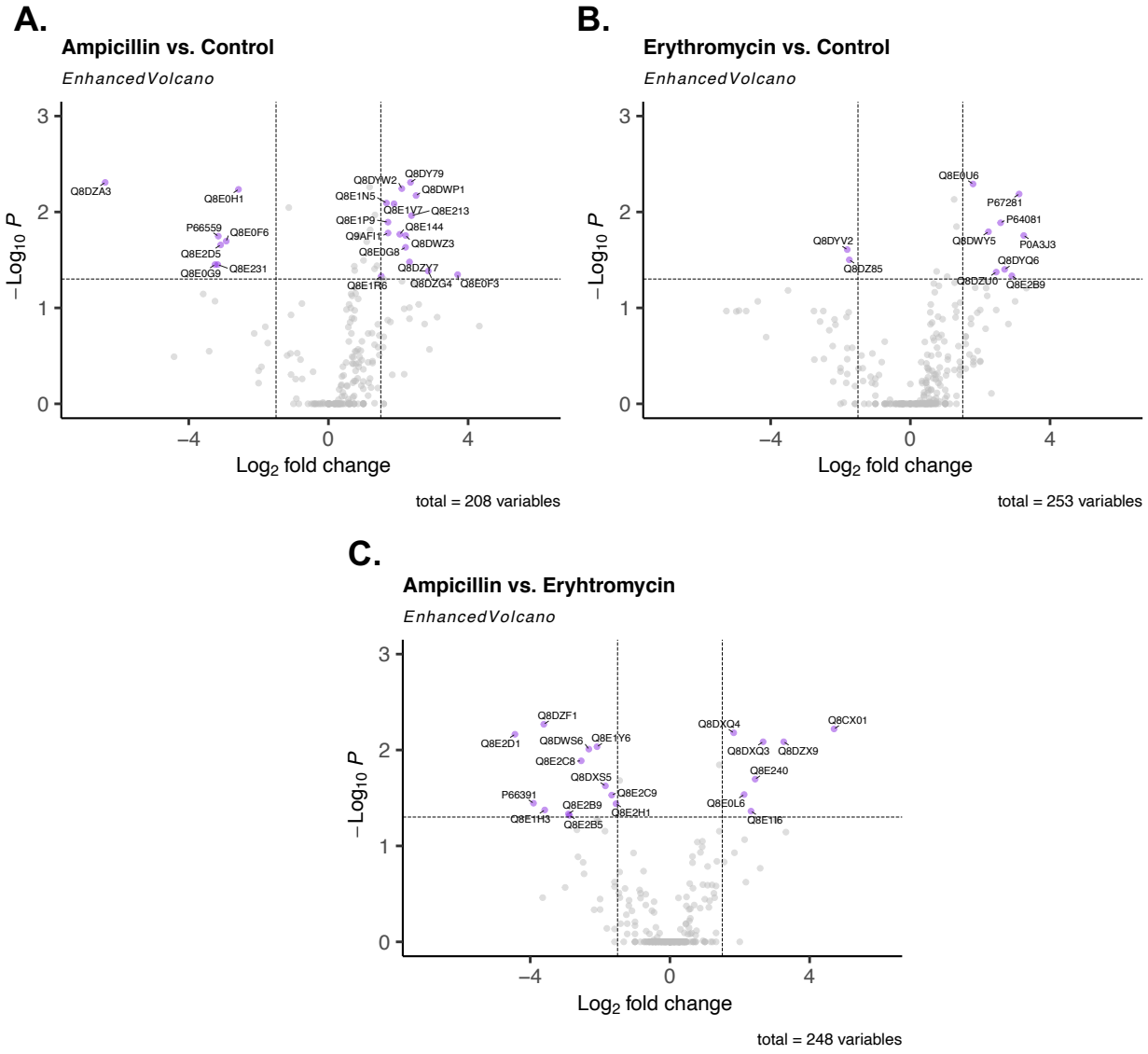

Figure 3.9: Some MV proteins are differentially abundant across treatment groups. Volcano plots display differential abundance of proteins shared between A) ampicillin and control MVs, B) erythromycin and control MVs, and C) ampicillin and erythromycin MVs. Pairwise Kruskal wallis tests were performed to assess differences in protein abundance. Resulting p-values are plotted along the y-axis (-Log_{10} scale) for which a p-value <0.05 was deemed significant. Log_2 fold change values of mean spectral counts (x-axis) were calculated for each comparison relative to the first treatment group listed. For example, the fold change for the ”Ampicillin vs. Control” (A) group was relative to the mean spectral counts of ampicillin MVs such that a Log_2-fold-change >1 represents proteins that are upregulated in ampicillin. A log_2 fold change cutoff of +/-1.5 (vertical dashed lines) and p-values <0.05 (horizontal dashed line) were used to identify proteins of significant interest (purple) that were differentially abundant in MVs between treatment groups, such proteins are labeled with their respective accession numbers on each plot.

By contrast, only 4% (n=10) of the proteins shared between the MVs from the erythromycin-treated and control samples were differentially abundant; most were upregulated in the former (n=8, 80%) with L_2FCs ranging from 3.25-1.79 (**Figure 3.6B**) and included the 50S ribosomal protein (RplF). Chaperone protein DnaK, which is a stress response protein, was also more abundant in the erythromycin MVs relative to control MVs, with the highest L_2FC value (3.25). While DnaK was also more abundant in MVs from the ampicillin-treated relative to control samples, the difference was not significant, as was true for other stress-associated proteins (e.g., CtsA) and a general stress protein (**Figure S3.2B**). In addition, 19 (7.6%) of the shared MV proteins were differentially abundant in the ampicillin- and erythromycin-treated samples (**Figure 3.6C**). Most (n=12, 63%) of these proteins were more abundant in the erythromycin MVs, including a cell-wall associated protein (PcsB), which was identified in MVs from all treatment groups, along with ten ribosomal proteins. The remaining seven (37%) proteins were more abundant in the ampicillin MVs compared to erythromycin MVs and included key proteins linked to virulence (e.g., cAMP factor) and cell wall integrity (e.g, LytR-cpsA-psr domain-containing protein) as well as penicillin-binding protein 1B (**Figure S3.2, 3.7**).

Increased abundance of antibiotic-specific targets highlights a protective role of MVs

Penicillin-binding proteins (PBPs) are not only important for peptidoglycan synthesis but are also the main targets for β-lactam antibiotics. In addition to the four PBPs that were significantly more abundant following ampicillin treatment, Pbp1A, another penicillin-binding protein, and FibA, a β-lactam resistance factor, were highly abundant in MVs (average = 22.42 spectral counts) from all three treatment groups (**Figure 3.7A**). Despite the observable differences in Pbp1A abundance between treatment groups, however, it was not significant. Although FibA was much less abundant overall (average = 3 spectral counts), it was similar across the three treatment groups. Of note, a sixth penicillin-binding protein, Pbp4, was identified in MVs from both antibiotic groups, but not in the control MVs. Increased abundance

of these PBPs along with the presence of other key cell-wall associated proteins demonstrate the protective potential of MVs against cell-wall targeting antibiotics, like ampicillin.

In contrast, erythromycin acts to inhibit protein synthesis by targeting the 50S ribosomal subunit. Indeed, a total of 22 50S ribosomal-related proteins were identified in MVs from all sample groups. Two of these proteins, RplX and RpmL, were exclusively observed in erythromycin MVs. Of those that were identified in MVs from all three treatment groups (n=12), five were significantly more abundant in erythromycin MVs relative to the control and/or ampicillin MVs (**Figure 3.7B**). Similar to the PBPs in the ampicillin MVs, the abundance of 50S ribosomal proteins in the erythromycin treatment group illustrates a protective function of MVs against antibiotics like the macrolides, which inhibit protein translation.

