EBSG, A NOVEL SURFACE PROTEIN, IS INVOLVED IN THE BIOLOGY OF LIPOTEICHOIC ACID IN *ENTEROCOCCUS FAECALIS*.

by

MEGAN KALTINGER

B.S., California State University, San Marcos, 2004

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2007

Approved by:

Major Professor
Dr. Helmut Hirt

Abstract

Enterococcus faecalis is one of the most frequently encountered enterococcal isolates and accounts for about 80% of enterococcal infections. Treatment of enterococcal infections has become increasingly difficult as this organism has a high incidence of antibiotic resistance. Lipoteichoic acid (LTA) is an essential amphiphilic polymer on the surface of most Gram positive bacteria. While the molecule's exact role is not yet fully understood, a role in cell-cell contact during conjugation enabling the spread of extrachromosomal elements has been discussed. LTA also has implications in regulating autolysis, sequestering cations to the cell surface, adhesion, biofilm formation, antibiotic resistance, UV sensitivity, acid tolerance, and virulence. The gene *ebsG* was identified in a mutant of *E. faecalis* with major alterations in LTA structure and decreased ability to act as a recipient in conjugative mating. *ebsG* codes for a 119 kDa protein with only weak homology to other surface proteins of Gram positive bacteria. Transcriptional linkage analysis indicated *ebsG* and its downstream genes are organized in an operon. LTA analysis reveals a higher glycosyl content of the molecule in the mutant during stationary phase. Compared to wild type OG1RF, the mutant is more sensitive to nisin, shows higher autolysis activity during stationary phase, and is better able to serve as a recipient in plasmid transfer. Our data indicate *ebsG* and the members of the operon play a role in LTA structure and may act to degrade LTA.

Table of Contents

List of Figures

List of Tables

Acknowledgements

The work presented in this thesis would not have been possible without the instruction and guidance of my major advisor, Dr. Helmut Hirt. He was always available to discuss any questions I had and to resolve any problems that came my way. I would also like to thank the other members of my committee: Dr. Lynn Hancock, Dr. Sherry Fleming, and Dr. Michael Kanost. Thank you all for your patience and guidance during my years at KSU.

I would also like to thank my mom Kathy, whose encouragement got me through all the tough times. Without her constant support and long phone calls, I would not have been as successful as I am today.

I am grateful for having a great pillar of support in the members of the Hirt and Hancock labs. I will never forget my talks with Torrey and listening to Darin sing to the radio every day. Thank you for making my time at KSU that much more enjoyable. To Vinai and Lance, I wish you both the best in your futures and I appreciate all the time you took out for me to help me with experiments. I couldn't have done it without your help. Paul, thanks for your help in the lab. We value your time and appreciate the service you provide for all of the members of the Hirt lab. Good luck with your studies.

To all my friends who have been with me during this process, I am eternally grateful. I know you probably didn't understand one word of what I was talking about, but the fact that you were there to listen made all the difference to me. To my best friend Alyssa, your support and friendship means the world to me and I'm so lucky to have found such an amazing friend.

I would also like to thank Dr. Melinda Wilkerson for her help with the flow cytometry work presented here. I also acknowledge the Kansas State University Biology Microscopy Facility, especially Dan Boyle, for assistance with TEM and imaging.

Literature Review

Introduction

When the first known antibiotic became available during World War II, it was considered a medical miracle. The biggest killer among war victims was infected wounds and without a reliable and effective treatment option, many soldiers died of infections that today would be considered not life-threatening. Penicillin, the first antibiotic discovered in 1928 by Alexander Fleming, is a diffusible β-lactam antibiotic produced by the soil mold *Penicillium notatum* that has antibacterial properties (84). However, four years after mass production of penicillin began in 1943, microorganisms were isolated that could resist its effects.

The first bacterium to overcome penicillin was *Staphylococcus aureus*. The introduction of methicillin in 1959 and 1960 was thought to solve the problem of penicillin-resistant *S. aureus*, but resistance to methicillin occurred almost immediately (12). In 1967, penicillin-resistant pneumonia caused by *Streptococcus pneumoniae* emerged in a remote village in Papua New Guinea. At the same time, American soldiers in Southeast Asia were acquiring penicillin-resistant *Neisseria gonorrhoeae* from prostitutes and by 1976, the returning soldiers brought this new strain of *N. gonorrhoeae* to the United States.

By 1953, antibiotics such as chloramphenicol, neomycin, terramycin, tetracycline, and cephalosporins were in use and were effective in treating bacterial infections resistant to penicillin. With the overuse and misuse of these and other antibiotics, more and more microbes are showing resistance. Vancomycin is an antibiotic that is considered for many Gram-positive bacterial infections, the drug of "last resort". When all other antibiotics fail, patients are treated with vancomycin. The first strain of vancomycin-resistant enterococci was discovered in 1988 (102).

Enterococci have been recognized not only as an important cause of nosocomial infections but also because of their remarkable and increasing resistance to antimicrobial agents, the most significant being vancomycin. Vancomycin-resistant *E. faecalis* and *E. faecium* (VRE) were first reported in England in 1988 (102) and quickly spread

throughout the United Kingdom, France, and the United States (11). Because the discovery of novel antimicrobial agents has slowed, researchers must find new ways to effectively target pathogenic bacteria. One approach is to develop analogs to current antibiotics in use that have activity against resistant organisms. Another strategy to overcome resistance is to develop new drugs which inhibit novel microbial targets (93).

Most low G+C Gram-positive bacteria have an anionic membrane-associated polymer on their surface called lipoteichoic acid (LTA). Other Gram-positive bacteria lacking the classical LTA molecule have anionic derivatives referred to as macroamphiphiles, lipoglycans, or cell surface glycolipids which have been extensively reviewed (95). As of yet, no mutants have been isolated that lack LTA so it is presumed to be essential for survival.

A variety of potential functions have been described for LTA including, regulation of autolysis, maintenance of cation homeostasis, biofilm formation, antibiotic resistance, UV sensitivity, acid tolerance, and virulence (75). A logical approach for new antimicrobial therapies would be LTA as a whole or to target the specific building blocks used to synthesize it. Since LTA and its derivatives are essential for bacterial survival, compounds that either interfere with synthesis or block it from adhering to host tissues would be reasonable avenues of research to pursue. One drawback is that the genes involved in LTA biosynthesis are not well characterized and not much is understood about the importance of this molecule to the bacterium.

Enterococci and *E. faecalis*

Enterococcus faecalis is a Gram-positive, non-motile, commensal bacterium which inhabits the gastrointestinal tract of mammals. Enterococci were once included in the genus *Streptococcus*, but the results of DNA-DNA hybridization studies demonstrated that fecal streptococci were only distantly related to other streptococci so the new genus *Enterococcus* was created (89). The bacterium is normally found in pairs or short chains. It is a facultative anaerobe that produces lactic acid as the major end product of metabolism. Enterococci can endure temperatures up to 60°C for 30 minutes, low pH, and desiccation, and have high endogenous resistance to high salinity, antibiotics, and bile acids (77).

The *E. faecalis* genome has been sequenced for strain V583, which is the first reported clinical isolate resistant to vancomycin in the United States (87). There are a total of 3337 predicted open reading frames on the chromosome. The plasmids present in *E. faecalis* V583 are pTEF1 and pTEF2 which are structurally similar to the pheromone-responsive plasmids pAD1 and pCF10, and pTEF3, which belongs to the family of pAMβ1 broad host range plasmids. Interestingly, over a quarter of the genome consists of DNA which is either mobile or exogenously acquired (77). There are a variety of plasmids previously described for *E. faecalis*, but the plasmids that are of particular interest are the pheromone-responsive conjugative plasmids (22).

Infections Caused by Enterococci

Although the genus *Enterococcus* includes 27 species (http://www.atcc.org), *E. faecalis* and *E. faecium* account for most clinical infections in humans (71). With the increase in antibiotic resistance, enterococci are more notably recognized as a dangerous nosocomial pathogen that can be difficult to treat. In hospital settings, the isolation of enterococci which are multi-drug resistant has become more common. According to the National Nosocomial Infections Surveillance (NNIS) data from January to December 2003, more than 28% of enterococcal isolates from participating hospital ICU's were vancomycin resistant (Fig. 1).

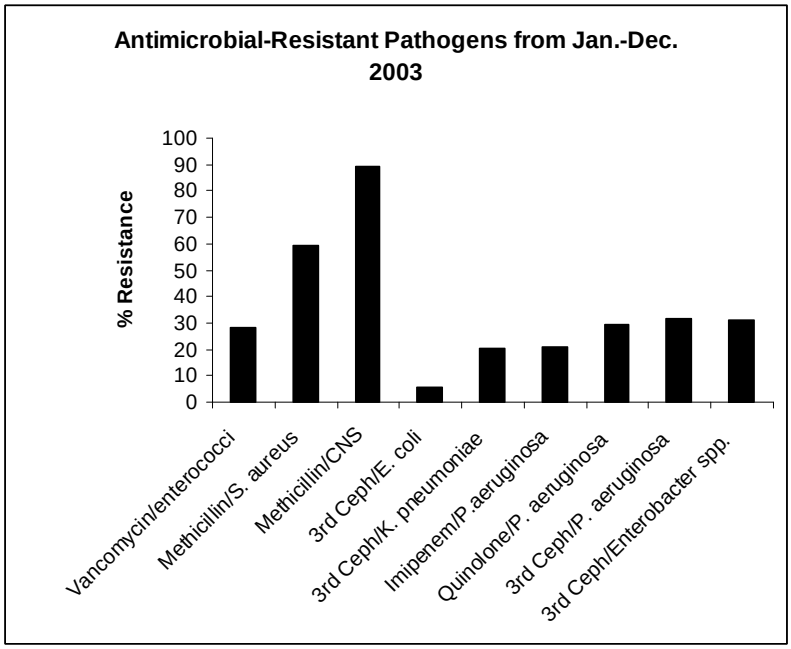

Figure 1. Antimicrobial-resistant pathogens associated with nosocomial infections in ICU patients, comparison of resistance rates from Jan.-Dec. 2003 (Adapted from 74). Vancomycin-resistant enterococci account for 28.5% of enterococcal nosocomial infections.

Enterococci are currently the third leading cause of nosocomial bacteremia in the United States (74). Many cases of bacteremia occur through the use of intravenous lines, abscesses, and urinary tract infections, but a large percentage have an unidentifiable origin and presumably begin in the intestinal tract. In these cases, bacteria are engulfed by intestinal epithelial cells or intraepithelial leukocytes. The bacteria can exit on the apical side of epithelial cells or migrate in phagocytes to the lymph nodes, where they proliferate and spread throughout the body by using the blood stream (48). The use of antibiotics such as cephalosporins, which have little antienterococcal activity, can cause enterococcal superinfection and are associated with enterococcal bacteremia (50, 112).

The ability of enterococci to bind to endocardial tissue or matrix components is critical to cause endocarditis. Experimental models of endocarditis have shown the vegetations on heart valves are mainly composed of fibrin, platelets, and fibronectin, providing a place for bacteria to bind (59, 86). Patients who are at risk of developing

endocarditis are those that have congenital heart malformations, acquired valvular defects, prosthetic valves, and previous bacterial endocarditis (30).

Urinary tract infections (UTIs) are commonly caused by enterococci in patients with abnormalities of the urinary tract or those that have indwelling catheters. Infections usually occur through organisms ascending the urethra and ureters from bacteria found in the fecal flora and can infect the bladder, prostate, and kidneys (48).

Treatment

The treatment of enterococcal infections usually requires a combination approach, for example, using penicillin with vancomycin, ciprofloxacin with ampicillin, or novobiocin with doxycycline (10). While the traditional combination approach uses a cell-wall active antibiotic and an aminoglycoside, this has been shown to provide no special benefit to patients and can lead to nephrotoxicity (25). An increase in the amount of clinical isolates that are vancomycin resistant has made enterococcal infections increasingly difficult to treat. New antibiotics are under investigation, including fluoroquinolones, streptogramins, oxazolidinones, semisynthetic glycopeptides, and glycylcyclines (45). While some of these antibiotics are effective against certain strains of enterococci, there is no specific drug that can treat all multi-drug resistant enterococci. The main drawback of treating these infections with a broad spectrum approach is that the more organisms are exposed to the drug (both commensals and pathogens), the more opportunities arise for resistance to evolve. Broad spectrum antibiotics affect both disease-causing organisms and commensals present in numbers large enough to generate resistance by otherwise rare mutations or genetic exchange.

Historically, physicians have relied on broad spectrum antibiotic therapy to treat infections but the current development and introduction of rapid diagnostic techniques may allow for a more focused approach to treating infectious diseases. Because of the complex interactions between host and pathogen during the establishment of infection, rapid and accurate diagnosis is required. If therapeutics are developed which target these specific interactions, the diverse commensal flora should essentially be left unaffected and the targeted population would be restricted to the relatively small numbers of

disease-causing organisms. As a result, the development of resistance would be less of a statistical probability (93).

Virulence Factors

Several virulence factors have been discovered which make enterococci, and specifically *E. faecalis*, such an important nosocomial pathogen. The ability of *E. faecalis* to transfer virulence factors and antibiotic resistance genes through the use of pheromone-responsive plasmids has become important in understanding how bacteria continue to evolve and evade the immune system. Aggregation substance (AS), an important virulence factor, is now being characterized for the role it plays in virulence and the establishment of bacterial attachment to host tissues which leads to infection. There has been evidence for AS to play a role in mediating enterococcal adherence to renal epithelial cells (56), but AS is not involved in colonizing the urinary tract (49). These virulence factors are discussed in detail below.

Gelatinase, a secreted zinc-containing metalloproteinase, is encoded by *gelE* on the chromosome of *E. faecalis*. It has been shown to hydrolyze gelatine, collagen, fibrinogen, casein, hemoglobin, insulin, and certain *E. faecalis* sex-pheromone-related peptides (64) and plays a role in enterococcal endocarditis (16, 34). Enterococcal hemolysin is also an important virulence factor as it has been shown to lyse both erythrocytes and a variety of Gram-positive bacteria (3, 6) and, when associated with AS, contributes to virulence in experimental animal endocarditis (14). Enterococcal surface protein, Esp, is another virulence factor of *E. faecalis* and has been shown to play a role in adherence and colonization of the host bladder tissue but does not cause any histopathological changes associated with UTIs (91).

Sex Pheromones and Gene Transfer

Enterococci are well known for their tendency to acquire and distribute antibiotic resistance genes. A group of plasmids called pheromone-responsive plasmids have been identified and studied for over 30 years and are a mechanism by which gene transfer occurs in *E. faecalis* (19). These plasmids represent the most extensively characterized class of plasmids in the world of Gram-positive bacteria.

Pheromone-inducible conjugation in *E. faecalis* starts with a donor cell, carrying a conjugative plasmid, which can detect the presence of plasmid-free recipient cells by a recipient-produced sex pheromone. The excreted pheromone binds to its plasmid-encoded receptor on the surface of the donor cell and activates transcription of conjugation factors, the most notable being AS. AS is an inducible surface adhesin protein that facilitates the attachment of the donor cell to a recipient cell by way of enterococcal binding substance (EBS) encoded on the chromosome (22). The cells form high-density aggregates which are very stable and allow the formation of a mating channel for plasmid transfer to occur (Fig. 2). Once a recipient cell acquires a copy of the plasmid, it now becomes a donor cell and can pass the plasmid on to a recipient cell in the presence of extracellular pheromone (22).

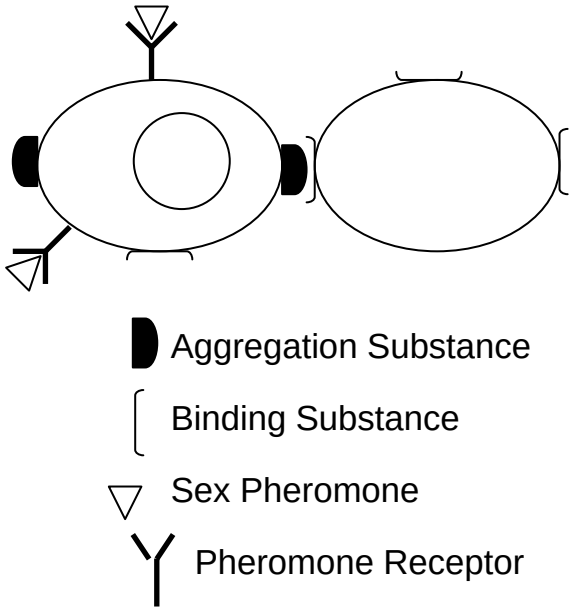

Figure 2. Model for mating pair formation via Asc10-EBS binding. See text for details. (Adapted from 76).

The two best characterized conjugative plasmids are pAD1 and pCF10 (21, 99) and their respective pheromones, cAD1 and cCF10, respectively. Considerable work has also been done on pPD1, and its cognate pheromone cPD1 (111). pAD1 and pPD1 carry the gene for hemolysin/bacteriocin production and pCF10 harbors Tn*925*, a transposon

similar to Tn*916*, encoding tetracycline resistance (22, 41). Pheromone-responsive plasmids can transfer to recipient cells at high frequency (10^{-3} to 10^{-1} transconjugants per donor) in broth matings (20) with a low pheromone concentration present in the culture supernatant (~5.0 x 10^{-12} M) (22). Two plasmids, pHKK702 and pHKK703, have been identified and confer high-level vancomycin resistance in clinical isolates of *E. faecium* (36), and are also closely related to pCF10. pCF10 was identified 15 years earlier from the same hospital as pHKK702 and pHKK703, which suggests the same plasmid has circulated around this environment for years and has picked up different antibiotic resistance genes over time. Because the horizontal transfer rate is so high in these plasmids, antibiotic resistance genes and other virulence factors are readily spread throughout enterococci populations, causing an increase in the prevalence of bacterial strains which are resistant to classical antibiotic therapies.

Aggregation Substance

As mentioned previously, AS is a surface protein encoded on the pheromone-responsive plasmids of *E. faecalis*. Its expression is induced by sex pheromones, which are heat stable, 7- to 8-amino acid hydrophobic peptides (97). AS expressed by the donor cell binds to EBS expressed by the recipient cell, and close cell contact mediates the conjugative transfer of the plasmid. The genes encoding AS from the three most-studied plasmids show high sequence similiarity and are named: *asa1* from pAD1, *asp1* from pPD1, and *prgB* from pCF10 (110). While their proteins are not identical, they show an overall sequence similarity between 75-85%. An example of a unique AS protein is Asa373 from the pheromone plasmid pAM373, which shows little similarity to the other AS proteins and appears to use a different mechanism for cellular aggregation (73).

Besides the role AS plays in the transfer of conjugative plasmids, other roles have been described which contribute to *E. faecalis* virulence. Previous studies identified a role for Asa1 in adherence to porcine renal tubular cells (56) and for adherence to human macrophages (94). *E. faecalis* bearing AS has been shown to resist killing in polymorphonuclear leukocytes (PMNs) despite these cells being activated (83). Asa1 and Asc10 have been shown to aid in the binding of *E. faecalis* to extracellular matrix components such as fibrin (40) thrombospondin, vitronectin, and collagen type I (86).

This important adherence property of *E. faecalis* bearing AS can mediate colonization and infection through injured epithelium and endothelium in host tissues where these extracellular components are exposed.

Many studies focus on the potential functions of AS, but few have focused on the properties of the protein that arbitrate these functions. One major problem with the use of conventional approaches is that the purified AS protein is highly unstable. Isolation of AS gives a 137 kDa product representing the full-length protein and a specific 78 kDa, N-terminal cleavage product (39). AS has an N-terminal signal sequence and a C-terminal LPXTG cell wall anchor motif, which is common in Gram-positive surface proteins. Asa1, Asp1, and Asc10 have two conserved Arg-Gly-Asp (RGD) motifs thought to be involved in binding to cell surface integrins (56, 91, 97). This was disproved by mutating the RGD motifs in Asc10 and examining their role in the internalization of *E. faecalis* into HT-29 enterocytes. This study showed the RGD motifs were not critical for efficient internalization into HT-29 enterocytes (107).

Scanning electron microscopy on Asa1 has found the N terminus is more exposed than the C terminus on the cell surface (39). Aggregation analysis has identified that the N- and C-terminal domains of Asc10 play an important role in aggregation and the variable region may also play a role in aggregation (106). The N-terminal domain has also been found to mediate clumping activity in Asa1 (72). Importantly, AS has also been found to bind LTA in a dose-dependent manner and a new N-terminal domain from amino acids 156 to 358 was found to be required for aggregation (108).

The Nature of the Binding Partner – Enterococcal Binding Substance

As previously mentioned, the receptor for AS is EBS, but it has not been well characterized and the component(s) are unknown. According to the model for mating pair formation (Fig. 2), both donor and recipient cells express EBS and it has been shown that donor cells can self-aggregate when induced to express AS (46). While the exact composition of EBS is unknown, several lines of evidence support a role for LTA involvement.

The work of Ehrenfeld *et al*. found purified LTA inhibited cell aggregation at relatively low concentrations, suggesting LTA could be part of the binding substance (23). A mutant created by Trotter *et al*. named INY3000, which will be discussed in

detail below, has been shown to be deficient in the ability to act as a recipient in conjugative matings, due to four Tn*916* insertions in the chromosome (100). When INY3000 LTA was examined, it was found to have a shorter polyglycerophosphate backbone and the fatty acids associated with the lipid anchor were shortened when compared to wild type *E. faecalis* LTA (100). This suggested LTA could be partially responsible for the decreased recipient ability observed. Finally, it has been shown that an N-terminal domain of Asc10 binds LTA in a dose-dependent manner (108), providing further evidence for the involvement of LTA in EBS.

Bacterial Adherence to Host Tissues

In order to initiate the infection process, bacterial adherence to host tissues is a critical first step. For enterococci living as commensals in the gastrointestinal tract, adhesins that bind to the mucosal surfaces of eukaryotic cells play a critical role in the maintenance of colonization. If enterococci didn't have a means of attachment, they would be eliminated by the flow of materials that normally pass through the intestines. As discussed previously, AS is an important bacterial adhesin molecule for *E. faecalis*. In addition to the transfer of pheromone plasmids carrying virulence traits and antibiotic resistance genes, AS plays a role in the adherence of enterococci to renal epithelial cells and to cardiac vegetations (14, 56). *E. faecalis* has also been found to bind to several extracellular matrix components (40, 86), which could aid in the establishment of infection to damaged tissues.

There are implications for LTA to act as an adhesin molecule, but not much work has been done on *E. faecalis* LTA to support this theory. *S. saprophyticus* LTA has been found to possibly act as an adhesin to uroepithelial cells (102). Chugh *et al.* found the lipid moiety of LTA plays a central role in the adherence of *S. epidermidis* to fibrin-platelet clots, an important factor in the establishment of bacterial endocarditis (15). LTA of *Lactobacillus johnsonii* La1has been shown to act as an adhesin factor for the attachment of these cells to Caco-2 human intestinal cells (32). The role of LTA to act as an adhesin molecule and the role it plays in enterococcal infections must continue to be investigated for a better understanding of how the infection process occurs.

Lipoteichoic Acid Structure, Biosynthesis, and Functions

The bacterial cell wall consists of a dynamic collection of molecules and proteins essential for survival, shape, and integrity. LTA is a membrane-associated polymer characteristic of most Gram-positive bacteria. Those Gram-positive bacteria that lack LTA have a similar molecule that performs much of the same functions as LTA (75). For example, *Micrococcus luteus* has the anion polymer lipomannan in place of LTA. *Mycobacterium leprae* and *Mycobacterium tuberculosis* have arabinomannan in place of LTA on their cell surface (95). To date, there is no mutant deficient for LTA which illustrates the importance of this molecule to the growth and survival of the organism.

Structure of LTA

LTA is an amphiphilic molecule that is anchored in the plasma membrane by a glycolipid anchor and extends through the peptidoglycan layer to the cell surface. The predominant type of LTA consists of a 1,3-linked polyglycerophosphate backbone attached to the glycolipid anchor by a phosphodiester bond (26), but variations to this structure have been reported. LTA from *Streptococcus pneumoniae* differs from the predominant structure by containing ribitol phosphate, galactosamine, glucose, and choline phosphate. Actinomycetes lack classical LTA but contain amphiphilic heteropolysaccharides such as lipomannans and lipoarabinomannans (96, 101).

LTA from *E. faecalis* has a hydrophilic polyglycerophosphate backbone that extends through the peptidoglycan layer which can be between 14 and 33 glycerophosphate units in length. This part of the molecule is substituted with D-alanine and kojibiose (α-1,2-glucose) moieties. The polyglycerophosphate backbone is linked to a hydrophobic phosphatidylkojibiosyl diacylglycerol anchor located in the cell membrane (Fig. 3) (28, 48). The LTA of enterococci has been shown to be identical to the group D antigen and therefore included in the group D streptococci (109).

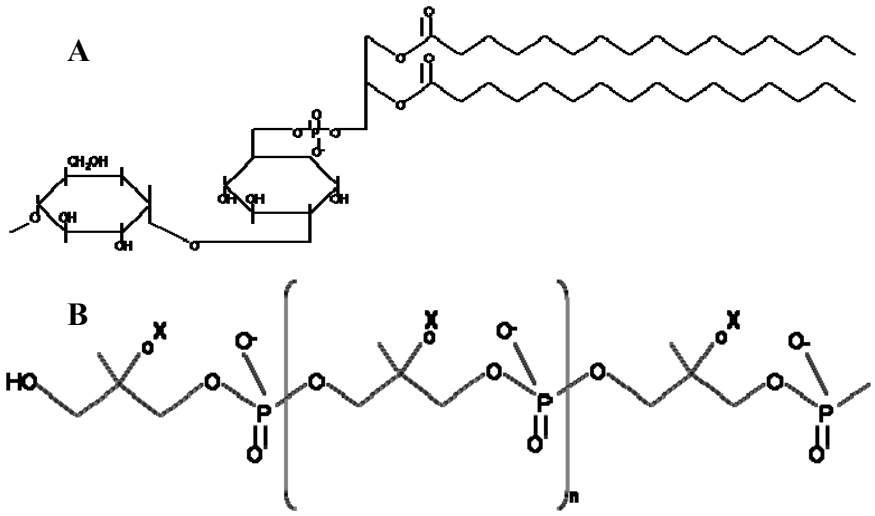

Figure 3. Lipoteichoic acid structure. (A) Glycolipid membrane anchor; (B) polyglycerophosphate backbone. X: D-alanyl or kojibiose substituents (Adapted from 75).

Biosynthesis of LTA

In 1975 studies were conducted that determined the lipid moiety of LTA from *Streptococcus faecalis* (later named *E. faecalis*) consists of phosphatidylkojibiosyl diacylglycerol. This lipid portion is linked to the polyglycerophosphate backbone via a phosphodiester bond (28). The same group later identified an enzyme in the cell membrane which catalyzes the transfer of glycerol phosphate from phosphatidylglycerol to phophatidylkojibiosyl diacylglycerol. Once the lipid anchor has been synthesized, the backbone is made by adding more glycerol phosphate units from phosphatidylglycerol to form the glycerol phosphate polymer (29).

Although there has been much work done on the structure of LTA, the genes required for LTA synthesis have not been well characterized. In *Staphylococcus aureus*, a gene was identified named *ypfP*, which encodes a processive glycosyltransferase required for glycolipid and LTA anchor synthesis in *Bacillus subtilis* and *S. aureus* (51, 53). Recently, a gene was identified in *S. aureus* named *ltaA*, which is involved in LTA biosynthesis (32). An *ltaA* mutant still synthesizes LTA, but a large portion of the LTA is anchored to diacylglycerol instead of diglycosyl-diacylglycerol, the normal LTA

anchor in *S. aureus.* LtaA is thought to function as a permease which transfers glycolipids across the cell membrane following a concentration gradient (32).

As mentioned previously, the polyglycerophosphate backbone of LTA in *E. faecalis* is substituted with either D-alanine or kojibiose moieties, which can alter the charge properties of the molecule. An operon has been identified which is necessary for D-alanylation of LTA in *S. aureus* and *S. pneumoniae* and homologs of these genes are found in *E. faecalis* (54, 55). The operon consists of four genes named *dltABCD*. The first gene, *dltA*, is the D-alanyl carrier protein ligase, which activates D-alanine with ATP. This activated complex is then transferred to the D-alanine carrier protein (Dcp) encoded by *dltC* with the help of the *dltD* gene. The *dltB* gene is predicted to be a transmembrane protein which may be involved in transporting the D-alanyl-Dcp complex across the cell membrane where D-alanine is added to the glycerol phosphate backbone of LTA (54, 73). Figure 4 depicts the model by which D-alanine is incorporated onto the backbone of LTA. To date, the genes involved in the addition of kojibiose residues to LTA have not been characterized.

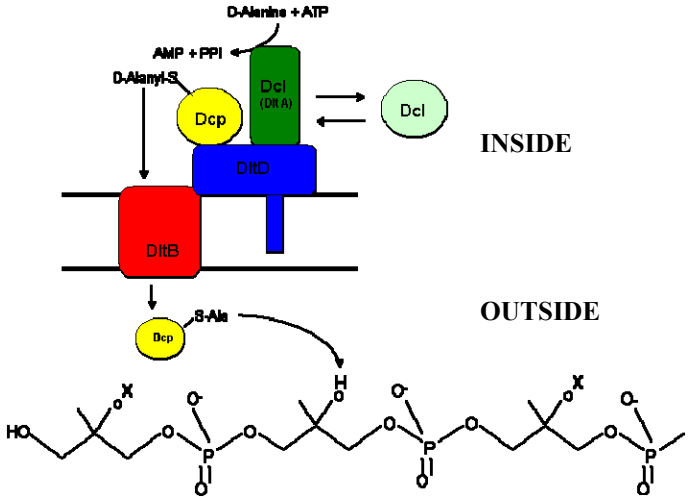

Figure 4. Model for the incorporation of D-alanine onto LTA (Adapted from 75).

Functions of LTA

While no one specific role has been assigned to LTA, it has an impact on many biological functions of the bacterial cell. LTA has been proposed to modulate the activities of autolysins, play a role in the maintenance of cation homeostasis, and to

define the electromechanical properties of the cell wall. The role LTA plays in plasmid transfer has already been explored and is an important contributor to *E. faecalis* virulence. Additional functions include a role in adhesion, biofilm formation, antibiotic resistance, UV sensitivity, acid tolerance, and virulence (75).

One important role of LTA is its postulated ability to regulate autolysins. LTA has been shown to be an inhibitor of autolysins in pneumococcus (43). It has been suggested that the degree of D-alanylation of LTA has an effect on its ability to regulate autolysis. In *E. faecalis*, the action of hemolysins and bacteriocins were inhibited by D-alanyl-wall teichoic acid and the removal of D-alanine residues abolished the inhibitory effect and autolysis was induced (17).

Because LTA has a net negative charge, it has been suggested that this molecule might be involved in the binding of cations and sequestering them to the surface of the cell. LTA has been shown to bind Mg^{2+} in *B. subtilis*, *Lactobacillus buchneri*, and *S. aureus* (2, 37, 60). It has also been observed that the degree of D-alanylation effects cation binding. When LTA from *S. sanguis* is stripped of D-alanine, one Mg^{2+} ion binds to LTA for every phosphodiester linkage in the backbone (85). Recently, it was discovered that the concentration of NaCl, $CaCl_2$, and $MgCl_2$ can inhibit the transcription of the *dlt* operon in *S. aureus*, which suggests bacteria are able to adjust the D-alanine content of LTA with respect to their environment. Interestingly, the concentration of divalent cations needed to alter transcription was much lower than the concentration of monovalent cations, suggesting divalent cations may play a more direct role in this process (54).

Another essential role LTA plays in the biology of Gram-positive bacteria is increased resistance to antimicrobial peptides. A study was initiated to determine if D-alanine esters on LTA affected sensitivity to cationic antimicrobial peptides in strains of *S. aureus* and *S. xylosus* which were devoid of D-alanine (78). Some of the antimicrobial peptides were host defense peptides such as human defensin HNP1-3, protegrin from porcine leukocytes, magainin II from amphibian skin, and bacteria-derived compounds such as gallidermin and nisin. All of these molecules have a net positive charge but differ in their structures. It was found that all the compounds had lower minimum inhibitory concentrations when the *dlt* operon was inactivated, suggesting that D-alanine

residues alter the surface charge of the bacteria and make it less susceptible to killing by cationic antimicrobial compounds (78).

LTA also functions as an inducer of complement, proinflammatory mediators, and immunogens. LTA has been shown to activate the classical complement pathway by binding to C1 protein and activating it, which then acts on its substrates C4 and C2. Specifically, LTA binds to the C1q subcomponent of C1 (63). The alternative complement pathway can also be activated by LTA and was shown to do so with *S. pneumoniae* LTA (44), although there are substantial differences in structure from *E. faecalis* LTA. Activation of the complement pathways by LTA is an important virulence factor because this can lead to damage of host cells. LTA can be released by bacteria and form micelles, which may consume complement (63). It can also be inserted into the cytoplasmic membrane of host cells and kill them by complement activation (44).

Recognition of LTA by the host initiates a cascade of inflammatory mediators, causes vascular and physiological changes, and recruits immune cells to the site of infection. LTA is able to induce the release of nitric oxide, IL-1, IL-6, and TNF-α by monocytes and macrophages and can also activate the oxidative burst in vitro (103). LTA can also activate the release of IL-12 through CD14 (15), which is a receptor on macrophages and monocytes that also binds lipopolysaccharide (LPS) from Gram-negative bacteria. LTA is also recognized by Toll-like receptors (TLR), a family of pattern recognition proteins that activate the release of cytokines and recruit macrophages and neutrophils to the site of infection. Specifically, LTA from *B. subtilis* and *S. aureus* have been shown to bind to TLR2 and TLR6 (38, 90).

While much work has been done to determine the biological effects of D-alanine residues on LTA, the role of sugar moieties has not been explored. It is important to examine the role these molecules play in the biology of bacteria to gain a better understanding of how bacteria, and specifically *E. faecalis*, establish an infection in the host.

Identification of Genes Involved in EBS Formation

In an attempt to identify genes involved in EBS, mutants were isolated that were defective in the ability to bind to donor cells in conjugative mating experiments. To do

this, random insertions of Tn*916* carrying a tetracycline resistance marker were introduced into the wild type *E. faecalis* OG1SSp chromosome. This group had previously isolated a lytic phage, NPV-1, capable of lysing *E. faecalis* OG1 wild type cells and its derivatives. To screen for conjugative-defective mutants (Con$^-$), Tn*916* transconjugants were subjected to NPV-1 to identify mutants that were not lysed by the phage, with the rationale that any alterations of the cell surface that left it phage-resistant could potentially affect mating ability. Mating experiments were then performed to identify Con$^-$ mutants that were unable to act as recipients and showed a 100- to 1000-fold reduction in recipient ability (100).

One of these mutants, named INY3000, carried insertions of Tn*916* at four different sites on the chromosome (100). Plasmid pAD1 and pCF500, a derivative of pCF10, were introduced separately into INY3000 to determine if plasmid transfer could occur if this mutant acted as a donor. No difference was seen between wild type donors and INY3000 acting as a donor, suggesting the decreased recipient ability of the mutant was due to a lack of effective cell-to-cell contact and not an inability to replicate the plasmid or produce AS (100).

Each of the four Tn*916* insertions was separated from the parent strain INY3000 to examine their individual effects on recipient ability. These four strains carrying a single copy of the transposon (INY3039, INY3040, INY3044, and INY3048) had normal recipient frequencies in broth matings, indicating no single insertion was responsible for the recipient deficient phenotype (100).