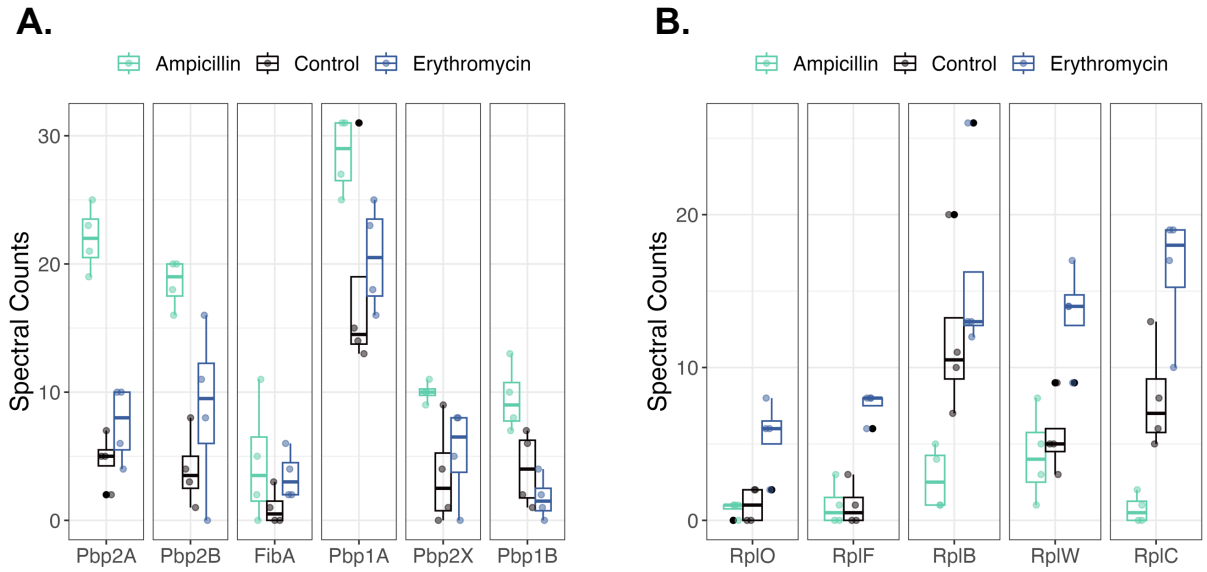

Figure 3.10: Differential abundance of A) β-lactam and B) Macrolide target proteins identified in MVs across ampicillin (turquoise), control (black) and erythromycin (blue) GBS treatment groups. Boxplots represent the summary of spectral counts (y-axis) across biological replicates (n=4) for each treatment group denoted by different colors, for each protein of interest (x-axis). Penicillin-binding proteins (PBPs) and beta-lactamase resistance factor FibA, are presented in panel A while 50S ribosomal proteins are presented in panel B. Pairwise Kruskal Wallis tests of mean spectral counts were performed to identify significant differences in MV protein abundances across treatment groups; p-values <0.05 (*) and <0.01 (**) were considered significant.

DISCUSSION

All prior studies of GBS MVs, including our previous work, have been conducted using standard broth conditions and have demonstrated roles of MVs in virulence through association with host cells and eliciting pro-inflammatory responses, as well as bacterial protection by reducing oxidative killing [21–23]. Consequently, we aimed to build upon this work by investigating MV biogenesis in the presence of antibiotics, important bacterial stressors that are commonly used to eradicate GBS from the genitourinary tract during pregnancy. Although antibiotics are the current treatment and prevention method for GBS neonatal disease and adverse pregnancy outcomes, evidence of antibiotic resistance and persistent GBS colonization threatens their effectiveness [10,11,17]. GB00112, belonging to the hypervirulent, ST-17 lineage, is one such isolate that persistently colonized the vaginal tract of a pregnant person despite IAP intervention. We have previously demonstrated that this isolate can escape antibiotic-mediated killing and produces MVs in the absence of antibiotics that contain a higher abundance of virulence factors compared to other GBS strains [15,23]. Through the experiments presented herein, we demonstrated differential production of MVs following exposure to two antibiotics commonly used for IAP. Importantly, these antibiotic-induced MVs varied in protein content that could impact virulence or protection from antibiotic-mediated killing.

Several studies in other bacterial species have demonstrated that MV production is associated with stressful conditions [25,28,59,60], including antibiotic stress [27,61]. The significant increase in MV production observed in both antibiotic-treated cultures suggest that antibiotic stress, regardless of the antibiotic type, affects MV production as well. In concordance with our findings, other studies have specifically reported that antibiotic exposure increases MV production across Gram-positive and Gram-negative species [24,25,29,31,32,62]. As GB00112 is susceptible to both ampicillin and erythromycin, cell death may, in part, account for the significant increase in MV production observed following antibiotic exposure. Indeed, we

observed a significant reduction in bacterial growth and viable CFUs in both antibiotic treatment groups relative to the untreated control (**Figure 3.1**). Although it has been reported that MV production requires metabolically active bacterial cells [63], weakening and lysis of the cell wall is thought to be a mechanism of MV release through the thick peptidoglycan layer in Gram-positive species [20,64,65]. The fact that ampicillin acts to thin and destroy the bacterial cell wall, is likely the reason for such a significant increase in the number of MVs released in the ampicillin-treated group even compared to the erythromycin-treated group. Distressed cell walls are evident in the thin-section TEM images of the ampicillin-treated GBS, as well as the presence of completely lysed cells resulting in non-viable "ghost cells" (**Figure 3.4**). A previous study observed similar damage to the peptidoglycan in *Bacillus subtilis* resulting in non-viable "ghost cells" along with an increase in MV production [26]. Moreover, many cell-wall associated proteins, including MltG and LytR-cpsA-psr (LCP), were significantly upregulated in the ampicillin MVs compared to MVs from the untreated and erythromycin-treated samples, respectively (**Figure S3.2C**). MltG, an endolytic murein transglycosylase important for cell wall remodeling and peptidoglycan metabolism [66], has been shown to mediate antibiotic resistance as well as virulence in *Streptococcus mutans* [67]. LCP family proteins are important for cell division, particularly cell envelope maintenance, and when depleted, contribute to decreased cell wall integrity and increased susceptibility to β-lactam antibiotics [68–70]. Similar investigations in other species provide evidence linking cell-wall weakening and MV release with β-lactam exposure [27,71]. Altogether these findings strongly suggest that ampicillin treatment of GBS releases excess MVs due to disruptions in the cell wall. Additional confirmation, however, is needed to determine this proposed mechanism and whether the ampicillin increases the formation of MVs, facilitates the release of more MVs, or both.

As erythromycin is not a cell-wall targeting antibiotic, the observed increase in MV production in erythromycin-treated GBS relative to control is unclear. Evidence of cell lysis and

ghost cells were observed in thin-section TEMs (**Figure 3.4**) of the erythromycin-treated GBS, which may explain some of the release. These were also observed to some extent in the control, however, and does not fully justify the significant increase in MVs from erythromycin-treated GBS. As a bacteriostatic antibiotic, another possible explanation for increased MV release from erythromycin treatment could be at cell division septa where cell growth was incomplete or stalled. The unique cell wall pattern observed in the thin-section TEMs of the erythromycin-treated GBS suggests abnormal cell division (**Figure 3.4**). Support for this suggestion comes from a study of *Salmonella*, which demonstrated MV release from cell division septa and showed that these MVs were larger in size and contained different proteins compared to MVs released along the cell body [72]. Intriguingly, we identified several cell division proteins in the MVs from all three treatment groups, some of which were highly abundant in both the ampicillin and erythromycin MVs (**Figure S3.2D**). The presence of larger particles was also located along streptococcal chains in the SEM images of erythromycin-treated GBS (**Figure S3.1**) that may be indicative of MV release from cell division septa. More comprehensive microscopy analyses across samples exposed at different timepoints and growth phases are needed to confirm this finding.