The cell envelope was then examined to determine what effects the Tn*916* insertions had on the surface of the cell. When LTA from INY3000 and its derivatives was examined and compared to wild type, the polyglycerophosphate backbone of INY3000 LTA was shorter in chain length compared to wild type and had an increase in the percentage of short chain (12-carbon) unsaturated fatty acids. Strains carrying only one transposon insertion had a normal fatty acid profile. Cell membrane fatty acids were also examined and no difference was seen between wild type OG1SSp and any of the strains carrying Tn*916* insertions. This indicates that the changes seen in fatty acid composition were specific to the fatty acids of LTA rather than changes in all the cellular lipids (100).

Because INY3000 is deficient in mating ability and that deficiency is due to a defect in binding substance expression, the DNA flanking the Tn*916* insertions was cloned into *E. coli* and characterized to identify the genes involved (4). No stable construct was obtained from INY3044 cloned fragments. For INY3039 and INY3040, excision of the transposon resulted in fragments of 3.6 and 9.5 kb respectively. For INY3048, excision of the transposon led to the deletion of the chromosomal insert and only left cosmid DNA. However, a few constructs did produce a 2.0 to 2.3 kb fragment after excision (4).

The excision fragment from INY3039 was sequenced because complementation studies determined this region of DNA was able to partially restore the recipient ability of INY3000. Three open reading frames were identified and named *ebsA*, *ebsB*, and *ebsC*. EbsA showed 27% identity to the N-terminus of *lktD* from *Actinobacillus actinomycetemcomitans*, which is homolgous to leukotoxin or hemolysin protein (33). EbsB showed 28% identity to the catalytic segment of the cell wall hydrolase *N*-acetlymuramoyl-L-alanine amidase, an autolysin from *Bacillus licheniformis*. EbsC shows 36% identity to a protein from *Salmonella typhimurium* which may suppress transcription of the silent gene *ushA*^{o}, a nonexpressed gene homologous to *ushA* from *E. coli*, which encodes a UDP-sugar hydrolase. A fourth open reading frame designated *orfD* shows 45% identity to a dehydroquinate dehydratase from *E. coli* (4).

It is interesting to note that the excision fragments from INY3044 and INY3048 were inconsistent in size, and this could be due to high instability or lethality in *E. coli*. It has also been proposed that these fragments are unstable in *E. faecalis* and could possibly rearrange (4).

DNA mapping of three of the four regions in INY3000 that play a role in binding substance expression has determined these regions are not contiguous on the chromosome of *E. faecalis* and the insertions in INY3039 and INY3048 are approximately 600 kb apart (70). According to the published genome sequence of *E. faecalis* V583, the *ebsABC* gene cluster and the Tn*916* insertion in INY3048 are just over 944 kb apart. The difference of roughly 300 kb in V583 compared to OG1RF is most likely due to insertions in the V583 chromosome. Analysis of the excision fragment of INY3048 has shown the Tn*916* insertion to be just upstream of a previously unidentified

cluster of five genes that may play a role in LTA structure and could be responsible for the changes in LTA observed in the INY3000 mutant.

The INY3048 Locus

There are 5 closely linked open reading frames (ORFs) just downstream of the Tn*916* insertion in INY3048 whose organization is depicted in Fig. 5. Almost all of the ORFs are novel genes that do not have homologs in other species. The fourth gene, named *ebsJ*, has similarity to a glycerophosphoryl diester phosphodiesterase, which may function in *E. faecalis* to release glycerophosphate moieties from the unsubstituted polyglycerophosphate backbone, an enzymatic activity which has been described in *Bacillus pumilus* (58). The proximity of the genes in an apparent operon close to one of the original insertions of Tn*916* in INY3000, and the presence of *ebsJ*, whose gene product could potentially be involved in LTA biosynthesis, prompted us to investigate the first ORF in the locus, named *ebsG*. The role of *ebsG* is unknown and is the subject of this research project.

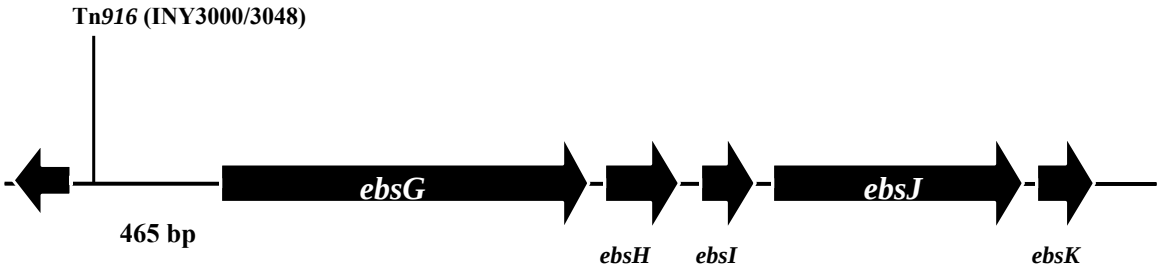

Figure 5. Organization of the *ebsG* operon and location of Tn*916* insertion in INY3000/INY3048. There are 463 bp between the predicted start site of *ebsG* and ORF *EF0774* upstream of *ebsG*.

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. faecalis* was grown without shaking at 37°C in Todd-Hewitt broth or Brain heart infusion (BHI) broth (Difco). *E. coli* strains were grown in BHI at 37°C with shaking. Agar plates contained 1.5 % agar. The following antibiotic concentrations were used: for *E. coli*, kanamycin at 50 μg/ml and erythromycin at 100 μg/ml; for *E. faecalis*, kanamycin at 1 mg/ml and erythromycin at 10 μg/ml.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strain		
E. coli		
EC1000	Km^r, cloning host for *repA*-dependent plasmids	59
E. faecalis		
OG1RF	Rif^r, fucidic acid-resistant wild type strain	18
OG1RF::*ebsG*	Rif^r, Erm^r	This study
Plasmids		
pMSP3535	Nisin-inducible cloning shuttle vector	6
pMSP7551	Nisin-inducible *ebsG* in pMSP3535	H. Hirt, unpublished
pTCV-*lac*	Erm^r, Km^r, shuttle vector with β-galactosidase reporter	78
pMJK203[a]	233bp *EcoRI-BamHI* fragment containing *ebsG* promoter	This study
pMJK204[a]	316 bp *EcoRI-BamHI* fragment containing *ebsG* promoter	This study

[a]These plasmids are derivatives of pTCV-*lac*, which contain the inserts described in the text for use in promoter fusion studies.

DNA Manipulations

Plasmid DNA from *E. coli* was isolated from overnight cultures using the QIAprep Spin Miniprep Kit (QIAGEN). Restriction endonuclease digestions were performed according to the manufacturer's instructions. A typical digestion contained at least 10 μl of DNA, 1 μl of each restriction enzyme, 2 μl of the correct 10X buffer, and dH_2O for a total volume of 20 μl. The clean up of restriction endonuclease digestions, ligation reactions, and PCR reactions was achieved using the QIAquick PCR Purification kit (QIAGEN).

Electrophoresis on 1% agarose gels was performed to screen plasmid DNA, PCR products, and digested DNA. DNA samples were mixed with 10X DNA Loading Dye (50% glycerol, 0.1M EDTA pH 7.5, 1.0% SDS, 0.1% bromophenol blue) and loaded into individual wells on the gel. Gels were stained in a solution containing 0.5 µg/ml ethidium bromide.

DNA ligations were performed at room temperature for 3 hours using T4 DNA ligase (Promega). A typical reaction had a volume of 20 µl and an insert to vector ratio of 3:1. Ligations were cleaned up as previously described above.

DNA sequencing was performed using appropriate primers on an Applied Biosystems 3730 DNA Analyzer with Applied Biosystems Big Dye chemistries. Sequences were compared to the *E. faecalis* V583 sequenced genome from TIGR (www.tigr.org).

Isolation of Chromosomal DNA

An overnight culture of *E. faecalis* OG1RF was prepared in THB and grown at 37°C without shaking. Equal amounts of overnight culture and 15% Chelex 100 resin (Bio-Rad) were mixed and incubated in boiling water for 10 mins. Cellular debris was pelleted in a table top centrifuge for 5 mins at maximum speed. The chromosomal DNA-containing supernatant was stored at -20°C (105).

PCR

Primers were acquired from IDT Integrated DNA Technologies, Inc. (Coralville, IA) and are listed in Table 2. Primers were diluted to 2 pmol/µl and stored at -20°C. PCR reactions were performed using GoTaq Flexi DNA Polymerase (Promega) according to the manufacturer's instructions. A typical reaction would contain: 2 µl template DNA, 10 µl forward primer (2 pmol/µl), 10 µl reverse primer (2 pmol/µl), 100 µM total dNTPs, 2.5 mM $MgCl_2$, 10 µl 5X reaction buffer, 0.5 µl GoTaq enzyme, and 11.5 µl dH_20 for a total reaction volume of 50 µl. Reactions were performed on a Mastercycler (Eppendorf) for 35 cycles at annealing temperatures appropriate for the primer pair used.

Table 2. Primers used in this study[a]

Primer name and function	Sequence (5' to 3')
Promoter fusion constructs	
GlacupR-21	ATATAGGATCCTAAACATTTTAATCCTCC (*Bam*HI)
Glacup224	ATATAGAATTCAACCAACTTTGTCAGTGG (*Eco*RI)
Glacup307	ATATAGAATTCGTAAAAGGACTGTAAAGG (*Eco*RI)
Glacup386	ATATAGAATTCCAGGCTAAACTATTCAGC (*Eco*RI)
Glacup399	ATATAGAATTCTTGTTGAAATAAACAGGC (*Eco*RI)
qRT-PCR	
EbsGRTF	GAAGTGGTTCAAGGCTTATCTG
EbsGRTR	TCGTTAAATCTCCGTGTGTTTG
DltARTF	CGGAAGAATTAACAGAAATGATGATGC
DltARTR	ATGAGCCACCTAACGCCAATG
GyrBRTF	CAAGCCAAAACAGGTCGCC
GyrBRTR	ACCAACACCGTGCAAGCC
RT-PCR gene linkage analysis	
0774-0775F	CACTCATTCATCGTATTCACTCCTAC
0774-0775R	CGTATTGGTCATTCCTTCTGTATCTC
0775-0776F	CCAACAAGCCAACCGTAACAC
0775-0776R	CGAAATCCTGACAATCTGAACAG
0775-0778F	CCAATGACTAATACAACAGTAAATCCAC
0775-0778R	GCTACCGAAATTAAGATGCTCACAC
0778-0779F	AAGGAACTATTGATGAGCCAGTC
0778-0779R	AATTCAGGTTATTAAACGAAAGATAAGG
0779-0780F	TATGCTACCAAGTGTGGCTTCTC
0779-0780R	ACTGCTAATTCTTCTTTCATTTCACG
0780-0781F	GCCAAACAAGCCCAAGGAAAAG
0780-0781R	AATACATCATTGCCATCTTCACCTG

[a]Underlined nucleotides indicate restriction cleavage sites not present in the template sequence and were employed to facilitate cloning. The restriction enzyme is shown in parentheses after the primer sequence.

Preparation of Competent Cells

E. coli competent cells were prepared according to established protocols. *E. faecalis* competent cells were prepared as previously described with modifications (92). Briefly, a 5 ml culture of *E. faecalis* OG1RF was grown for 12-15 hours at 37°C in M17 broth (Difco), diluted 1:100 in 100 ml of SGM17 media containing 5% glycine, and grown overnight at 37°C. Cells were collected at 4°C and washed two times with ice-cold electroporation buffer (0.5M sucrose, 10% glycerol). After the second wash, cells were resuspended in a minimum volume of electroporation buffer and 40 µl aliquots were either used immediately or stored at -80°C.

Electroporation Conditions

In preparation for electroporation, *E. coli* and *E. faecalis* cells were thawed on ice for 5 minutes along with 2 mm electroporation cuvettes and the resuspension media. Plasmid DNA was added (2-3 µl) to each aliquot and allowed to chill for 5 additional minutes. The cell/DNA suspension was transferred to a pre-chilled cuvette and pulsed at 25 µF, 200 Ohms, at 2.5 kV using a Bio-Rad Gene Pulser Xcell electroporator. After pulsing, the cell/DNA suspension was resuspended in 400 µl of BHI and incubated on ice for 5 minutes. The cells were transferred to 37°C for 2 hours and spread on plates containing the appropriate antibiotics for selection.

Construction of Promoter-β-galactosidase fusions in pTCV-*lac*

The primers listed in Table 2 were used to amplify regions of the OG1RF genome encoding the predicted promoter region of *ebsG* for fusion to a promoterless *lacZ* reporter. The PCR products were digested with *Eco*RI and *Bam*HI and cloned into pTCV-*lac*, immediately 5' to the promoterless *lacZ* gene. Reporter fusions were propagated in *E. coli* and correct fragment insertion was identified by colony PCR using primers Vlac1 and Vlac2 (80), which amplify across the cloning site. Plasmid DNA was isolated as described and introduced into *E. faecalis* OG1RF by electroporation. Transformants were plated on BHI agar supplemented with erythromycin and X-Gal (150 µg/ml) for the detection of β-galactosidase expression.

β-Galactosidase Activity Assay

β-galactosidase assays were performed according to the method of Miller with some modifications (67). After growing cells in respective media conditions, cells were harvested and cell pellets were washed once with 1.5 ml Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) and then resuspended in 1.5 ml Z buffer. The cell suspension was added to a 2.0 ml screw cap tube which contained 0.5 ml volume of 0.1 mm diameter glass beads (BioSpec Products, Inc.). Cells were disrupted for 60 seconds at 4,800 rpm in a mini-beadbeater (BioSpec Products, Inc.). The tubes were centrifuged briefly to pellet beads and the aqueous layer removed and centrifuged for 10 min at maximum speed in a tabletop centrifuge. Five hundred microliters was removed

and used for the β-galactosidase assay and the remaining liquid was used to assay total protein content using the BCA Protein Assay kit (Pierce). For the Miller assay, 100 µl of ONPG substrate (4 mg/ml *o*-nitrophenyl-β-D-galactoside in Z buffer) was added to 0.5 ml of supernatant and the reaction was allowed to develop for 30 minutes in a 37°C water bath. The reaction was stopped by adding 0.25 ml of 1 N Na_2CO_3. The color change was quantified using a microtiter plate reader at 405 nm. Samples were assayed in triplicate and data is expressed as fold induction over empty vector.

RNA Isolation and Real-time quantitative RT-PCR

E. faecalis OG1RF total RNA was collected from cells grown between 2 and 6 hours in BHI at 37°C using the RNeasy Mini kit (QIAGEN). Quantification of total RNA was spectrophotometrically determined using a Nano-Drop ND-1000 spectrophotometer (NanoDrop). RNA concentrations from each time point were standardized to 500 ng/µl. cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad Laboratories) using random primers. Each reaction contained: 4 µl 5X reaction mixture, 1 µl iScript reverse transcriptase, 2 µl RNA template (1 µg), and dH_20 for a total reaction volume of 20 µl.

For Real-time quantitative RT-PCR, the iQ SYBR Green Supermix (Bio-Rad Laboratories) was used with a typical reaction containing the following: 1 µl from each 20 µl cDNA reaction, 150 nM of the appropriate forward and reverse primers, 10 µl of iQ SYBR Green Supermix, and dH_20 for a total reaction volume of 20 µl, according to the manufacturer's instructions on an iCycler iQ real-time thermocycler (Bio-Rad Laboratories). Fluorescence detection was visualized using iCycler iQ real-time detection software. For use as a positive control, primers were designed to amplify a 110 bp target of *gyrB*. As additional controls, reactions containing no template were used to measure interference from primer dimer formation and reactions containing no reverse transcriptase were used to assess genomic DNA contamination.

Gene Linkage Analysis

Gene linkage analysis was done using cDNA from OG1RF cells grown for 4 hours. Primer pairs specific for each intergenic junction were used to amplify across

these junctions by PCR. The following is the predicted sizes for the PCR products of each junction: product of the *EF0774-ebsG* junction (610 bp); product of the *ebsG-ebsH* junction (474 bp); product of the *ebsG-I* junction (829 bp); product of the *ebsI-ebsJ* junction (320 bp); product of the *ebsJ-ebsK* junction (214 bp); and the product of the *ebsK-EF0781* junction (481 bp).

Production of Rabbit Polyclonal Antibodies

A 780 bp fragment of *ebsG* excluding the signal peptide was cloned into pET28B. Purification was performed according to the manufacturer's instructions. Purified protein was injected into New Zealand white rabbits according to standard protocols (H. Hirt, personal communication).

Transmission Electron Microscopy

From an overnight culture, 1.5 ml of cells were collected by centrifugation, washed 3 times with PBS, and resuspended in PBS + 5% goat serum. The cells were incubated with prepared primary antibody solution (1:50 in PBS) for 2 hours at RT and washed 3 times with PBS + 5% goat serum. The cells were then incubated with 1:40 12 nm Colloidal Gold-AffiniPure goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) for 1 hour at RT. Cells were washed 3 times in PBS and collected by centrifuging at low speed. Cells were fixed in a buffer containing 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2-7.4) overnight at RT with constant rotation. The samples were washed 3 times in 0.1 M sodium cacodylate buffer and were post-fixed with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1-2 hours until the sample was black or amber in color. The sample was washed 3 times in 0.1 M sodium cacodylate buffer, 3 times in 0.2 M sodium acetate buffer (pH 5.2), and pre-embedded with 2% uranyl acetate in 0.2 M sodium acetate buffer for 1 hour, protected from the light. The samples were again washed 3 times in 0.2 M sodium acetate buffer and dehydrated with a sequential treatment of 50, 60, 70, 80, 90, 95, and 100% ethanol. Polymerization was carried out at 60°C in EMBED 812/Araldite resin.