The presence of stress-related proteins observed in MVs also proposes their role in the GBS response to stress. In order to persist under stressful conditions, bacteria can induce the stringent response, which is designed to slow growth and limit non-essential processes [73–75]. MV production has been linked to stress responses in other species [25,59] and may contribute to the transition of this survival state. Indeed, we identified DltS, an environment signaling molecule [76] in only the antibiotic MVs, and found other stress-associated proteins including DnaK [77], Gls24 [78], carbon starvation protein CstA, and a “general stress protein” to be more abundant in antibiotic-induced MVs compared to control MVs (**Figure S3.2B**). Additionally, MVs may facilitate a stringent response by expelling non-essential factors like virulence-associated proteins. We identified virulence factors in MVs that were also observed in other studies of GBS

MVs under standard broth conditions [21,23], including CAMP factor and hyaluronate lyase that were upregulated (**Figure S3.2A**) or observed exclusively in the MVs from antibiotic-treated groups (**Table S3.1**). While antibiotic stress may trigger the export of non-essential components through MVs to enter a dormant survival state, antibiotic-treated GBS producing MVs with high abundances of virulence factors also demonstrates that these MVs are even more cytotoxic to host cells compared to those produced in the absence of stress, and on their own, can cause damage to host cells, as has been observed in other species [21,28]. Measuring protein content in MVs following exposure to host tissues or models that mimic the host environment are needed to determine how protein content differs. These factors may be important for survival in addition to virulence as some studies have demonstrated a lack of virulence activity for CAMP and alternative roles for hyaluronate lyases [79,80]. Due to their role in survival, these factors may inadvertently affect virulence in this context.

Importantly, studies have shown that MVs can mediate resistance to antibiotics by 1) acting as decoys by carrying antibiotic targets away from the bacterial cell [27,36,81,82], 2) transporting antibiotics out of the cell [83], 3) carrying and delivering antibiotic-degrading enzymes [84–86], and 4) disseminating antibiotic resistance genes from antibiotic-resistant strains [87]. Based on our observations that both ampicillin- and erythromycin-targeting molecules were found in MVs from antibiotic-treated GBS, we suspect that these MVs can facilitate protection from antibiotic-mediated killing via the decoy strategy. Indeed, we identified six PBPs, which are known targets of β-lactam antibiotics like ampicillin. Five of these PBPs were most abundant in the MVs from the ampicillin-treated cells and four were significantly more abundant relative to control or erythromycin MVs (**Figure 3.7A**). Additionally, one PBP, Pbp4, was exclusively identified in MVs from antibiotic-treated MVs. Similarly, we identified 21 50S ribosomal proteins packaged in the GBS MVs that are known targets of erythromycin. Two of these ribosomal proteins were only found in the erythromycin MVs, and among those that were shared in MVs from all groups (n=5), they were most abundant in MVs from erythromycin-

treated GBS (**Figure 3.7B**). These findings, along with similar observations in other studies, demonstrate that GBS MVs may serve as a vehicle for exporting antibiotic targets outside and away from the bacterial cell, promoting escape of antibiotic-mediated killing. Other studies have shown that MVs facilitate antibiotic resistance through disseminating antibiotic resistance factors to neighboring bacteria [34,35]. We identified FibA in all MVs, which is classified as a β-lactam resistance factor as it is involved in peptidoglycan crosslinking and mediates resistance via PBP alteration [88]. The overall abundance of FibA was relatively low, however, and not significantly different across treatment groups (**Figure 3.7A**). Together these findings suggest that antibiotic-induced MVs enhance GBS survival of antibiotic stress by serving as decoys, such that the antibiotics will bind to the targets carried by the MVs rather than the bacterial cell.

An important caveat in assessing protein composition and abundance in these MVs is that a given sample may not be representative of the entire MV population, and we expect there to be variation across strains. The higher number of MVs produced by the ampicillin-treated GBS may explain some of the increases in protein abundance observed in these MVs compared to the control and erythromycin groups. To limit this confounding variable, however, we normalized the total amount of protein across the three treatment groups; hence, it is likely that these differential abundances are comparable but may be underrepresented across groups. It is also worth noting that our assessment of GBS MV outputs after four hours of exposure to very high concentrations of antibiotics likely overestimates the effect, relative to what is occurring *in vivo* and during IAP. Current IAP recommendations for GBS-positive pregnant people consist of administering antibiotics intravenously for a minimum treatment duration of four hours in order to reach antibiotic levels in the amniotic fluid, cord and neonatal blood that are above the MIC [9,89]. Nonetheless, in many cases, it is not feasible to complete the four hours of treatment due to variable laboring times. This, in addition to fact that the concentration of GBS in the vaginal tract is highly variable, makes it challenging to mimic this effect *in vitro*. Therefore, the treatment methods used in this study serve as a starting point for investigating

GBS MVs in a more clinically relevant context. As this is the first study to examine GBS MVs following antibiotic exposures, more work is needed to investigate this effect across a wider range of antibiotic concentrations and treatment durations.

In summary, findings from this work have enhanced our understanding of GBS MVs in a more clinically relevant context. We have demonstrated that antibiotic treatment of GBS not only induces excess production of MVs but results in MVs that carry key proteins to promote bacterial survival in the presence of antibiotics. Furthermore, we identified proteins that were unique to MVs from specific antibiotic treatments as well as those that were shared but differentially abundant across MVs from all three treatment groups. Such findings highlight that MV production and composition in GBS is distinctive to antibiotic exposure as well as the specific type of antibiotic, thus suggesting that GBS MVs are responsive to and reflective of surrounding environmental factors. Importantly, the abundant presence of antibiotic targets, PBPs and 50S ribosomal proteins, in MVs from the ampicillin and erythromycin treated groups, respectively, suggests that MVs can mediate bacterial escape of antibiotic-mediated killing. These results provide support for the hypothesis that MVs contribute to the ability of hypervirulent strains, like GB00112, to persist in the host despite antibiotic treatment. As the first study to explore GBS MVs in the presence of antibiotics, we have only scratched the surface of understanding the impact of MVs in this context, yet our findings highlight important clinical implications. Further work is needed to fully understand the mechanism behind MV production in the context of antibiotics to unravel the full extent to which MVs threaten the effectiveness of current GBS treatment and prevention methods.