Sample blocks were trimmed and silver to gold thin sections were cut on a Reichert Ultracut S ultramicrotome (Leica). Thin sections were placed on 200 mesh

copper grids and viewed on a CM100 (FEI Company) transmission electron microscope at 100 kV. Images were captured using a Hamamatsu digital camera (C8484 and Advanced Microscopy Techniques Corp.) with image capture engine software version 5.4.2.22B.

EbsG Expression using Flow Cytometry

Overnight cultures were inoculated 1:100 in 5 ml fresh media and grown for 6 hours, with samples taken every 30 minutes between 2 and 6 hours. Cells were collected at 4°C and resuspended in 5 ml 0.9% NaCl. A concentration of 1.0 x 10^6 cells was taken from each time point and resuspended in 200 µl of prepared primary antibody solution (1:50 in 0.9% NaCl) for 1 hour at room temperature with rotation. Cells were collected, resuspended in 100 µl 0.9% NaCl, and incubated for 1 hour at room temperature in the dark with 10 µl of Alexa Fluor 488 goat anti-rabbit IgG (100 µg/ml concentration, Invitrogen). Cells were again collected, resuspended in 1 ml 0.9% NaCl, and kept on ice. Flow cytometry analysis of EbsG expression was performed on a Becton Dickinson FACSCalibur flow cytometer for a minimum of 30,000 gated events. OG1RF::pMSP7551 was used as a positive control, with stepwise increases in nisin concentration from 0-25 ng/ml. OG1RF::*ebsG* was used as a negative control and unstained OG1RF cells were used to subtract out any autofluorescence of the bacterial cells. The same experiment was performed with cells grown in serum, which were washed 3 times with 0.9% NaCl before labeling.

SDS-PAGE and Western Blot Analysis

Overnight cultures were inoculated 1:100 in fresh media and samples were taken every hour between 2 and 5 hours for wild type OG1RF. For the *ebsG* mutant and OG1RF::pMSP7551 uninduced and induced with 20 ng/ml nisin, cells were grown for 4 hours. The samples were collected by centrifugation, washed 1-2 times with PBS, and resuspended in a small volume (typically 100 µl) of extraction buffer (5 mg/ml lysozyme, 0.5 mM PMSF, 50 mM Tris pH 8.0, 25 mM EDTA pH 8.0) for 1 hour with rotation at RT. The extracted cells were collected by centrifugation and the protein concentrations were determined using a Nano-Drop ND-1000 spectrophotometer (NanoDrop). The

extracts were separated by sodium dodecyl sulfate-8% PAGE by loading equal amounts of protein per lane.

The proteins were transferred to a BA 85 nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.) and blocked overnight in PBS-T + 10% milk powder. The membrane was rinsed with PBS-T and incubated with anti-EbsG antibody (1:1000) for 2 hours at RT with shaking. The membrane was washed 6 x 5 mintues in PBS-T and incubated with HRP-goat anti-rabbit IgG (Invitrogen) for 1 hour at RT. Development of the blot was performed using the SuperSignal West Pico Chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

Lipoteichoic Acid Purification and Analysis

LTA purification was performed according to the butanol extraction method (68). Briefly, 4 l of media (THB supplemented with 1% glucose) was inoculated with a 5.0 ml overnight culture and incubated at 37°C for 12 hours. Cells were harvested for 15 mintues at 6000 rpm and the pellet was resuspended in 20 ml ice-cold 0.1 M sodium citrate (pH 4.0). Bacterial cells were disrupted with 0.1 mm glass beads in a beadbeater (Glen Mills, Inc.) 2 times for 2 minutes each. The cell suspension was extracted with an equal volume of butanol for 30 minutes at room temperature with vigorous stirring. After centrifugation for 30 minutes at 4000 rpm, the aqueous phase was removed and the extraction procedure was repeated by adding 0.1 M sodium citrate (pH 4.0) to the interphase and butanol. The aqueous phases were combined and centrifuged for 20 minutes at 17,000g. The supernatant was lyophilized and resuspended in a minimum volume of 0.1 M sodium acetate buffer (pH 4.7, 15% propanol). The lysate was subjected to hydrophobic interaction chromatography on an octyl-Sepharose column equilibrated with 0.1 M sodium acetate buffer. Elution was performed using a linear gradient (15 to 80% propanol) in 0.1 M sodium acetate buffer and aliquots of 5 ml were collected, with every 3rd fraction being assayed for phosphate content according to previously described methods (13). Fractions containing phosphate were pooled and dialyzed against water in Spectra/Por 3500 Da cut-off dialysis tubing (Spectrum Laboratories) and the resulting material was lyophilized.

The resulting lyophilized material was hydrolyzed with 48% hydrofluoric acid at 2°C for 48 hours followed by vacuum drying over KOH at 2°C. Phase separation was performed in cholorform/methanol/water at 1:1:0.9 v/v. The aqueous and organic phases underwent 2.0 M HCl hydrolysis at 100°C for 2.5 hours and were neutralized with NaOH.

The components of LTA were analyzed as follows. Glyerol content was determined using Glycerol Reagent A (Zen-Bio, Inc.). Glucose was determined using the Glucose Assay kit (Sigma). D-alanine was determined by the D-amino acid oxidase method (5). Phosphate was determined as previously described (13).

Autolysis Assay

Cell autolysis was determined by modifications of previously described methods (81). Five-milliliter cultures of OG1RF, OG1RF::*ebsG*, and OG1RF::pMSP7551 with or without 25 ng/ml nisin were inoculated 1:100 in THB and grown to either mid-log or stationary phase. The cells were washed 3 times with dH_20 at 4°C, and resuspended in 5 ml of 10 mM sodium phosphate buffer (pH 6.8) with trypsin (0.5 μg/ml). The suspension was incubated in a 37°C water bath and the OD_{675} was measured every 30 minutes for 4 hours.

Nisin Challenge

Five-milliliter cultures of OG1RF, OG1RF::*ebsG*, and OG1RF::pMSP7551 with or without 25 ng/ml nisin were inoculated 1:100 in THB and grown at 37°C for 150 minutes, with the OD_{600} being measured every 30 minutes. Nisin was added to the cultures so they each contained a final concentration of 250 ng/ml and the OD_{600} was measured for an additional 4 hours.

Mating Assay

Mating assays were performed according to previously described methods (69). Briefly, overnight cultures of donor (OG1SSp::pCF10) and recipient (OG1RF and OG1RF::*ebsG*) cells were inoculated 1:10 into fresh THB for 2 hours at 37°C. In the case of the donor strain, 10 ng/ml of pheromone cCF10 was added. After 2 hours, 1 part donor culture was added to 9 parts recipient culture and mating was allowed to proceed for 10 minutes at 37°C. The mixture was then plated in triplicate on media selective for

the donors (1 mg/ml streptomycin) or for the transconjugants (200 μg/ml rifampicin + 10 μg/ml tetracycline).

Mating assays with concanavalin A were performed as above, with concanavalin A (100 ng/ml) added just before the 10 minute mating period. The mating suspension was plated as above to select for donors and recipients.

Microtiter Plate Assay for Biofilm Formation

Biofilm formation was assayed according to previously described methods with some changes (24). Briefly, Costar 3590 polystyrene 96-well plates (Corning Inc.) were filled with 180 μl of tryptic soy broth (TSB) without the addition of dextrose and 20 μl of overnight culture and incubated at 37°C for 24 hours. The plates were read in a microtiter plate reader at OD_{630}, the culture medium was discarded, and the plates were washed 3 times with PBS. The plates were dried for 1 hour at 60°C and stained for 2 minutes with 2% crystal violet. The stain was removed by rinsing the plate with tap water, and the plates were dried for 10 minutes at 60°C. The OD_{630} was determined and biofilm formation was normalized to growth with the biofilm index, which is calculated as OD of biofilm x (0.5/OD of growth) (18). Each strain was tested in triplicate.

Silicone Elastomer Disk Assay for Biofilm Formation

Silicone elastomer sheets were obtained from Cardiovascular Instrument Corp. (Wakefield, MA.). As per the manufacturer's instructions, the material was cleaned by washing in hand soap and water and rinsed in dH_2O. Flat circular disks, 9 mm in diameter, were obtained by cutting with a cork borer (57) and were sterilized by autoclaving. Biofilm formation was measured by previously described methods with modifications (57, 82). A sterile silicone disk was placed at the bottom of each well in a Costar 3524 24-well culture plate (Corning, Inc.). Overnight cultures of OG1RF, OG1RF::pMSP3535, OG1RF::*ebsG*, and OG1RF::pMSP7551 uninduced and induced with nisin were inoculated 1:100 in fresh THB and 1 ml of each cell suspension was added to the wells and incubated at 37°C for 24 hours. Each strain was tested with three disks. The broth was removed and replaced with 1 ml 0.9% NaCl, and the wells were shaken for 30 minutes. The saline was discarded to remove any planktonic organisms.

The disks were then individually placed in 15-ml polystyrene tubes containing 5 ml of 0.9% NaCl. The tubes were sonicated for 45 seconds and vortexed for 15 seconds. Samples of the cell suspensions were plated in triplicate and the numbers of colonies per milliliter were counted.

E. faecalis Adherence to Extracellular Matrix Molecules (ECM)

Bacterial adherence to ECM was measured using the ECM Cell Adhesion Array kit (Chemicon) with some modifications. Overnight cultures of OG1RF, OG1RF::*ebsG*, or OG1RF::pMSP7551 induced with nisin were inoculated 1:100 in fresh media and allowed to grow until late exponential/early stationary phase. After rehydrating the appropriate number of plate strips with PBS, 100 µl of the cell suspensions were added to each well in a strip and incubated at 37°C for 2 hours. The media was removed and the wells were washed 2 times with Assay Buffer (provided in kit). The Cell Stain Solution (provided in kit) was added to each well for 5 minutes at RT and then the strips were washed gently with dH_2O 3-5 times. Extraction Buffer (provided in kit) was added to each well with gentle rotation until the cell-bound stain was completely solubilized, about 10 minutes. Absorbance was determined on a microtiter plate reader at 540-570 nm.

Manduca sexta Animal Model

Overnight cultures of OG1RF or OG1RF::*ebsG* were diluted to a concentration of approximately 1.0×10^6 cells in 50 µl of PBS. Ten fifth-instar *Manduca sexta* larvae were injected for each strain, incubated at 26°C, and monitored daily for survival.

Results

EbsG is a novel surface protein of *E. faecalis*

The first open reading frame in the operon (Fig. 5), named *ebsG*, encodes a protein of 962 amino acids. It contains an LPXTG motif characteristic of other Gram-positive surface proteins. Genes *ebsH* and *ebsI* encode proteins of 127 and 169 amino acids, respectively, and are similar to other hypothetical proteins of Gram-positive bacteria. The next open reading frame, named *ebsJ*, encodes a protein of 595 amino acids and is included in the glycerophosphoryl diester phosphodiesterase (GPDP) protein family. This protein family is involved in fatty acid and phospholipid metabolism and hydrolyzes deacylated phospholipids to glycerol-3-phosphate and corresponding alcohols. The last open reading frame in the operon, *ebsK*, encodes a peptide of 135 amino acids and is a member of the MutT/nudix protein family. This protein family is a superfamily of Mg^{2+}-requiring enzymes that catalyze the hydrolysis of nucleoside diphosphates linked to other moieties (66).

Upon examination of the amino acid sequence of *ebsG*, we discovered there are four repeats of 154 amino acids in strain OG1RF (H. Hirt, personal communication). Interestingly, there are only three of these repeats in strain V583. The V583 protein sequence of *ebsG* is 154 amino acids shorter than the protein sequence from OG1RF. The function and importance of these repeats is unknown.

BLASTp analysis (www.tigr.org) of the EbsG amino acid sequence from *E. faecalis* V583 reveals this gene is a novel surface protein with weak homology to other surface proteins in Gram-positive bacteria. A hypothetical protein from *S. epidermidis* shows 25.6% identity to the region of EbsG containing the 151 amino acid repeats and appears to contain three 148 amino acid repeats similar to the organization in EbsG. EbsG also shows 32.3% identity to a putative collagen adhesion protein from *B. cereus*. It is interesting to note that EbsG shows 24.3% identity to Asa1 on plasmid pTEF1 of *E. faecalis* and also shows 22.3% and 22.1% identity to two putative AS proteins located on the V583 chromosome.

The N-terminus of *ebsG* (amino acids 77-246) shows homology to von Willebrand Factor (vFW) type A domain, which in humans is a glycoprotein that participates in platelet adhesion to sites of vessel wall injury (52). The A domain of vWF is involved in binding to the platelet receptor glycoprotein Ib through conformational changes exposing the domain after other regions of vWF bind collagen on the subendothelium. This domain also contains distinct binding sites for heparin and sulphatides (47). The protein also contains a sequence motif representing a metal-ion-dependent adhesion site (MIDAS) that confers divalent metal-dependent binding to ligands (79). Interestingly, amino acids 166-331 of *ebsG* have homology to the central region of DltD. As discussed previously, DltD brings the activated D-alanine and the Dcp in close proximity to each other to allow the complex to be transferred across the membrane by DltB. This may be important to note since it is hypothesized that *ebsG* and its downstream genes may play a role in LTA structure.

Analysis of LTA

After purification of LTA from stationary phase *E. faecalis* wild type, the *ebsG* mutant, and cells carrying pMSP7551 induced with nisin, the resulting material was hydrolyzed and analyzed for glycerol, phosphate, glucose, and D-alanine content. For each strain, the glycerol to phosphate ratio was 1:1 as expected (Table 3). We observed glucose to glycerol ratios of 0.72 in the *ebsG* mutant compared to 0.32 in wild type cells, which is an increase of over 2-fold (Fig. 6). In cells carrying pMSP7551 induced with nisin, we observed a glucose to glycerol ratio of 0.19, which is nearly a 2-fold decrease compared to wild type. There was no difference in the ratio of glycerol to D-alanine in any of the strains tested. This data suggests *ebsG* may be involved in removing the glucose moieties from LTA when cells are in stationary phase.

Table 3. LTA components in *E. faecalis* strains (mmol/ml).

Strain	Glycerol	Phosphate	Glucose	D-alanine
OG1RF	2.16	2.03	0.69	1.00
OG1RF::*ebsG*	2.04	1.97	1.46	0.82
OG1RF::pMSP7551	2.04	2.01	0.41	0.94

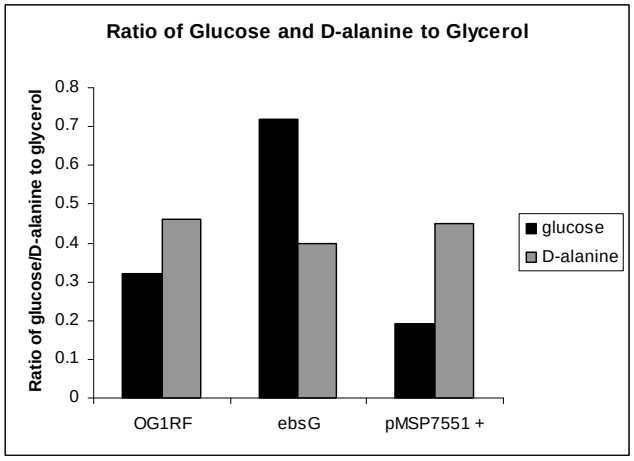

Figure 6. Ratio of glucose to glycerol and D-alanine to glycerol in *E. faecalis* strains (n = 1).

Transcriptional linkage of *ebsG* and its downstream genes

To confirm the transcriptional linkage of *ebsG* and its downstream genes, cDNA was synthesized from OG1RF total RNA and PCR was employed with primer pairs spanning the junction between each of the open reading frames throughout this region (Fig. 7, top panel). Parallel reactions using RNA as the template to control for genomic DNA contamination failed to yield an amplification product. As shown in the bottom panel of Fig. 7, a product of the expected size was seen for PCR spanning each of the intergenic regions from *ebsG* to *ebsK*, demonstrating the transcriptional linkage of these genes. The primer pairs spanning the junctions upstream of *ebsG* and downstream of *ebsK* did not produce a PCR product, confirming these genes are not transcriptionally linked to those within the *ebsG* operon. It can therefore be concluded that the genes EF0775 (*ebsG*) through EF0780 (*ebsK*) form an operon.

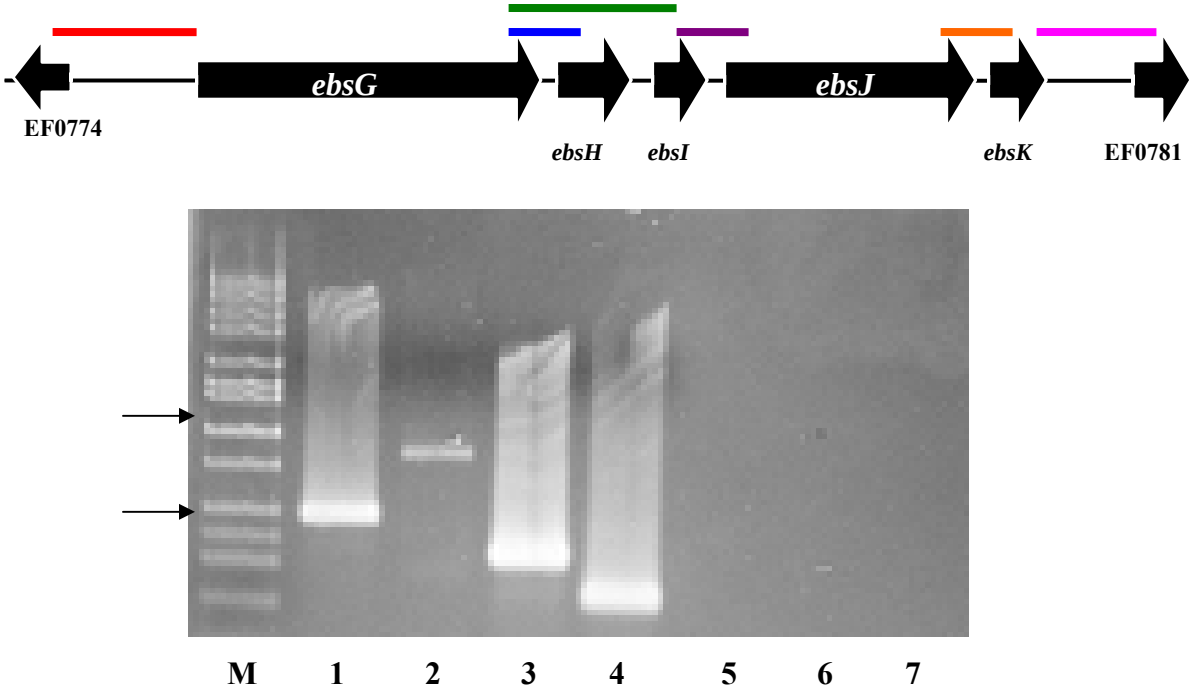

Figure 7. Transcriptional linkage of the genes downstream of *ebsG*. Primer pairs specific to each intergenic junction were used to amplify across these junctions by PCR, shown in the diagram above. The base pairs in parentheses are the predicted sizes for the PCR products and the colors correspond to the location of the PCR product with respect to the operon. Lane 1, PCR product of the *ebsG-ebsH* junction (474 bp, blue); lane 2, product of the *ebsG-ebsI* junction (829 bp, green); lane 3, product of the *ebsI-ebsJ* junction (320 bp, purple); lane 4, product of the *ebsJ-ebsK* junction (214 bp, orange); lane 5, product of the EF0774-*ebsG* junction (481 bp, red); lane 6, product of the *ebsK*-EF0781 junction (481 bp, magenta); and lane 7, RNA control using primer pair for *ebsG-ebsH* junction. The molecular ladder is shown on the far left (M). Arrows indicate 1000 bp (upper) and 500 bp (lower) respectively.

EbsG is located on the cell surface of *E. faecalis*

To confirm EbsG is located on the cell surface, transmission electron microscopy (TEM) was performed using a polyclonal anti-EbsG antibody for labeling, along with a

12-nm-diameter gold particle-labeled secondary antibody. Labeling was successful using OG1RF::pMSP7551 induced with nisin, as shown in Fig. 8C. *E. faecalis* OG1RF cells grown for four hours were also examined but very little labeling could be seen on the cell surface (Fig. 8A), suggesting the protein is not expressed high enough to detect by TEM at this time point. The *ebsG* mutant showed no labeling on the cell surface (Fig. 8B). Uninduced pMSP7551-carrying cells were used as a negative control and did not show any labeling (Fig. 8D).

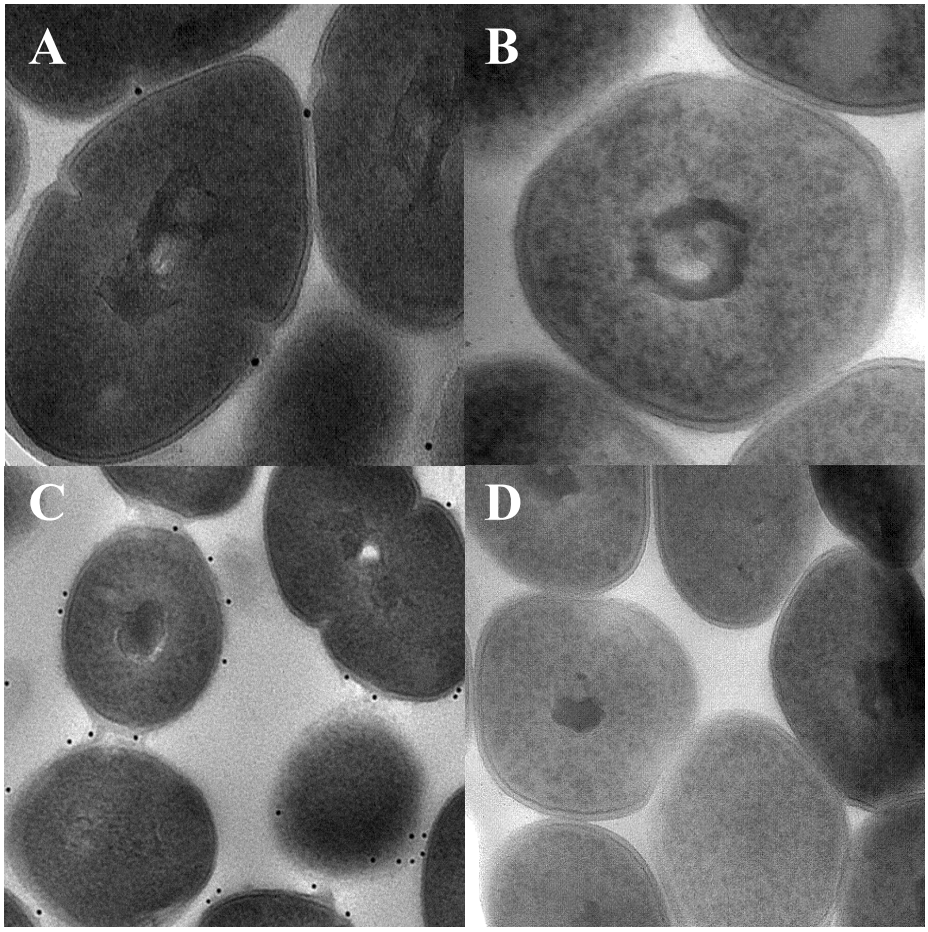

Figure 8. Electron microscopy of EbsG on the surface of *E. faecalis*. A primary rabbit polyclonal antibody against EbsG was used, followed by a 12-nm diameter gold particle-labeled secondary antibody (see Materials and Methods). (A) OG1RF; (B) OG1RF::*ebsG*; (C) OG1RF::pMSP7551 induced with 25 ng/ml nisin; (D) OG1RF::pMSP7551, no nisin induction.

Expression of EbsG in *E. faecalis* by Western blot analysis

The expression of EbsG in *E. faecalis* was investigated by Western blot analysis of cell surface extracts with anti-EbsG antibodies. We wanted to determine when EbsG is expressed during growth of wild type OG1RF cells. We were not able to determine when the protein is expressed by Western blot analysis because the primary polyclonal antibody was not specific enough and multiple bands were detected in every lane on the blot (Fig. 9). We expected to see a band corresponding to EbsG at 119 kDa, the predicted molecular weight of the protein in OG1RF. When EbsG is overexpressed in cells carrying pMSP7551 induced with nisin, we observed two bands with molecular weights of about 90 kDa and 35 kDa (Fig. 9, lane 1). These same bands appeared very faint in uninduced cells carrying pMSP7551 (Fig. 9, lane 2). This could be a degradation product of EbsG. These same bands present in both induced and uninduced pMSP7551 cells indicates the promoter is not silent and some protein product could still be made despite no nisin induction. There didn't appear to be any major bands missing in the *ebsG* mutant compared to wild type cells and induced cells carrying pMSP7551 (Fig. 9, lane 3). The attempt to remove unspecificity of the antibody by absorption to OG1RF::ebsG cells was unsuccessful (data not shown).

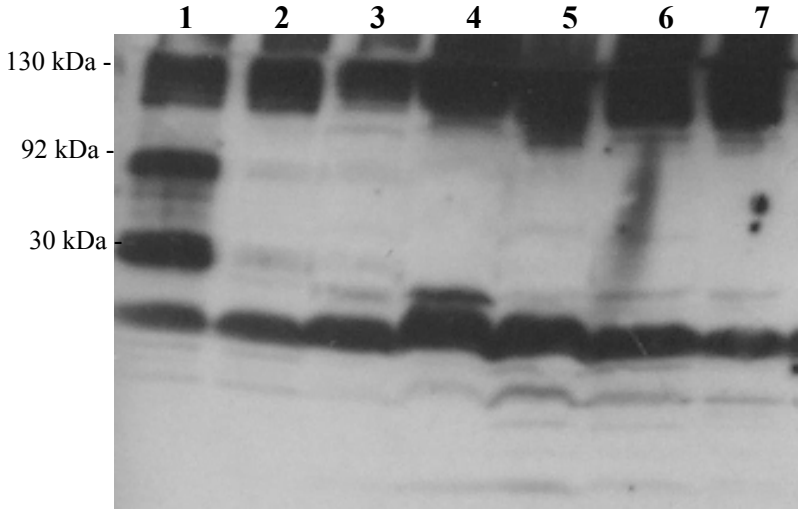

Figure 9. Expression of EbsG from *E. faecalis*. Western blot analysis was performed with a polyclonal antibody against EbsG. The protein concentrations in all lanes are identical. Lane 1, OG1RF::pMSP7551 induced with 20 ng/ml nisin; lane 2, OG1RF::pMSP7551 uninduced; lane 3, OG1RF::*ebsG*; lane 4, OG1RF – grown for 2 hrs; lane 5, OG1RF – 3 hrs; lane 6, OG1RF – 4 hrs; lane 7, OG1RF – 5 hrs.

Because we observed labeling using the antibody directed against EbsG in TEM experiments, but did not see specificity with the Western blot, we hypothesized the antibody recognizes an epitope only displayed when the protein is in its native conformation. To assess this possibility, we performed a Western blot using a native gel under non-denaturing conditions. This was unsuccessful as we were not able to detect any proteins on the blot.

EbsG expression examined by flow cytometry

Flow cytometry was employed to analyze the expression of EbsG during *E. faecalis* growth. *E. faecalis* OG1RF cells were grown for 2-6 hours and samples were taken every half hour. The cells were labeled with anti-EbsG antibody and then labeled with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody and the percent positive for EbsG was measured. Maximal expression was seen during 2-3 hours of growth (Fig. 10, bottom panel). At 2.5 hours of growth, a drop in expression occurred, with partial

recovery at 3 hours of growth. We did not observe any labeling for *ebsG* mutant cells. When EbsG was overexpressed using a step-wise nisin induction scheme, the percentage of positive cells was maximal at 20 ng/ml of nisin, with 80.41% of cells labeling positive for EbsG (Fig. 10, top panel).

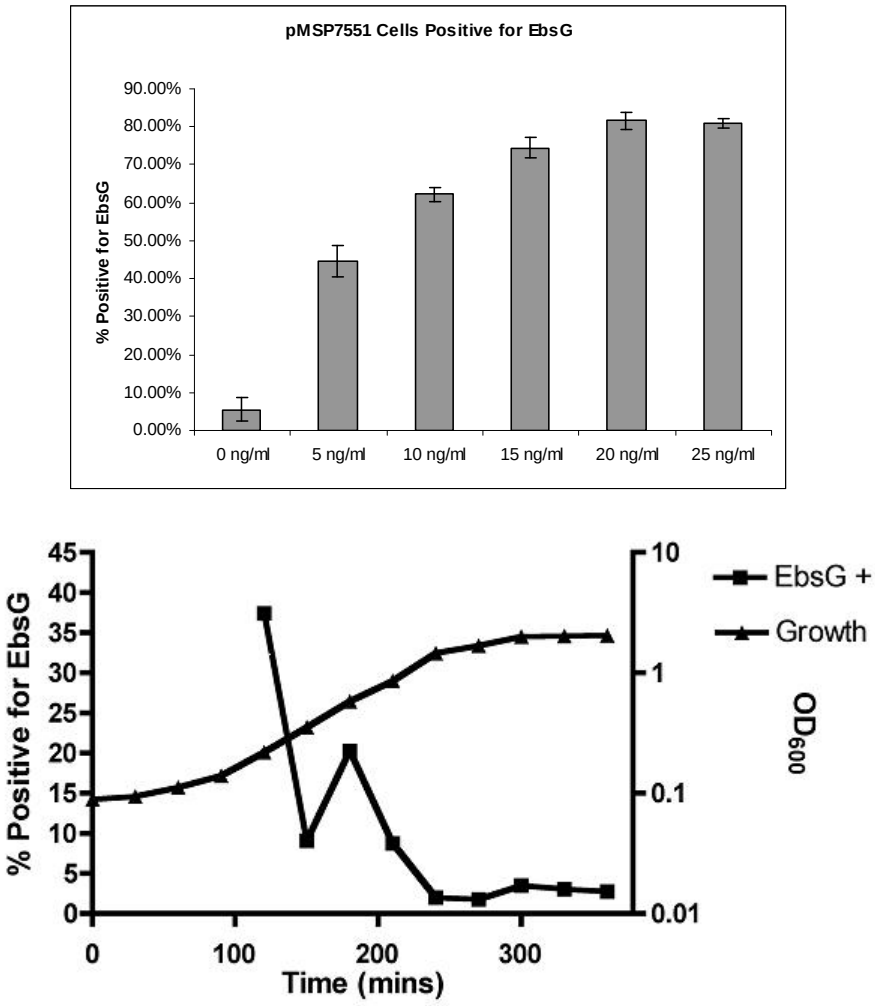

Figure 10. Flow cytometry analysis of EbsG on the surface of *E. faecalis* during growth. Top panel: step-wise nisin induction of cells carrying pMSP7551 show an increase in the percent positive for EbsG as the nisin concentration increases (n = 3). Bottom panel: growth was monitored at 30 minute intervals (right axis) and the percent of cells positive for EbsG was measured at 30 minute intervals between 2 and 6 hours (left axis) (n = 3).

Expression of the *ebsG* operon during growth

To detect the promoter activity of the *ebsG* operon, regions upstream of *ebsG* were cloned in front of a promoterless *lacZ* reporter gene in pTCV-*lac* (80). A PCR fragment spanning a 185 bp region upstream of the predicted start codon of *ebsG* was cloned into pTVC-*lac* to make pMJK201. This vector was transformed into *E. faecalis* and the resulting transformants were assessed for promoter activity as reflected by reporter β-galactosidase activity. No activity over basal levels was detected, suggesting all the elements required for the promoter were not cloned into the vector.

A new vector, pMJK202, was constructed to include 440 bp upstream of the predicted *ebsG* start codon. This vector includes the entire region between *ebsG* and an upstream ORF orientated in the opposite direction, EF0774. *E. faecalis* cells electroporated with pMJK202 produced no blue colonies on plates containing X-gal. DNA sequencing revealed this construct was unstable and therefore was not used to assess promoter activity.

Two more constructs were made, pMJK203 and pMJK204 described in Table 1, that included 233 and 316 bp upstream of the *ebsG* start codon, respectively. These constructs produced white colonies on plates containing X-gal in *E. faecalis* and their sequences were confirmed by DNA sequence analysis. Promoter activity was assayed over time by growing the cells in THB, as shown in Figure 11. Activity is expressed as fold induction over the empty vector pTCV-*lac*. Each of these constructs produced activity only 1- to 2-fold over the empty vector at each of the time points tested. This suggested the operon might not be expressed when cells are grown in normal media.

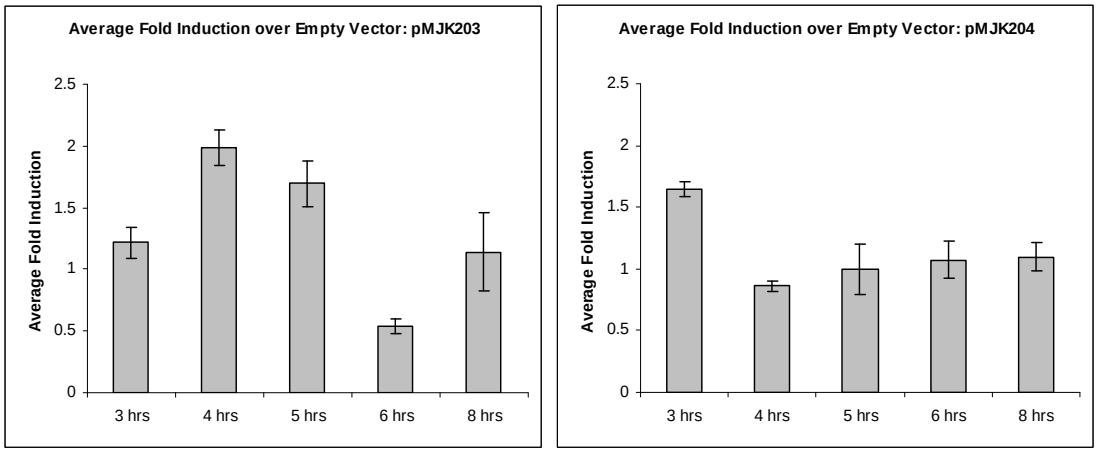

Figure 11. Activity of the *ebsG* promoter in OG1RF carrying either pMJK203 (left panel) or pMJK204 (right panel) over time. Promoter activity was determined by constructing fusions of the upstream region of the *ebsG* operon with a promoterless *lacZ* gene in pTCV-*lac*. Data is presented as the average fold induction over the empty vector (n = 3).

Because we saw only a 1- to 2-fold induction of expression over the empty vector for cells grown in THB, we tried to stress the cells by growing them in diluted media to determine if an environment with limited nutrients initiates transcription. We grew cultures of *E. faecalis* carrying either pMJK203 or pMJK204 in normal THB, ½ X THB, ¼ X THB, or 1/8 X THB and assayed them for β-galactosidase activity. We did not see a difference in expression with cells grown in these media conditions compared to cells grown in normal strength THB (Fig. 12). We also tried growing cells in serum to determine if the *ebsG* operon is a serum-induced gene cluster. We did not see an increase in expression compared to normal or diluted THB (data not shown). This data indicated this operon may be expressed in response to a novel environmental condition.