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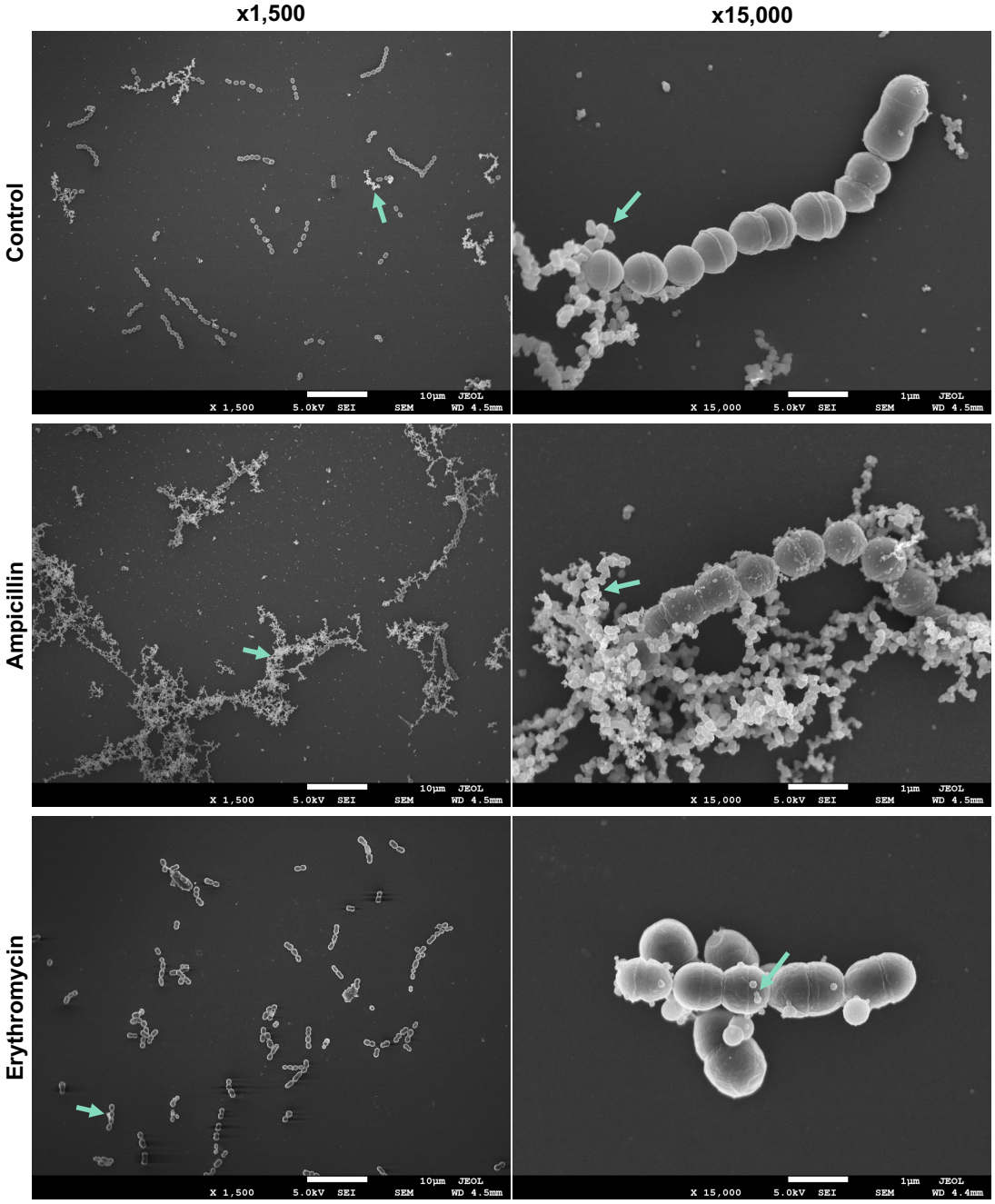

Figure S3.7: Scanning electron microscopy (SEM) images of GB00112 after antibiotic treatment. GB00112 cultures collected after treatment with no antibiotics (control, top), ampicillin (middle), or erythromycin (bottom) were imaged. Scale bars represent 10μm and 1μm for 1,500x (left) and 15,000x (right) magnifications, respectively. Chains of streptococci are identified as GBS bacteria while the smaller extracellular particles (turquoise arrows) are hypothesized to be membrane vesicles.

Table S3.6: MV proteins identified in both ampicillin and erythromycin treatment groups.

Accession Number	Protein Description
Q8E1Z9	30S ribosomal protein S15 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpsO PE=3 SV=1
Q8DXM8	5-bromo-4-chloroindolyl phosphate hydrolysis protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1822 PE=4 SV=1
Q8E2C6	50S ribosomal protein L16 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rplP PE=3 SV=1
Q8E2C7	50S ribosomal protein L22 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rplV PE=3 SV=1
Q8DYU0	50S ribosomal protein L35 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpmI PE=3 SV=1
Q8DYY5	ABC transporter, ATP-binding/permease protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1337 PE=4 SV=1
Q8DYY4	ABC transporter, ATP-binding/permease protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1338 PE=4 SV=1
Q8DWT3	Amino acid ABC transporter, amino acid-binding protein/permease protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2130 PE=3 SV=1
Q8DZZ5	Amino acid ABC transporter, permease protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0947 PE=3 SV=1
Q8DYA1	Amino acid permease, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1589 PE=4 SV=1
Q8E1F7	Biotin transporter OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0398 PE=3 SV=1
Q8E1Q1	Cell cycle protein GpsB OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=gpsB PE=3 SV=1
Q8E185	Cell division protein DivIB OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=divIB PE=3 SV=1
Q8E184	Cell division protein FtsA OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=ftsA PE=3 SV=1
Q8E183	Cell division protein FtsZ OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=ftsZ PE=3 SV=1
Q8CX14	Cell division protein, FtsW/RodA/SpoVE family OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=ftsW PE=4 SV=1
Q8DWS2	Cyclic-di-AMP phosphodiesterase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2141 PE=3 SV=1
Q8E0Q9	CylA protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=cylA PE=4 SV=1
Q8E160	DegV family protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0502 PE=4 SV=1
Q8E051	Diadenylate cyclase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=dacA PE=3 SV=1
Q8E261	DUF2207 domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0137 PE=4 SV=1
Q8DYR4	Glycosyl transferase, group 1 family protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1410 PE=4 SV=1
Q8DYR3	Glycosyl transferase, group 2 family protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1411 PE=4 SV=1
Q8DYQ1	Glycosyl transferase, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1423 PE=4 SV=1
Q8E0L0	Histidine kinase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0720 PE=4 SV=1
Q8E0P9	Hyaluronate lyase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0677 PE=4 SV=1
Q8DYT1	Iron compound ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1392 PE=4 SV=1
Q8DYF2	Manganese ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1532 PE=4 SV=1
Q8DZB5	Membrane protein, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1194 PE=3 SV=1
Q8DYR1	Membrane protein, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1413 PE=4 SV=1
Q8DWX4	Membrane protein, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2087 PE=4 SV=1
Q8E152	MurM protein, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0510 PE=3 SV=1
Q8E252	Penicillin-binding protein 4, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0146 PE=3 SV=1
Q8E0E0	Permease, GntP family OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0794 PE=4 SV=1
Q8DZV5	Phosphate transport system permease protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0991 PE=3 SV=1
Q8DZV9	Phosphate-specific transport system accessory protein PhoU OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=phoU PE=3 SV=1
Q8DXD6	Phosphatidate cytidylyltransferase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=cdsA PE=3 SV=1
Q8DZE1	Polysaccharide biosynthesis protein CpsK(V) OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=cpsK PE=4 SV=1
Q8DYT3	Polysaccharide biosynthesis protein, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1390 PE=4 SV=1
Q8DY62	Potassium uptake protein, Trk family, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1631 PE=4 SV=1
Q8DXQ8	Sensor protein DltS OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=dltS PE=3 SV=1
Q8E003	Transcriptional regulator, GntR family OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0938 PE=4 SV=1
Q8E0C7	tRNA 5-hydroxyuridine methyltransferase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=trmR PE=3 SV=1
P67633	Tyrosine recombinase XerS OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=xerS PE=3 SV=1
Q8E050	YbbR-like protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0886 PE=4 SV=1
Q8DZT3	YkuD domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1014 PE=4 SV=1
Q8E243	Zinc ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0155 PE=4 SV=1

Table S3.7: Proteins uniquely identified in MVs from each treatment group.