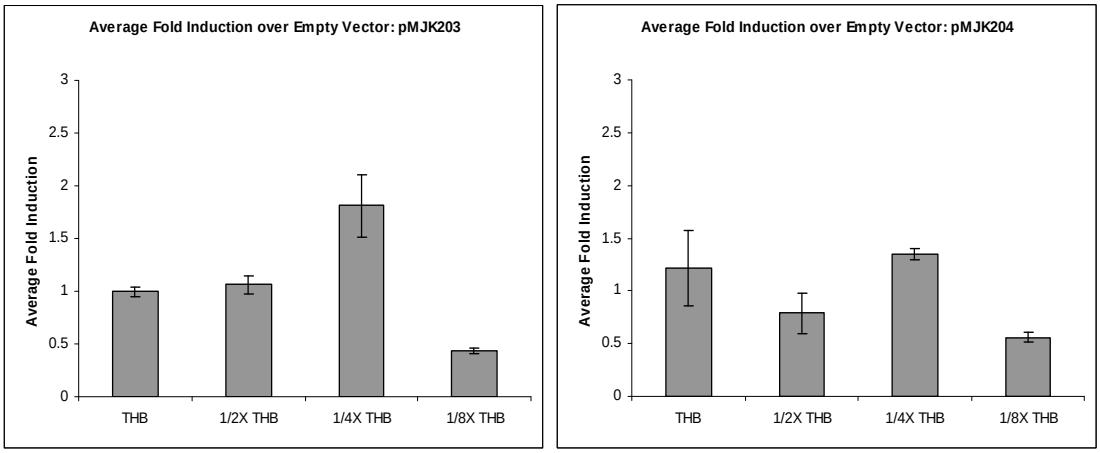

Figure 12. Activity of the *ebsG* promoter in OG1RF carrying either pMJK203 (left panel) or pMJK204 (right panel) grown in diluted media. Data is presented as the average fold induction over the empty vector (n = 3).

In an attempt to identify a factor limiting expression of the *ebsG* operon, pMJK203 and pMJK204 were electroporated into 12 different response regulator (RR) mutants previously identified in *E. faecalis* (35). Response regulators are part of two-component systems which sense an extracellular signal by phosphorylating a histidine kinase, and then transfering the phosphoryl group to the response regulator, which can act as a transcriptional regulator and modulate gene expression (42). No transconjugants were obtained by electroporating pMJK203 into RR10, RR14, and RR18 and no transconjugants were obtained by electroporating pMJK204 into RR14 and RR18. For mutants carrying pMJK203, the highest activity was detected in RR04, with expression being 2-fold over the empty vector (Fig. 13, top graph). For mutants carrying pMJK204, the highest activity was detected in RR10, with expression being almost 2.5-fold over the empty vector (Fig. 13, bottom graph). It is possible this operon could be under the control of RR14 or RR18, but that could not be determined at this time.

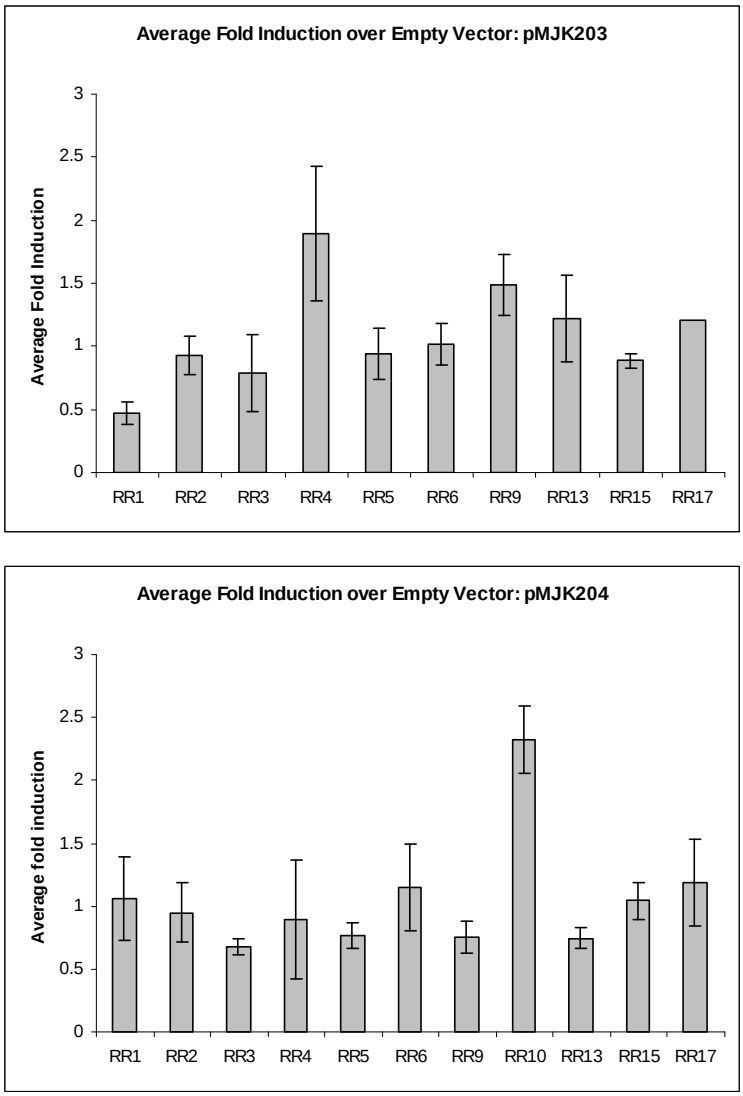

Figure 13. Activity of the *ebsG* promoter in response regulator mutants carrying either pMJK203 or pMJK204. Data is presented as the average fold induction over the empty vector (n = 3).

Cations have been shown to alter LTA structure by changing the level of expression of the genes in the *dlt* operon (54). Therefore, to determine if expression of the *ebsG* operon was up- or down-regulated by extracellular cations, OG1RF::pMJK203 and OG1RF::pMJK204 were grown on plates containing X-gal and 5 sterile 6 mm blank paper disks (Becton, Dickinson and Company) were placed equidistant apart on the plates. Ten milliliters of 1.0 M $MgSO_4$, KCl, $CaCl_2$, NaCl, and $CuSO_4$ were pipetted onto each of the 5 disks and plates were incubated at 37°C. None of the compounds

tested had an effect on promoter activity (data not shown). This experiment was repeated using cationic peptides such as nisin and polymyxin B and other compounds such as EDTA and a high concentration of bile salts. Again, none of the compounds tested had an effect on promoter activity (data not shown).

The same experiment was done testing antibiotic disks containing tetracycline, penicillin, ampicillin, vancomycin, and ciprofloxacin (Becton, Dickinson and Company) to see if these molecules had an effect on expression of the operon. Compared to the background, a slight increase in blue color was seen at the edge of the zone of inhibition for penicillin and ampicillin (data not shown). When cells were challenged with increasing concentrations of penicillin and ampicillin in liquid culture and assayed for β-galactosidase activity, no change in expression was seen over the empty vector.

In order to determine when the *ebsG* transcript appears during *E. faecalis* growth, we used quantitative RT-PCR. After two and three hours of growth, we saw maximal expression of *ebsG* with cycle thresholds (Ct) of 23.33 and 23.13 respectively (Fig. 14). As cells moved from late-exponential to stationary phase, a decrease in the amount of transcript was observed, with an ending Ct of 29.8 at 6 hours of growth. Interestingly, when we examined *ebsG* transcript expression in INY3048 grown for 3 hours, we observed a Ct of 17.26, resulting in an increase of 5.87 cycles compared to wild type expression at the same time point. This data suggests the presence of the Tn*916* insertion upstream of *ebsG* may play a role in the observed INY3000 phenotype.

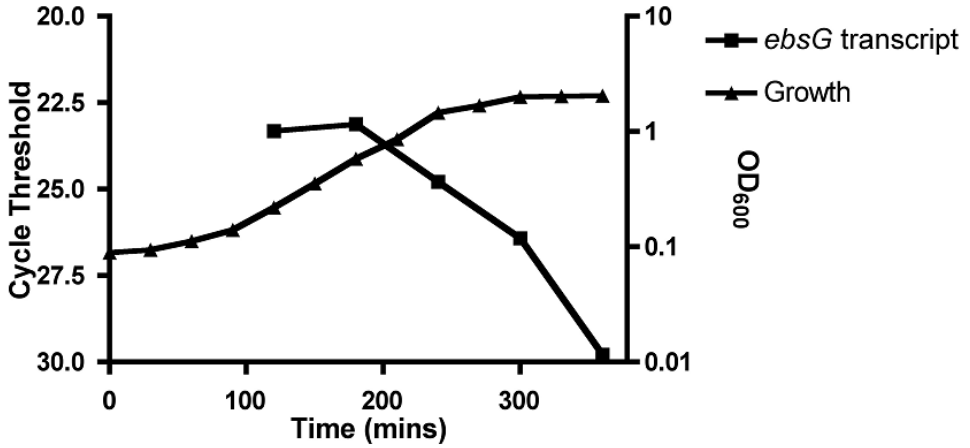

Figure 14. Expression of *ebsG* using qRT-PCR. Abundance of transcript is expressed as cycle threshold on the left axis. Culture growth is expressed as OD_{600} on the right axis.

Autolytic properties of the *ebsG* mutant

An important phenotype seen in mutants that express altered LTA is a change in autolytic activity. In order to detect whether changes in LTA structure have an effect on the autolytic activity of *E. faecalis* cells, cultures of wild type OG1RF, the *ebsG* mutant, and the EbsG overexpressing strain OG1RF::pMSP7551 induced and uninduced with nisin from different growth phases (exponential phase and stationary phase) were used in an autolysis assay. No difference was seen in cultures grown to exponential phase (Fig. 15, top graph). In cultures grown to stationary phase, the *ebsG* mutant lysed faster than wild type and the overexpression of EbsG by strain OG1RF::pMSP7551 induced with nisin showed a slower rate of autolysis (Fig. 15, bottom graph). This suggests that the increased sugar content of LTA from the *ebsG* mutant has an effect on cell autolysis.

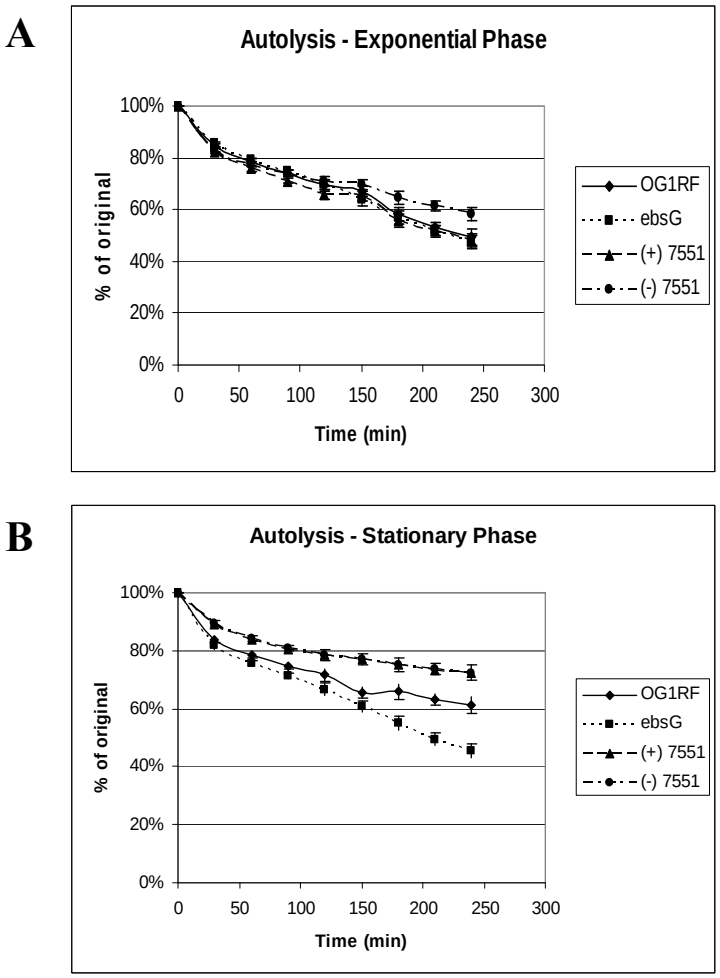

Figure 15. Autolysis assay of exponential (A) and stationary phase cells (B) of *E. faecalis*. Autolysis was monitored at 30 minute intervals for 4 hours in cells grown to mid-exponential phase and stationary phase (n = 3).

The *ebsG* mutant recovers slower when challenged with nisin

Nisin is a small, positively charged, 34-amino acid antimicrobial peptide produced by *Lactococcus lactis* and is used in dairy fermentations and as a food preservative. Nisin is representative of a class of antibiotics that is characterized by the presence of lanthionine, an uncommon amino acid (8). This peptide forms pores in cell membranes and is primarily active against Gram-positive bacteria (65). An increase in the anionic charge of LTA, produced by the removal of D-alanine or kojibiose residues, would result in increased sensitivity to cationic peptides. To test this, we challenged cells

with nisin after 2.5 hours of growth and their rate of recovery was recorded at 30 minute intervals. The *ebsG* mutant recovered slower than wild type OG1RF cells, with growth commencing after 90 minutes for wild type and 150 minutes for the mutant after nisin addition (Fig. 16). This could be explained by the changes on the cell surface due to the altered LTA structure of the *ebsG* mutant. When EbsG is overexpressed, there does not appear to be any effect on growth. Cells carrying uninduced pMSP7551 mirror wild type with respect to recovery, suggesting EbsG must be induced prior to the challenge in order for recovery to occur at a faster rate.

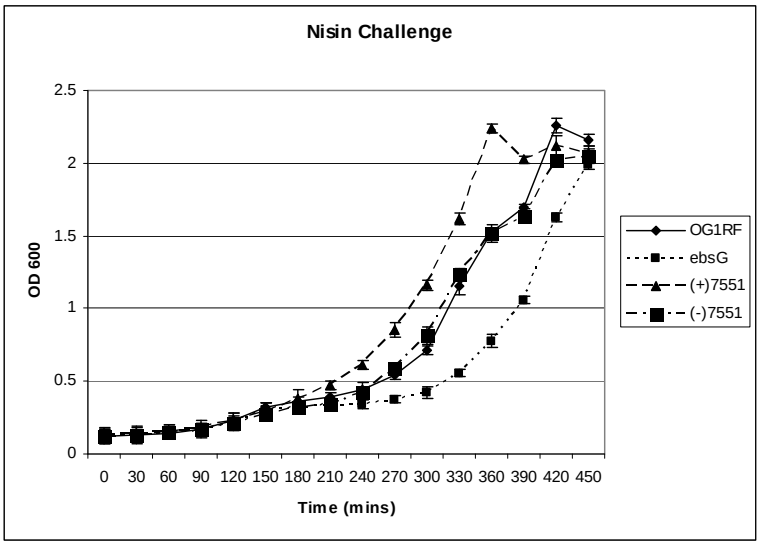

Figure 16. Cells were challenged with 250 ng/ml nisin and recovery was monitored at 30 minute intervals.

Mating

Mating assays were performed to determine if changes in LTA structure or *ebsG* play a role in plasmid transfer. Compared to wild type, the *ebsG* mutant was a better recipient in broth matings by 1.25 fold. In cells where EbsG is overexpressed, a decrease in recipient ability of over 3-fold compared to wild type was seen (Table 4). This suggests that a higher glycosylation ratio of LTA confers an increase in mating ability.

Concanavalin A is a carbohydrate-binding protein (lectin) originally extracted from the jackbean (*Canavalia ensiformis*). Concanavalin A has been used to isolate *E.*

faecalis LTA using affinity chromatography by its ability to bind the kojibiose moieties substituted on the LTA backbone (62). We therefore decided to investigate whether concanavalin A could block mating by binding to the sugar moieties on LTA. In wild type cells, mating decreased by over 10-fold when concanavalin A was added to recipient cells prior to mating (Table 4). In the *ebsG* mutant, a decrease of about 30-fold was seen with the addition of concanavalin A compared to normal mating conditions. When concanavalin A was added to cells overexpressing EbsG, a decrease of 2-fold was seen compared to normal mating conditions. A closer look at the number of transconjugants obtained during mating with concanavalin A revealed *ebsG* mutant cells were impaired as recipients compared to wild type and cells overexpressing EbsG were better recipients.

Table 4. Recipient abilities of *E. faecalis* strains

Recipient strain	Transfer frequency/recipient
OG1RF[a]	4.54×10^{-2}
OG1RF::*ebsG*[a]	5.73×10^{-2}
OG1RF::pMSP7551[a] (induced)	1.40×10^{-2}
OG1RF[b]	3.85×10^{-3}
OG1RF::*ebsG*[b]	1.67×10^{-3}
OG1RF::pMSP7551[b]	6.47×10^{-3}

[a]Broth matings were performed with *E. faecalis* OG1SSp::pCF10 as a donor. Donors were induced for 2 hr with pheromone cCF10, combined 1:10 with recipients, and allowed to mate for 10 mins. [b]Broth matings with concanavalin A.

Biofilm formation is not affected by EbsG

Alterations to cell surface molecules such as LTA can affect several bacterial properties such as adherence and biofilm formation. *E. faecalis*::pMSP3535, the *ebsG* mutant, and OG1RF::pMSP7551 uninduced and induced with nisin were compared regarding formation of biofilm on polystyrene surfaces. No difference was seen between the wild type OG1RF::pMSP3535 and the *ebsG* mutant (Fig. 17). Over-expression of EbsG resulted in a 3.5-fold increase in biofilm density, which suggests that the sugar moieties of LTA do not play a role in the formation of *E. faecalis* biofilms.

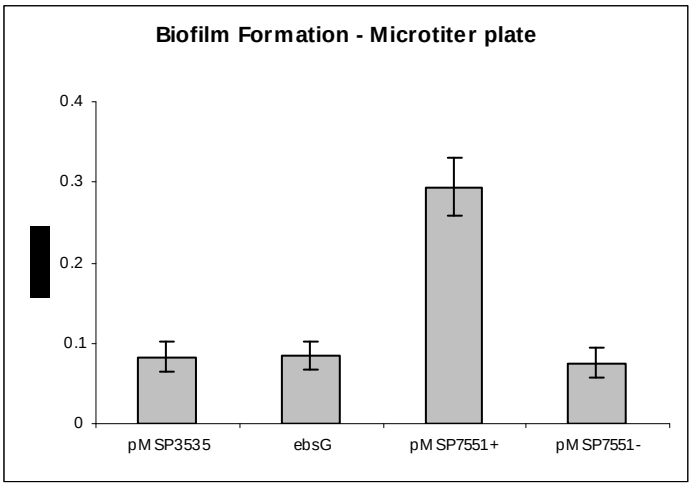

Figure 17. *E. faecalis* strains were tested for biofilm formation in polystyrene microtiter plates. The amount of biofilm is expressed as the biofilm index (Deighton). Error bars represent standard errors of the means (n = 3).

Because enterococci have the ability to adhere to indwelling catheters and form biofilms (31), it was important to examine this property of *E. faecalis* and to determine if *ebsG* and LTA play a role in bioprosthetic colonization. Silicone disks were inoculated with wild type OG1RF, the *ebsG* mutant, OG1RF::pMSP3535, or OG1RF::pMSP7551 uninduced and induced with nisin and biofilms were allowed to form for 24 hours. The disks were washed and sonicated and the cell suspensions were plated in triplicate to determine the number of colony forming units (cfu's) per disk. The *ebsG* mutant had little effect on biofilm formation compared to wild type OG1RF and OG1RF::pMSP3535 (Fig. 18). However, an increase of over 4-fold in biofilm formation was seen with OG1RF::pMSP7551 induced with nisin. If the *ebsG* operon functions to degrade LTA and *ebsG* functions in the removal of sugar moieties from the LTA, this data suggests the sugar moieties are not important in biofilm formation.

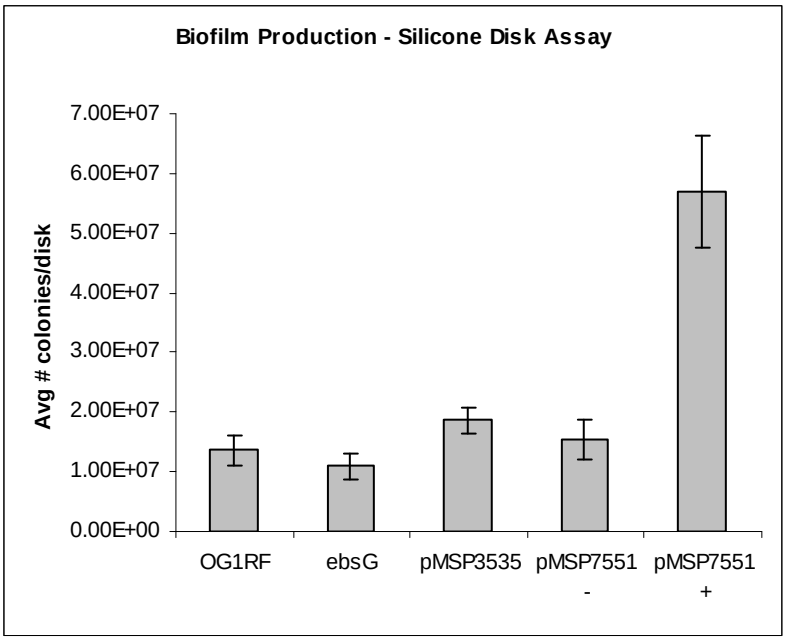

Figure 18. *E. faecalis* strains were tested for their ability to colonize bioprosthetical silicone disks. Error bars represent standard errors of the means ($n = 3$).

Role of EbsG in adherence to Extracellular Matrix Molecules (ECMs)

The adhesion of bacterial cells to ECMs is an important first step in the establishment of an infection. To test whether changes in LTA have an effect on bacterial binding to ECMs, wild type OG1RF, the *ebsG* mutant, and OG1RF::pMSP7551 cells induced with nisin were tested for their ability to bind selected ECMs. The wild type OG1RF did not show strong binding to any of the ECMs tested (Fig. 19). We did not see a difference between the wild type and the *ebsG* mutant in their ability to bind the selected ECMs. Overexpression of EbsG showed slightly higher binding to all ECMs tested compared to wild type. Interestingly, when EbsG is overexpressed, these cells bound 1.5 times more collagen IV than wild type, suggesting EbsG may be involved in binding collagen.

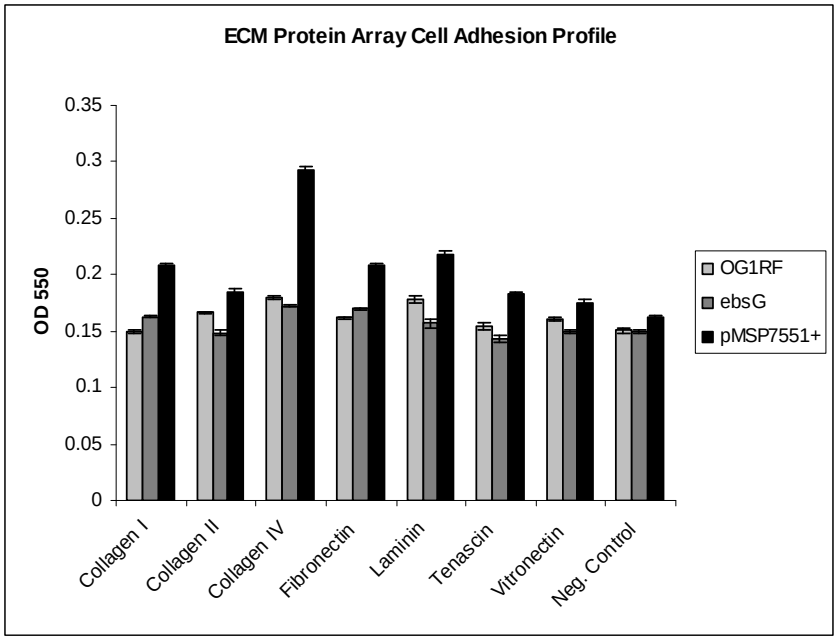

Figure 19. ECM protein cell adhesion array. All strains were tested twice for binding to the selected ECM (n = 2).

Manduca sexta virulence model

M. sexta, the tobacco hornworm, has been used as an insect virulence model because it is easy to produce in large numbers, has a large size which facilitates inoculation, and virulence can be monitored through death (27). This insect model was explored to determine if *ebsG* or changes in LTA structure had an effect on virulence. The first deaths for the *ebsG* mutant occurred on day two, with all worms dying by day 14. For the wild type, the first death occurred on day two and out of 10 worms, one survived and pupated, but did not hatch. There was no significant difference (Logrank test, $p = 0.7648$) in attenuation of virulence between the wild type OG1RF and the *ebsG* mutant (Fig. 20), which suggests *ebsG* does not play a role in virulence.

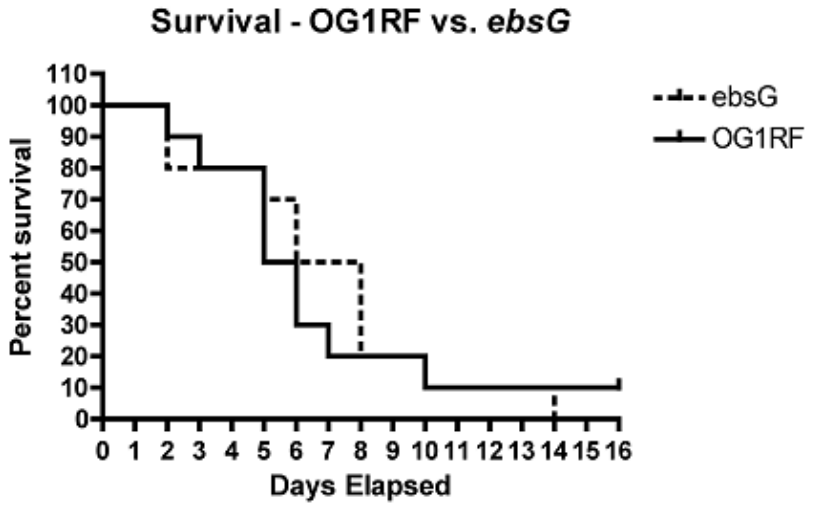

Figure 20. *M. sexta* insect model for OG1RF and OG1RF::*ebsG* virulence. Data is presented as the percent survival vs. the number of days elapsed.

Discussion

Enterococci are becoming increasingly prevalent in hospital-acquired infections. With the emergence of isolates resistant to all antibiotics, including vancomycin, there is great need to develop new therapies which can effectively target these resistant microbes. One potential approach is to target LTA, which is present on the surface of many clinically relevant Gram-positive bacteria. To date, there is no mutant which does not synthesize LTA, so it is assumed this molecule is essential for survival. Unfortunately, the genetic elements involved in LTA structure are not well characterized and little is known about the importance of this molecule to microorganisms.

We have identified a novel operon in *E. faecalis* involved in LTA structure. We decided to focus our research on the first gene in the operon, *ebsG*. Bioinformatics data suggests EbsG is a surface protein with weak homology to other surface proteins in Gram-positive bacteria. We confirmed surface localization with gold-labelled antibodies against EbsG (Fig. 8), however, the amount of labeling was low in wild type cells, giving us a clue that the protein is not expressed at a high level at 4 hours of growth. We did not observe the same level of labeling between wild type cells and cells carrying pMSP7551 uninduced. The reason for this could be that *ebsG* is downregulated in the presence of erythromycin (1), which is needed for pMSP7551 stability.

It is interesting to note that while ORF SE1500 from *S. epidermidis* does not show high sequence identity to EbsG, it does contains three long amino acid repeats similar to the organization of the repeats in EbsG. The functional importance of these repeats is unknown, although it is interesting that EbsG from *E. faecalis* V583 contains only 3 of the 4 repeats. The importance of these repeats to EbsG will have to be investigated.

EbsG shows homology to a collagen binding protein from *B. cereus*, AS proteins from *E. faecalis*, and homology to the A domain of vWF. While these proteins are not involved in LTA structure, they are involved in ligand binding, indicating EbsG may have some adhesin properties or may bind specific sites on LTA. The MIDAS domain present in vWF may suggest EbsG requires the presence of a cation to function properly. The source of these cations could be from LTA on the surface of the cell, since this

molecule is involved in cation sequestering. It was interesting to see EbsG shows homology to DltD. Since DltD is involved in adding D-alanine to LTA, and we propose EbsG is involved in LTA structure, it is feasible the gene products of the *ebsG* and *dlt* operons may work together to alter LTA structure.

Because of the close proximity *ebsG* and its downstream genes have to one another, we hypothesized they might be organized into an operon. Transcriptional linkage analysis has determined *ebsG* and its downstream genes are in fact in an operon (Fig. 7). This suggests these gene products all work together to degrade LTA when the cell needs to alter its LTA structure. The importance of altering LTA structure to the cell has not yet been determined.

One of the genes in the operon, *ebsJ*, is homologous to a GPDP, which has been shown to degrade the unsubstituted glycerophosphate backbone of LTA in *B. pumilus* (58). It is therefore possible this gene could perform the same function in *E. faecalis* and supports our hypothesis that this operon degrades LTA. The last gene in the operon, *ebsK*, belongs to the MutT/nudix protein family, which requires Mg^{2+} to catalyze the hydrolysis of nucleoside diphosphates (66). A role for this protein remains unclear.

The changes in LTA structure in INY3000 and the proximity of the Tn*916* insertion upstream of the *ebsG* operon prompted us to investigate the structure of LTA from strains where *ebsG* is inactive and overexpressed. We observed an increase of over 2-fold in the glucose to glycerol ratio in the *ebsG* mutant compared to wild type (Table 3, Fig. 6). Overexpression of *ebsG* causes a decrease in the glucose to glycerol ratio. This suggests EbsG may be responsible for removing the glucose moieties from LTA. This fits with our hypothesis that this operon alters LTA structure by degrading it. We have proposed the possible function of *ebsJ* is to breakdown the unsubstituted LTA backbone because of sequence alignment to the GPDP family of proteins. If this is the case, EbsG would be needed to remove the glucose units from the backbone before degradation by EbsJ can occur.

The ratios of glucose and D-alanine to glycerol do not add to 1 and there are several possible explanations. The first is that LTA molecules are not homogeneous, meaning some can have more sugar or D-alanine substituents than others. Another possibility is that all the possible sites for glucose or D-alanine to be linked to the

backbone are not occupied, which would explain ratios that are less than 1. In the case of *ebsG* mutant cells, the total ratio of glucose and D-alanine to glycerol is 1.2. This could be possible if glucose and D-alanine are bonded together and then linked to the backbone. The fact that kojibiose is a disaccharide alters the glucose ratio, making it half of what is calculated since the assay is measuring monosaccharides. For the *ebsG* mutant, this would reduce the ratio from 0.72 to 0.36.

Along with our analysis of the components of LTA, we wanted to determine the length of the LTA backbone. To determine the chain length of LTA, the glycerol content from the aqueous phase (after acid hydrolysis) is divided by the glycerol content from the organic phase (after acid hydrolysis). Due to low sensitivity of the assays we performed to determine LTA content, we can not be confident our calculations are correct and therefore did not include this data.

Our attempts to examine when EbsG is expressed during *E. faecalis* growth were hindered for a couple of reasons. Western blot analysis produced multiple bands in each lane and we could not identify which band corresponded to EbsG (Fig. 9). There did not appear to be any major bands missing in the *ebsG* mutant compared to the other lanes. We did, however, find two prominent bands in cells overexpressing EbsG that were smaller than its predicted protein size, which could be degradation products of EbsG. Our attempts to absorb the unspecific antibodies using the *ebsG* mutant produced no bands on the gel. This suggested our antibody may recognize EbsG in its native conformation.

Since we observed labeling using this antibody in TEM experiments, we performed a Western blot under non-denaturing conditions to determine whether the antibody would better recognize EbsG in its native conformation. Our attempts were unsuccessful. This may have been due to the low expression of EbsG, which we observed in TEM experiments and *ebsG* promoter fusion experiments. This could also be due to the fact that the antibody was made with only a portion of the protein. The portion of the protein used may fold differently than full-length EbsG, resulting in an antibody that recognizes an eptitope not present in the normal EbsG structure. To alleviate this problem, the primary antibody will most likely have to be remade.

We constructed promoter fusions to a promoterless *lacZ* gene to determine the expression levels of the operon during growth. We saw a 1- to 2-fold induction over the empty vector for all conditions tested when using regions that included 233 and 316 bp upstream of the *ebsG* start site (Fig. 11). This suggested we might be missing an important element for initiation of transcription or the growth conditions were not optimal for the operon to be expressed, which correlates with our TEM data. Our attempts to clone the entire region upstream of *ebsG* into the expression vector were hindered because of plasmid instability. When the insert was sequenced, we observed a shorter DNA sequence than what we had cloned. A recombination event could have occurred in *E. coli* resulting in this shorter product that produced limited activity as seen in the reporter assays.

We also tried growing the cells in different media conditions, with the thoughts that expression might increase if the cells were stressed. Growth of cells in dilute media (Fig. 12) or serum, and exposure of cells to cations, cationic peptides, and antibiotics did not produce an increase in expression. This suggests the operon might respond to a unique environmental condition which requires the sugar content of LTA to be altered.

In an attempt to increase expression, we hypothesized the operon might be under the control of a RR from *E. faecalis* (35). We transformed our reporter constructs into RR mutants but did not see an increase in expression (Fig. 13), indicating our operon is not under the control of these RR. We did, however, see a slight increase in reporter activity in an RR10 mutant background. This RR has been shown to be involved in stress tolerance and virulence in *E. faecalis* (98), which may implicate a role for EbsG in these processes and correlates with the hypothesis that the operon might be induced under a specific environmental condition. We were not able to obtain any transconjugants by transforming RR14 and RR18 with the reporter constructs, so there is still a possibility the *ebsG* operon might be under the control of one of these RR.

Since we could not accurately detect EbsG by Western blot but we did see labeling using TEM (Fig. 8C), we decided to examine expression using flow cytometry. To our knowledge, flow cytometry has not been used to label bacterial surface proteins. Even though expression was not high, 40% of cells were positive for EbsG at 2 hours, and expression appeared to decrease as cells moved into late-exponential and log phase

(Fig. 10). In stationary phase, only 2-3% of cells labeled positive for EbsG. It is interesting to note that expression was maximal during the earlier stages of growth. Based on LTA structure data, we determined there was no difference between the glucose content of wild type and *ebsG* mutant cells during exponential phase (unpublished data), only during stationary phase, leading us to believe the operon is expressed in stationary phase. We did observe a decrease in expression for cells grown for 2.5 hours, which partially recovered at 3 hours of growth, which we could not explain.

Since we could not accurately determine when EbsG is expressed, we decided to use RT-PCR to determine when the *ebsG* transcript is made during the growth of *E. faecalis*. This data correlates with what we observed using flow cytometry, with maximal expression occurring at 2-3 hours of growth (Fig. 14). As cells move from exponential to stationary phase, a decrease in the amount of transcript was observed. The data obtained by flow cytometry also showed a decrease in expression as cells entered stationary phase. Overall, there does not appear to be an abundance of transcript made at any time during *E. faecalis* growth, which indicates either the operon is not expressed in the growth conditions we used or the gene products are only required in limited quantities. Further examination is necessary to accurately determine when EbsG and the rest of the gene products in the operon are expressed.

Upon examination of *ebsG* transcript expression in INY3048, we observed an increase in the amount of transcript present when compared to wild type. This was interesting because the Tn*916* insertion is just upstream of *ebsG*, which would make one hypothesize the amount of transcript would decrease compared to wild type. This means the transcript is most likely reading out from the end of the transposon and could be partially responsible for shortened LTA phenotype observed in INY3000 (4).

As discussed in the introduction, LTA is proposed to be involved in many cellular processes. Since we observed an altered LTA structure in cells where *ebsG* is inactive, we wanted to investigate what this means to the biology of the cell. LTA has been shown to inhibit autolysins in pneumococcus (43) and the D-alanine content of LTA can inhibit the action of hemolysins and bacteriocins in *E. faecalis* (75). We investigated what effect an increase in glucose content of LTA would have on the rate of autolysis. In cells grown to exponential phase, we did not observe a difference in the rate of autolysis between

wild type, *ebsG* mutant cells, and cells overexpressing EbsG (Fig. 15). When cells were grown to stationary phase, *ebsG* mutant cells lysed at a higher rate compared to wild type cells. Overexpression of EbsG resulted in a slower rate of autolysis. This data suggests the sugar moieties may play a role in regulating autolysis when cells are in stationary phase.