Accession Number	Protein Description	Treatment Group*
P66443	30S ribosomal protein S16 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpsP PE=3 SV=1	Ampiicillin
Q8E0P0	ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0688 PE=3 SV=1	Ampiicillin
Q8DWP3	ABC transporter, permease protein, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2172 PE=4 SV=1	Ampiicillin
Q8DWT4	Amino acid ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2129 PE=4 SV=1	Ampiicillin
Q8E0L4	Amino acid ABC transporter, permease protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0716 PE=3 SV=1	Ampiicillin
Q8DX48	Antitoxin OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2009 PE=3 SV=1	Ampiicillin
Q8E181	Cell division protein SepF OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=sepF PE=3 SV=1	Ampiicillin
Q8E1M0	Cysteine synthase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=cysK PE=3 SV=1	Ampiicillin
Q8DZG6	Gls24 protein, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1135 PE=3 SV=1	Ampiicillin
Q8DYZ5	Histidine kinase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1327 PE=4 SV=1	Ampiicillin
Q8DYS9	Iron compound ABC transporter, permease protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1394 PE=3 SV=1	Ampiicillin
Q8E052	Lipid II isoglutaminyl synthase (glutamine-hydrolyzing) subunit MurT OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=murT PE=3 SV=1	Ampiicillin
Q8E060	Magnesium transporter, CorA family OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0875 PE=3 SV=1	Ampiicillin
Q8DX11	Methylenetetrahydrofolate reductase (NAD(P)H) OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2048 PE=4 SV=1	Ampiicillin
Q8E0B3	Phosphocarrier protein HPr OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=ptsH PE=3 SV=1	Ampiicillin
Q8DYR2	Polysaccharide biosynthesis protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1412 PE=4 SV=1	Ampiicillin
Q8E1E6	R3H domain protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0410 PE=4 SV=1	Ampiicillin
Q8E1N6	Serine/threonine phosphatase, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0318 PE=4 SV=1	Ampiicillin
Q8DYB3	Thymidylate kinase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=tmk PE=3 SV=1	Ampiicillin
Q8DY17	Universal stress protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1677 PE=3 SV=1	Ampiicillin
Q8DWX0	UPF0297 protein SAG2091 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2091 PE=3 SV=1	Ampiicillin
P66500	30S ribosomal protein S19 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpsS PE=3 SV=1	Erythromycin
Q8E2C3	50S ribosomal protein L24 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rplX PE=3 SV=1	Erythromycin
Q8E268	50S ribosomal protein L28 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpmB PE=3 SV=1	Erythromycin
Q8DZC2	ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1187 PE=4 SV=1	Erythromycin
Q8E1A0	ABC transporter, ATP-binding/permease protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0461 PE=4 SV=1	Erythromycin
Q8DY00	Alkaline shock protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1694 PE=3 SV=1	Erythromycin
Q8DWP9	Arginine/ornithine antiporter OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=arcD PE=3 SV=1	Erythromycin
Q8CX00	Chaperonin GroEL OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=groEL PE=3 SV=1	Erythromycin
Q8E1N4	DUF2154 domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0320 PE=4 SV=1	Erythromycin
Q8DXT3	Glutamine synthetase I alpha OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=glnA PE=3 SV=1	Erythromycin
Q8DYQ2	Glycosyl transferase, group 2 family protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1422 PE=4 SV=1	Erythromycin
Q8DZY3	L-lactate dehydrogenase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=ldh PE=3 SV=1	Erythromycin
Q8DZK7	NAD kinase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=nadK PE=3 SV=1	Erythromycin
Q8DZK9	Phosphate acetyltransferase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1092 PE=3 SV=1	Erythromycin
Q8E0H2	Phosphoenolpyruvate carboxylase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=ppc PE=3 SV=1	Erythromycin
Q8DWQ7	Putative sugar uptake protein SAG2157 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2157 PE=3 SV=2	Erythromycin
Q8E1F9	Queuine tRNA-ribosyltransferase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=tgt PE=3 SV=1	Erythromycin
Q8E0K4	Signal recognition particle receptor FtsY OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=ftsY PE=3 SV=1	Erythromycin
Q8DXM7	Toxic anion resistance protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1823 PE=3 SV=1	Erythromycin
Q8E1K6	3-oxoacyl-[acyl-carrier-protein] reductase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=fabG PE=3 SV=1	Control
Q8DYY9	5'-nucleotidase family protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1333 PE=3 SV=1	Control
Q8E188	50S ribosomal subunit assembly factor BipA OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=bipA PE=3 SV=1	Control
Q8DWP4	ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2171 PE=4 SV=1	Control
Q8E1H1	Abhydrolase_3 domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0383 PE=4 SV=1	Control
P65886	Adenylosuccinate synthetase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=purA PE=3 SV=1	Control
Q8DYK6	Amidase family protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1474 PE=4 SV=1	Control
Q8E2G9	Aminotransferase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0019 PE=3 SV=1	Control
Q8DWW0	Arginine repressor OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=argR PE=3 SV=1	Control
Q8DWV5	Aspartate--tRNA ligase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=aspS PE=3 SV=1	Control
Q8DY26	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=gatB PE=3 SV=1	Control
Q8E174	ATP-dependent Clp protease, ATP-binding subunit OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0488 PE=3 SV=1	Control
Q8DZF9	ATP-dependent DNA helicase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=pcrA PE=3 SV=1	Control
Q8E2I6	Beta sliding clamp OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=dnaN PE=3 SV=1	Control
Q8DYV9	Bifunctional protein PyrR OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=pyrR PE=3 SV=1	Control
Q8E1K2	Biotin carboxylase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=accC PE=4 SV=1	Control
Q7ZAL4	Chromosome partition protein Smc OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=smc PE=3 SV=1	Control
Q8E0A2	Coagulase domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0832 PE=4 SV=1	Control
Q8E161	DNA repair protein RecN OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=recN PE=3 SV=1	Control
Q8DZE8	DNA topoisomerase 4 subunit A OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=parC PE=3 SV=1	Control
Q8DZE7	DNA topoisomerase 4 subunit B OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=parE PE=3 SV=1	Control
Q8DZB1	dTDP-glucose 4,6-dehydratase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rfbB PE=3 SV=1	Control
Q8DZ04	DUF1836 domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1318 PE=4 SV=1	Control
Q8DYD5	DUF31 domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1552 PE=4 SV=1	Control
P65274	Elongation factor 4 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=lepA PE=3 SV=1	Control
Q8DXX6	Endonuclease MutS2 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=mutS2 PE=3 SV=1	Control
Q8E284	Glutamate--tRNA ligase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=gltX PE=3 SV=1	Control
Q8DZZ7	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=glmS PE=3 SV=3	Control
Q8DY25	Glutamyl-tRNA(Gln) amidotransferase subunit A OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=gatA PE=3 SV=1	Control
Q8E1E8	Glycerol-3-phosphate dehydrogenase [NAD(P)+] OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=gpsA PE=3 SV=1	Control
Q8E1T3	Glycine--tRNA ligase beta subunit OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=glyS PE=3 SV=1	Control
Q8DY73	GTPase Der OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=der PE=3 SV=1	Control
Q8DYI1	GTPase Era OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=era PE=3 SV=1	Control
Q8DYL0	GTPase Obg OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=obg PE=3 SV=1	Control
P67486	Histidine--tRNA ligase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=hisS PE=3 SV=1	Control
Q8DZB2	Hyaluronidase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1197 PE=3 SV=1	Control
Q8E2H3	Hypoxanthine-guanine phosphoribosyltransferase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=hpt PE=3 SV=1	Control
Q8E0I1	Lysine--tRNA ligase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=lysS PE=3 SV=1	Control
Q8E1K7	Malonyl CoA-acyl carrier protein transacylase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=fabD PE=3 SV=1	Control
Q8DYD0	Methionine--tRNA ligase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=metG PE=3 SV=1	Control
Q8E1Q7	NH(3)-dependent NAD(+) synthetase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=nadE PE=3 SV=1	Control
Q8DZ91	Nitroreductase family protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1220 PE=4 SV=1	Control
Q8DYQ8	Nucleotide sugar dehydratase, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1416 PE=4 SV=1	Control
Q8E0G1	Peptide chain release factor 3 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=prfC PE=3 SV=1	Control
Q8E066	Phenylalanine--tRNA ligase alpha subunit OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=pheS PE=3 SV=1	Control
Q8E2A3	Phosphoglycerate mutase family protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0092 PE=4 SV=1	Control
Q8E0M4	Proline dipeptidase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=pepQ PE=1 SV=1	Control
Q8DXD8	Proline--tRNA ligase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=proS PE=3 SV=1	Control
Q8DXS4	Pur operon repressor OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=purR PE=3 SV=1	Control
Q8DZD0	Purine nucleoside phosphorylase DeoD-type OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=deoD-1 PE=3 SV=1	Control
Q8DZK1	Redox-sensing transcriptional repressor Rex OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rex PE=3 SV=1	Control
Q8DY64	Ribosomal RNA small subunit methyltransferase G OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rsmG PE=3 SV=1	Control
Q8DZW8	Sensor histidine kinase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0977 PE=4 SV=1	Control
P67566	Serine--tRNA ligase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=serS PE=3 SV=1	Control
Q8DYX2	Surface antigen-related protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1350 PE=4 SV=1	Control
Q8DYI9	TGc domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1491 PE=4 SV=1	Control
Q8E0L9	Threonine--tRNA ligase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=thrS PE=3 SV=1	Control
Q8E1Q0	THUMP domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0303 PE=4 SV=1	Control
Q8DZX6	Transcriptional regulator, GntR family OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0968 PE=4 SV=1	Control
Q8E0H0	Triosephosphate isomerase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=tpiA PE=3 SV=1	Control
P0A3F1	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=mnmG PE=3 SV=1	Control
Q8DXM5	tRNA-dihydrouridine synthase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1825 PE=3 SV=1	Control
P66979	tRNA-specific 2-thiouridylase MnmA OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=mnmA PE=3 SV=1	Control
Q8E241	Tyrosine--tRNA ligase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=tyrS PE=3 SV=1	Control
P65465	UDP-N-acetylenolpyruvoylglucosamine reductase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=murB PE=3 SV=1	Control
Q8E091	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=murA1 PE=3 SV=1	Control
Q8E069	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=murA2 PE=3 SV=1	Control
Q8DY77	UDP-N-acetylmuramate--L-alanine ligase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=murC PE=3 SV=1	Control
Q8DXY6	UvrABC system protein A OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=uvrA PE=3 SV=1	Control
Q8DZL5	Xanthine phosphoribosyltransferase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=xpt PE=3 SV=1	Control

*Specifies the treatment group in which the given protein was uniquely identified

Table S3.8: Pairwise comparisons of MV protein abundance across treatment groups.

Accession Number	Protein Description	Comparison*	P-value**	log2FC***	Ampicillin	Erythromycin	Control
Q8E0G9	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=gpmA PE=3 SV=1	A & C	0.035	-3.25	0.50	1.50	4.75
P66559	30S ribosomal protein S3 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpsC PE=3 SV=1	A & C	0.018	-3.15	2.50	14.00	22.25
Q8E231	Acetate kinase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=ackA PE=3 SV=1	A & C	0.035	-3.17	0.50	1.00	4.50
Q8E0F3	Acyltransferase family protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0780 PE=4 SV=1	A & C	0.045	3.70	6.50	6.00	0.50
Q8E2D5	Aldehyde-alcohol dehydrogenase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=adhE PE=3 SV=1	A & C	0.022	-3.09	1.00	2.50	8.50
Q8E1V7	Amino acid ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0244 PE=3 SV=1	A & C	0.008	1.88	28.50	13.50	7.75
Q8E144	Cell division ATP-binding protein FtsE OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=ftsE PE=3 SV=1	A & C	0.017	2.04	8.25	3.25	2.00
Q8DZA3	Conserved domain protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1206 PE=4 SV=1	A & C	0.005	-6.39	0.50	6.25	42.00
Q8E0F6	DEAD-box ATP-dependent RNA helicase CshA OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=cshA PE=3 SV=1	A & C	0.020	-2.93	1.25	7.75	9.50
Q8E0H1	Elongation factor Tu OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=tuf PE=3 SV=1	A & C	0.006	-2.57	5.75	13.00	34.25
Q8DY79	Endolytic murein transglycosylase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=mltG PE=3 SV=1	A & C	0.005	2.35	25.50	10.50	5.00
Q8DZG4	Gls24 protein, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1137 PE=3 SV=1	A & C	0.041	2.86	14.50	1.75	2.00
Q8DYW2	HlyD_D23 domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1361 PE=3 SV=1	A & C	0.006	2.10	36.50	15.25	8.50
Q8E1P9	Mid-cell-anchored protein Z OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=mapZ PE=3 SV=1	A & C	0.013	1.71	18.75	9.75	5.75
Q8E1N5	Non-specific serine/threonine protein kinase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0319 PE=4 SV=1	A & C	0.008	1.66	42.75	18.50	13.50
Q8E213	Oligopeptide ABC transporter, oligopeptide-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0187 PE=4 SV=1	A & C	0.011	2.38	13.00	9.50	2.50
Q8DWZ3	Penicillin-binding protein 2A OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=pbp2A PE=4 SV=1	A & C	0.018	2.21	22.00	7.50	4.75
Q8E0G8	Penicillin-binding protein 2b OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0765 PE=3 SV=1	A & C	0.023	2.21	18.50	8.75	4.00
Q8E1R6	Penicillin-binding protein 2X OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=pbpX PE=3 SV=1	A & C	0.047	1.51	10.00	5.25	3.50
Q8DWP1	Serine protease OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2174 PE=1 SV=1	A & C	0.007	2.51	32.75	7.25	5.75
Q8DZY7	Sugar ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0955 PE=4 SV=1	A & C	0.033	2.32	18.75	17.25	3.75
Q9AFI1	Tyrosine-protein kinase CpsD OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=cpsD PE=3 SV=2	A & C	0.017	1.71	12.25	7.75	3.75
Q8DXS5	30S ribosomal protein S12 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpsL PE=3 SV=1	A & E	0.024	-1.85	3.75	13.50	8.50
P66391	30S ribosomal protein S13 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpsM PE=3 SV=1	A & E	0.036	-3.91	0.50	7.50	0.75
Q8DWS6	30S ribosomal protein S4 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpsD PE=3 SV=1	A & E	0.010	-2.32	6.00	30.00	20.00
Q8E1Y6	30S ribosomal protein S9 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpsI PE=3 SV=1	A & E	0.009	-2.09	1.00	4.25	3.50
Q8E2B5	50S ribosomal protein L15 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rplO PE=3 SV=1	A & E	0.048	-2.87	0.75	5.50	1.00
Q8E2C8	50S ribosomal protein L2 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rplB PE=3 SV=1	A & E	0.013	-2.54	2.75	16.00	12.00
Q8E2C9	50S ribosomal protein L23 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rplW PE=3 SV=1	A & E	0.030	-1.67	4.25	13.50	5.50
Q8E2D1	50S ribosomal protein L3 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rplC PE=3 SV=1	A & E	0.007	-4.44	0.75	16.25	8.00
Q8E2B9	50S ribosomal protein L6 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rplF PE=3 SV=1	A & E	0.046	-2.91	1.00	7.50	1.00
Q8DXQ3	Amino acid ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1797 PE=3 SV=1	A & E	0.008	2.67	22.25	3.50	7.50
Q8DXQ4	Amino acid ABC transporter, permease protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1796 PE=3 SV=1	A & E	0.007	1.83	19.50	5.50	10.25
Q8CX01	cAMP factor OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=cfb PE=4 SV=1	A & E	0.006	4.70	13.00	0.50	1.75
Q8DZX9	Extracellular protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0963 PE=4 SV=1	A & E	0.008	3.26	33.50	3.50	12.50
Q8E1I6	LytR_cpsA_psr domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0368 PE=3 SV=1	A & E	0.043	2.32	3.75	0.75	1.50
Q8E2H1	PcsB protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=pscB PE=4 SV=1	A & E	0.036	-1.54	9.00	26.25	17.25
Q8E240	Penicillin-binding protein 1B, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0159 PE=4 SV=1	A & E	0.020	2.44	9.50	1.75	4.00
Q8E0L6	Peptidase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG0714 PE=4 SV=1	A & E	0.029	2.13	12.00	2.75	9.75
Q8DZF1	Ribosomal protein S1 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rpsA PE=4 SV=1	A & E	0.005	-3.61	2.25	27.50	8.50
Q8E1H3	Translation initiation factor IF-2 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=infB PE=3 SV=1	A & E	0.042	-3.58	1.50	18.00	18.00
Q8E2B9	50S ribosomal protein L6 OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rplF PE=3 SV=1	E & C	0.046	2.91	1.00	7.50	1.00
Q8DWY5	ABC transporter, ATP-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG2076 PE=4 SV=1	E & C	0.016	2.24	6.00	8.25	1.75
P0A3J3	Chaperone protein DnaK OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=dnaK PE=3 SV=1	E & C	0.018	3.25	13.50	19.00	2.00
P64081	Enolase OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=eno PE=3 SV=1	E & C	0.013	2.58	10.00	27.00	4.50
Q8DZ85	Host cell surface-exposed lipoprotein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1227 PE=4 SV=1	E & C	0.032	-1.75	6.75	3.50	11.75
Q8DZU0	Iron-compound ABC transporter, iron-compound-binding protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1007 PE=4 SV=1	E & C	0.042	2.46	4.00	16.50	3.00
Q8DYV2	PGA_cap domain-containing protein OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1371 PE=3 SV=1	E & C	0.025	-1.81	2.50	1.00	3.50
Q8DYQ6	Polysaccharide biosynthesis protein, putative OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=SAG1418 PE=4 SV=1	E & C	0.040	2.70	5.00	6.50	1.00
P67281	Ribonuclease Y OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=rny PE=3 SV=1	E & C	0.006	3.12	8.00	19.50	2.25
Q8E0U6	Septation ring formation regulator EzrA OS=Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) OX=208435 GN=ezrA PE=3 SV=1	E & C	0.005	1.79	15.50	27.75	8.00

"Ampicillin", "Erythromycin", and "Control" columns display average spectral counts across 4 replicates for a given protein
*Pairwise comparisons include: Ampicillin vs. Control (A&C), Ampicillin vs. Erythromycin (A&E), Erythromycin vs. Control (E&C)
**p-value calculated from pairwise Kruskal Wallis statistical test
***Log_2 fold change

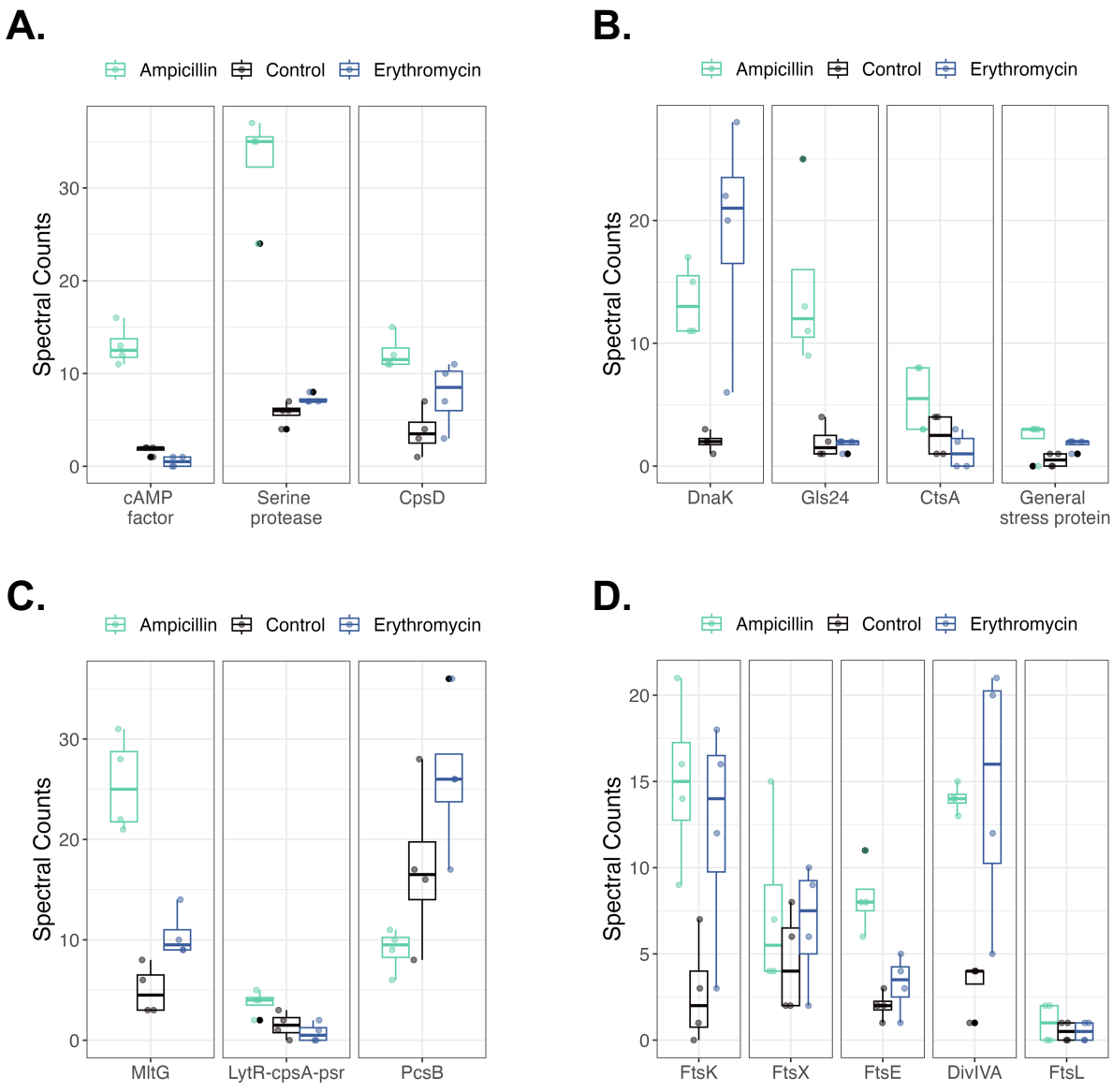

Figure S3.8: Abundance of key proteins differ across MVs from each treatment group. Differential abundance of A) virulence, B) stress-associated, C) cell-wall associated, and D) cell division proteins identified in MVs across ampicillin (turquoise), control (black) and erythromycin (blue) GBS treatment groups. Boxplots represent the summary of spectral counts (y-axis) across biological replicates (n=4) for each treatment group denoted by different colors, for each protein of interest (x-axis). Pairwise Kruskal Wallis tests of mean spectral counts were performed to identify significant differences in MV protein abundances across treatment groups, of which p-values <0.05 (*) and <0.01 (**) were deemed significant and very significant, respectively.

CHAPTER 4:
CONCLUSIONS AND FUTURE DIRECTIONS

Current treatment and prevention strategies for GBS disease involving antenatal screening of GBS colonization and administration of IAP have limited success as only the incidence of EOD in neonates has declined since its implementation [1]. The rise in antibiotic resistance along with evidence of persistent colonization despite IAP, threatens its effectiveness. Increasing observations of persistent GBS vaginal colonization is concerning as it suggests that these isolates can survive the presence of antibiotics and recover after IAP treatment. Moreover, as colonization of the vaginal tract with GBS is the main risk factor for severe EOD and LOD in neonates as well as GBS-associated preterm births and stillbirths [2–5], persistent colonization poses an even greater risk, especially for subsequent pregnancies. Prior studies of GBS isolates collected from pregnant people before and after IAP found that the prophylaxis itself was significantly associated with the persistent colonization phenotype [6,7]. Interestingly, specific genotypic lineages were more likely to persist despite IAP, including the hypervirulent ST-17 lineage and ST-19s. This finding suggests that IAP may select for more resilient strains that can survive in the presence of antibiotics. While it is known that ST-17 isolates contain unique virulence factors that enhance their ability to cause invasive infections, the underlying mechanisms of persistent colonization after IAP are not well understood. In other pathogens, the production of membrane vesicles (MVs) is one such strategy that promotes survival in the presence of stress, including antibiotics. Although we previously demonstrated that ST-17 GBS strains produce MVs with a distinct protein composition when grown in standard broth conditions [8], their biogenesis, contents, and function in the presence of antibiotics has yet to be examined. The overarching goal of this dissertation was to understand how GBS survives IAP to persistently colonize the vaginal tract. Thus, I addressed these critical knowledge gaps in two main studies.

In the first study (Chapter 2), I investigated the impact of IAP on GBS genomic evolution and persistence by employing WGS analyses across 97 clinical isolates obtained before and after IAP treatment. Examining isolates obtained from the same subject over time, before and

after antibiotic treatment, presented a unique and valuable opportunity to assess genomic evolution *in vivo*. Indeed, we observed evidence of significant genetic variation between some persistent isolate (prenatal-postpartum) pairs after IAP, highlighting the presence of mutations in important genes that promote survival including those encoding attachment proteins and factors important for stress responses. Notably, acquisition of antibiotic resistance genes was not observed, demonstrating the importance of tolerance mechanisms in persistent colonization. Furthermore, we discovered mutators in a subset of the persistent (postpartum) isolates, each representing three different lineages, or STs, with mutations in DNA mismatch repair systems. The identification of mutators in different lineages suggests that this is not an ST-dependent phenomenon, although some lineages are known to have higher genome plasticity [9], making them more vulnerable to genomic alterations. The discovery of mutators threatens the existing effectiveness of IAP treatment as they increase the likelihood of horizontal gene transfer and homologous recombination, potentially contributing to the spread of strains with antibiotic resistance or enhanced virulence. Indeed, a link between hypermutation and persistent infections has been observed, suggesting that persistent GBS populations may be a source for mutators as was demonstrated in other species [10]. Thus, the presence of GBS mutators may serve as a mechanism of enhanced survival and colonization in the GBS population. While most of the postpartum isolates were not classified as mutators, many had evidence of genes undergoing diversifying selection, thereby demonstrating microevolution in GBS as a result of IAP. Indeed, some genes that are needed for adherence had acquired point mutations including *pilA, fbsA*, and accessory Sec system-related genes, which may partly explain the increased biofilm production in over half of the persistent isolates. This finding further suggests that some mutations promote the emergence of phenotypes (e.g., biofilms) that increase the ability of GBS to persist and tolerate antibiotics in the vaginal tract. Altogether, findings from this study improve our understanding of GBS persistent colonization after IAP and brings awareness to GBS

mutators while highlighting the impact of evolutionary pressure on colonizing GBS isolates due to IAP.

As this study is the first, to our knowledge, to describe the mutator phenotype in GBS, further comparative genomic investigations of GBS isolates before and after IAP are needed to determine the frequency and impact of mutators in clinical GBS populations. Additional characterization of GBS base excision and mismatch repair machinery in mutators is also needed to elucidate the basis of mutator emergence in GBS populations. More comprehensive studies of the entire GBS vaginal population before and after IAP treatment would enhance our understanding of the impact of selective pressures due to IAP. Although these analyses led us to identify key genes that likely play a role in persistent colonization and survival during antibiotic exposure, further work is needed to test the functionality of these mutations in regard to colonization of host cells, antibiotic stress survival, and impact on pathogenesis. Overall, this study has provided a meaningful overview of the genomic adaptations in the presence of antibiotic stress and has generated many insightful hypotheses for the role of these genomic signatures in conferring long-term colonization in survival of GBS in the vaginal tract.

In the second study (Chapter 3), I investigated the impact of antibiotics on MV production and composition in a hypervirulent ST-17 clinical GBS isolate from a pregnant patient with persistent colonization. I hypothesized that GBS MVs are produced in response to antibiotics that can mediate bacterial survival in such stressful conditions. Indeed, we demonstrated that treating GBS with two different classes of antibiotics induces increased production of MVs regardless of the antibiotic class. We also showed that MVs produced by antibiotic-treated GBS have distinct protein compositions with an abundance of key proteins that elude to a role in bacterial survival in the presence of antibiotics. The identification of MV proteins that were unique to the type of antibiotic, and an abundance of these proteins in the antibiotic-treated cells relative to the untreated controls suggests GBS MVs are responsive to and reflective of surrounding environmental factors. Specifically, the high abundance of

antibiotic targets including penicillin-binding proteins and ribosomal proteins, in MVs from the ampicillin and erythromycin treated cells, respectively, indicates that MVs can mediate bacterial escape of antibiotic-mediated killing. These findings support the hypothesized role of MVs in contributing to GBS survival of antibiotic stress, which in turn, enhances their ability to persist in the host.

As the first study to explore GBS MVs in the presence of antibiotics, we have demonstrated important clinical implications including their potential ability to mediate resistance to antibiotic killing. Future experiments include an evaluation of GBS survival in the presence of antibiotics with or without excess MVs, for example, to confirm their hypothesized protective role. Further work is also needed to assess the physiological impact of these MVs on host cells by using *in vitro* tissue culture methods or more dynamic *ex vivo* models like organoids. Moreover, since we investigated GBS MV production in response to only two clinically relevant antibiotics at one concentration, more work is needed to investigate this effect across a wider range of antibiotic doses and treatment durations. The same is true for GBS strains, as we only examined responses to one ST-17 strain and differential responses are likely given the genotypic and phenotypic variation observed across strains. While other studies have described increased release of MVs due to cell-wall weakening of antibiotics like β-lactams, further studies are needed to define the mechanisms of excess MV biogenesis in GBS as a result of antibiotic exposure. This will be particularly important for those antibiotics that target other components of the cell besides the cell wall. Overall, findings from this study have unearthed hypotheses about potential mechanisms of excess MV release due to antibiotic exposure and possible roles of these MVs in GBS survival, which are needed to guide future expansions of this work.

In this dissertation, I have demonstrated that 1) antibiotic treatment impacts GBS evolution *in vivo* to promote persistent colonization by selecting for genes that enhance fitness in the presence of antibiotics and 2) antibiotic treatment increases production of MVs with distinct protein composition that suggests a role in GBS survival of antibiotic stress. Together,

these findings have enhanced our understanding of GBS adaptation in the face of antibiotics and identified potential mechanisms behind persistent colonization. The significance of these findings extends to GBS in the clinic. By understanding how resilient strains emerge and operate, we can develop new ways to treat them and more effectively prevent adverse outcomes of GBS disease in neonates and during pregnancy.

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