LTA is a negatively charged molecule and has been shown to bind cations in *B. cereus*, *S. aureus*, and *L. buchneri* (12, 37, 54, 60). If the charge of the cell becomes more negative by decreasing the amount of D-alanine substituents on LTA, an increase in sensitivity to cationic peptides has been observed (78). We wanted to investigate whether the sugar moieties of LTA affect cationic peptide binding by challenging cells with nisin. We observed a slower rate of recovery in *ebsG* mutant cells compared to wild type and faster rate of recovery when EbsG is overexpressed (Fig. 16). This data implies the sugar moieties may either directly bind cationic peptides or they mask the anionic charge of LTA, allowing positively charged molecules to bind to the cell surface.

Another important role for LTA in the biology of *E. faecalis* is involvement in EBS and mating aggregate formation. LTA has been shown to bind AS (108) and inhibit conjugative mating (23, 100). INY3000 is deficient in mating ability, which could partially be due to its shortened LTA backbone. We investigated whether the glucose content of LTA would alter the transferability of pheromone-responsive plasmids in *E. faecalis*. Compared to wild type, *ebsG* mutant cells were better able to act as recipients for plasmid pCF10. Conversely, overexpression of EbsG resulted in a decrease in recipient ability (Table 4). This suggests the increase in LTA glucose content confers better conjugative plasmid transfer. To confirm this, we performed mating experiments with concanavalin A, which has been used to isolate enterococcal LTA by binding to glucose moieties (62). We predicted that the binding of concanavalin A to LTA would block mating if the sugar moieties of LTA are involved in mating aggregate formation.

In wild type cells, the addition of concanavalin A blocked mating by 10-fold (Table 4). Cells that contain higher LTA glucose content, as seen in the *ebsG* mutant, had a decreased ability to act as recipients and cells containing a lower LTA glucose content (pMSP7551) were better able to act as recipients. This demonstrates the glucose

moieties on LTA play a role in plasmid transfer by possibly allowing donor and recipient cells to form tighter mating aggregates.

The ability of bacteria to form biofilms is associated with virulence in a number of pathogens, such as *Pseudomonas aeruginosa*, *Streptococcus mutans*, *S. aureus*, and *E. faecalis* (9). There have been implications LTA might play a role in biofilm formation and for this reason, we investigated whether an increase in sugar content of LTA altered the ability to form biofilms in *E. faecalis*. We did not observe a difference in biofilm formation on polystyrene plates or silicone elastomer disks for wild type and *ebsG* mutant cells (Fig. 17 and 18). Overexpression of EbsG resulted in a denser biofilm compared to wild type on both surfaces tested. While this data does not exclude the possibility LTA is involved in biofilm formation, it does indicate the sugar moieties on LTA are not required for biofilm formation.

The adherence of bacterial cells to ECMs is important in the successful establishment of an infection. Since LTA is a surface polymer that might be involved in adhesion, we investigated whether alterations in LTA had an affect on the cell's ability to bind ECMs. We did not see a difference between wild type and *ebsG* mutant cells in their ability to bind ECMs, but we did see a slight increase in the binding of cells overexpressing EbsG to all ECMs tested, and in particular, collagen IV (Fig. 19). This correlates with bioinformatics data indicating EbsG shares homology with a collagen binding protein from *B. cereus*. While this does not support a role for EbsG in LTA structure, it does indicate the protein might have some adhesive properties, which is supported by bioinformatics data.

LTA has been shown to be a virulence factor for Gram-positive bacteria by activating complement, proinflammatory mediators, and immunogens (15, 38, 63, 88, 96). We investigated whether *ebsG* contributes to *E. faecalis* virulence by injecting *Manduca sexta* with *ebsG* mutant or wild type cells and monitoring death. We did not see a significant difference in attenuation of virulence between the wild type and *ebsG* mutant (Fig. 20). Inactivation of the *dlt* operon resulted in attenuated virulence of the mutant compared to wild type (Allen et al., unpublished data) and we hypothesized inactivation of *ebsG* might have the same results. Our data indicates *ebsG* inactivation

does not attenuate virulence in this insect model and LTA glucose content does not play a role in *E. faecalis* virulence.

The data presented here indicate *ebsG* is involved in LTA structure and may alter the structure by removing the kojibiose moieties from the polyglycerophosphate backbone. We have shown an increase in glucose content of LTA plays a role in autolysis during stationary phase, cationic peptide sequestering, and the transfer of conjugative plasmids in *E. faecalis*. This altered LTA structure does not appear to be involved in biofilm formation, virulence, or adherence to ECMs.

We noticed some of the phenotypes we observed in the *ebsG* mutant such as data from autolysis assays and the LTA structural data only when the cells were in stationary phase. This led us to believe *ebsG*, and the rest of the genes in the operon, are expressed during stationary phase. Analysis of the data obtained by RT-PCR and flow cytometry however have shown the *ebsG* transcript and protein are expressed during the earlier stages of growth. This may indicate the gene products of the *ebsG* operon are made during exponential phase but are not functional until stationary phase.

EbsG is a novel suface protein in *E. faecalis*, along with the other members of its operon. It is unclear how the other members of the operon participate in LTA structure, except for *ebsJ*, which probably functions in the breakdown of the glycerolphosphate backbone. Since GPDP proteins have been shown to degrade the unsubstituted LTA backbone (58), this operon may break down LTA by first removing the kojibiose units and then degrading the backbone. EbsG is not crucial to survival and cells grow to levels similar to wild type when it is inactivated, suggesting the process of altering glucose content does not appear to be essential. The reason for altering LTA structure is not known, but may provide the cell with an additional level of defense against cationic peptides and may aid in preventing autolysis. This cellular event must be investigated further.

This would be the first set of genes described to alter the glycosyl content of LTA. The reason for altering LTA structure remains unclear. Studies have shown *S. aureus* can alter the D-alanine content of LTA in the presence of increased cation concentration (54). It is possible the glucose content can also be altered in response to an as yet undiscovered signal. The role LTA plays in the biology of Gram-positive bacteria is still unclear. By

identifying genes involved in LTA structure and determining how these genes are regulated, we can get a better idea of the function of LTA and its purpose not only in *E. faecalis*, but for other medically important Gram-positive species.

Bibliography

1. **Aakra, A., Vebo, H., Snipen, L., Hirt, H., Aastveit, A, Kapur, V., Dunny, G., Murray, B., and I. F. Nes.** 2005. Transcriptional response of *Enterococcus faecalis* V583 to erythromycin. Antimicrob. Agents Chemother. **49:** 2246-2259.
2. **Archibald, A. R., J. Baddiley, and S. Heptinstall.** 1973. The alanine ester content and magnesium binding capacity of walls of *Staphylococcus aureus* H grown at different pH values. Biochem. Biophys. Acta. **291:** 629-634.
3. **Basinger, S. F. and R. W. Jackson.** 1968. Bacteriocin (hemolysin) of *Streptococcus zymogenes*. J. Bacteriol. **96:** 1895-1902.
4. **Bensing, B. A., and G. M. Dunny.** 1993. Cloning and molecular analysis of genes affecting expression of binding substance, the recipient-encoded receptor(s) mediating mating aggregate formation in *Enterococcus faecalis*. J. Bacteriol. **175:** 7421-7429.
5. **Bergmeyer, H. U.** 1983. In *Methods of Enzymatic Analysis*, p. 337-340. Ed. By Bergmeyer, H. U. New York: Academic Press Inc.
6. **Brock, T. D., B. Peacher, and D. Pierson.** 1963. Survey of the bacteriocines of enterococci. J. Bacteriol. **86:** 702-707.
7. **Bryan, E. M., T. Bae, M. Keerebezem, and G. M. Dunny.** 2000. Improved vectors for nisin-controlled expression in Gram-positive bacteria. Plasmid. **44:** 183-190.
8. **Buchman, G. W., S. Banerjee, and J. N. Hansen.** 1988. Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. J. Biol. Chem. **263:** 16260-16266.
9. **Carinol, K., and M. S. Gilmore.** 2004. Signal transduction, quorum-sensing, and extracellular protease activity in *Enterococcus faecalis* biofilm formation. J. Bacteriol. **186:** 8161-8163.
10. **Caron, F., M. Pestel, M. Kitzis, J. F. Lemeland, G. Humbert, and L. Gutmann.** 1995. Comparison of different β-lactam-glycopeptide-gentamicin combinations for an experimental endocarditis caused by a highly β-lactam resistant and highly glycopeptide-resistant isolate of *Enterococcus faecium*. J. Infect. Dis. **171:** 106-112.

11. **Cetinkaya, Y., P. Falk, and C. G. Mayhall.** 2000. Vancomycin-resistant enterococci. Clin. Microbiol. Rev. **13:** 686-707.
12. **Chambers, H. F.** 1988. Methicillin-resistance Staphylococci. Clin. Microbiol. Rev. **1:** 173-186.
13. **Chen, P. S., T. Y. Toribara, and H. Wanner.** 1956. Microdetermination of phophorus. Anal. Chem. **11:** 1756-1758.
14. **Chow, J. W., L. A. Thal, M. B. Perri, J. A. Vazquez, S. M. Donabedian, D. B. Clewell, and M. J. Zervos.** 1993. Plasmid-associated hemolysin and aggregation substance production contributes to virulence in experimental enterococcal endocarditis. Antimicrob. Agents Chemother. **37:** 2474-2477.
15. **Cleveland, M. G., J. D. Gorham, T. L. Murphy, E. Tuomanen, and K. M. Murphy.** 1996. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-depedent pathway. Infect. Immun. **64:** 1906-1912.
16. **Coque, T. M., J. E. Patterson, J. M. Steckelberg, and B. E. Murray.** 1995. Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. J. Infect. Dis. **171:** 1223-1229.
17. **Davie, J. M., and T. D. Brock.** 1966. Effect of teichoic acid on resistance to the membrane-lytic agent of *Streptococcus zymogenes*. J. Bacteriol. **92**: 1623-1631.
18. **Deighton, M., and R. Borland.** 1993. Regulation of slime production in *Staphylococcus epidermidis* by iron limitation. Infect. Immun. **61:** 4473-4479.
19. **Dunny, G. M., and D. B. Clewell.** 1975. Transmissible toxin (hemolysin) plasmid in *Streptococcus faecalis* and its mobilization of a noninfectious drug resistance plasmid. J. Bacteriol. **124:** 784-790.
20. **Dunny, G. M., B. L. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. USA. **75:** 3479-3483.
21. **Dunny, G. M., C. Funk, and J. Adsit.** 1981. Direct stimulation of the transfer of antibiotic resistance by sex pheromones in *Streptococcus faecalis*. Plasmid. **6:** 270-278.

22. **Dunny, G. M., and B. A. Leonard.** 1997. Cell-cell communication in gram-positive bacteria. Annu. Rev. Microbiol. **51:** 527-564.
23. **Ehrenfeld, E. E., R. E. Kessler, and D. B. Clewell.** 1986. Identification of pheromone-induced surface proteins in *Streptococcus faecalis* and evidence of a role for lipoteichoic acid in formation of mating aggregates. J. Bacteriol. **168:** 6-12.
24. **Fabretti, F., C. Theilacker, L. Baldassarri, Z. Kaczynski, A. Kropec, O. Holst, and J. Huebner.** 2006. Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. Infect. Immun. **74:** 4164-4171.
25. **Falagas, M. E., D. K. Matthaiou, and I. A. Bliziotis.** 2006. The role of aminoglycosides in combination with a beta-lactam for the treatment of bacterial endocarditis: a meta-analysis of comparative trials. J. Antimicrob. Chemother. **57:** 639-647.
26. **Fischer, W.** 1988. Physiology of lipoteichoic acids in bacteria. Adv. Microb. Physiol. **29:** 233-302.
27. **Fleming, V. E. Feil, A. K. Sewell, N. Day, A. Buckling, and R. C. Massey.** 2006. Agr interference between clinical *Staphylococcus aureus* strains in an insect model of virulence. J. Bacteriol. **188:** 7686-7688.
28. **Ganfield, M. C. and R. A. Pieringer.** 1975. Phosphatidylkojibiosyl diglyceride. The covalently linked lipid constituent of the membrane lipoteichoic acid from *Streptococcus faecalis* (faecium) ATCC 9790.
29. **Ganfield, M. C., and R. A. Pieringer.** 1980. The biosynthesis of nascent membrane lipoteichoic acid of *Streptococcus faecium* (*S. faecalis* ATCC 9790) from phosphatidylkojibiosyl diacylglycerol and phosphatidylglycerol. J. Biol. Chem. **255:** 5164-5169.
30. **Giessel, B. E., C. J. Koenig, and R. L. Blake, Jr.** 2000. Management of bacterial endocarditis. Am. Fam. Physician. **61:** 1725-1732.
31. **Gray, J., P. J. Marsh, D. Stewart, and S. J. Pedler.** 1994. Enterococcal bacteraemia: a prospective study of 125 episodes. J. Hosp. Infect. **27:** 179-186.

32. Grundling, A., and O. Schneewind. 2007. Genes required for glycolipid synthesis and lipoteichoic acid anchoring in *Staphylococcus aureus*. J. Bacteriol. **189:** 2521-2530.

33. Guthmiller, J. M., E. Kraig, M. P. Cagle, and D. Kolodrubetz. 1990. Sequence of the *lktD* gene from Actinobacillus actinomycetemcomitans. Nucleic Acids Res. **18:** 5292.

34. Gutschik, E., S. Moller, and N. Christensen. 1979. Experimental endocarditis in rabbits. Significance of the proteolytic capacity of the infecting strains of *Streptococcus faecalis*. Acta. Pathol. Microbiol. Scand. **87:** 353-362.

35. Hancock, L. E., and M. Perego. 2004. Systematic inactivation and phenotypic characterization of two-component signal transduction systems of *Enterococcus faecalis* V583. J. Bacteriol. **186:** 7951-7958.

36. Heaton, M. P., L. F. Discotto, M. J. Pucci, and S. Handwerger. 1996. Mobilization of vancomycin resistance by transposon-mediated fusion of a VanA plasmid with an *Enterococcus faecium* sex pheromone-response plasmid. Gene. **171:** 9-17.

37. Heckels, J. E., P. A. Lambert, and J. Baddiley. 1977. Binding of magnesium ions to cell walls of *Bacillus subtilis* W23 containing teichoic acid or teichuronic acid. Biochem. J. **162:** 359-365.

38. Henneke, P., S. Morath, S. Uematsu, S. Weichert, M. Pfitzenmaier, O. Takeuchi, A. Muller, C. Poyart, S. Akira, R. Berner, G. Teti, A. Geyer, T. Hartung, T. Trieu-Cuot, D. L. Kasper, and D. T. Golenbock. 2005. Role of lipoteichoic acid in the phagocyte response to group B *Streptococcus*. J. Immunol. **174:** 6449-6455.

39. Hirt, H., G. Wanner, D. Galli, and R. Wirth. 1993. Biochemical, immunological and ultrastructural characterization of aggregation substances encoded by *Enterococcus faecalis* sex-pheromone plasmids. Eur. J. Biochem. **211:** 711-716.

40. Hirt, H., S. L. Erlandsen, and G. M. Dunny. 2000. Heterologous inducible expression of *Enterococcus faecalis* pCF10 aggregation substance Asc10 in *Lactococcus lactis* and *Streptococcus gordonii* contributes to cell hydrophobicity and adhesion to fibrin. J. Bacteriol. **182:** 2299-2306.

41. Hirt, H., D. A. Manias, E. M. Bryan, J. R. Klein, J. K. Marklund, J. H. Staddon, M. L. Paustian, V. Kapur, and G. M. Dunny. 2005. Characterization of the

pheromone response of the *Enterococcus faecalis* conjugative plasmid pCF10: complete sequence and comparative analysis of the transcriptional and phenotypic responses of pCF10-containing cells to pheromone induction. J. Bacteriol. **187:** 1044-1054.

42. **Hoch, J. A., and T. J. Silhavy.** 1995. Two-component signal transduction. ASM Press, Washington, D.C.
43. **Holtje, J., and A. Tomasz.** 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in pneumococcus. Proc. Nat. Acad. Sci. **72:** 1690-1694.
44. **Hummell, D. S., A. J. Swift, A. Tomasz, and J. A. Winkelstein.** 1986. Activation of the alternative complement pathway by pneumococcal lipoteichoic acid. Infect. Immun. **47:** 384-387.
45. **Huycke, M. M., D. F. Sahm, and M. S. Gilmore.** 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg. Infect. Dis. **4:** 239-249.
46. **Ike, Y., and D. B. Clewell.** 1984. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn*917* as an insertional mutagen. J. Bacteriol. **158:** 777-783.
47. **Jenkins, P. V., K. J. Pasi, and S. J. Perkins.** 1998. Molecular modeling of ligand and mutation sites of the type A domain of human von Willebrand factor and their relevance to von Willebrand's disease. **91:** 2032-2044.
48. **Jett, B. D., M. M. Huycke, and M. S. Gilmore.** 1994. Virulence of enterococci. Clin. Microbiol. Rev. **7:** 462-478.
49. **Johnson, J. R., C. Clabots, H. Hirt, C. Waters, and G. M. Dunny.** 2004. Enterococcal aggregation substance and binding substance are not major contributors to urinary tract colonization by *Enterococcus faecalis* in a mouse model of ascending unobstructed urinary tract infection. Infect. Immun. **72:** 2445-2448.
50. **Jones, R. N.** 1985. Gram-positive superinfections following beta-lactam chemotherapy: the significance of the enterococcus. Infection. **13**(Suppl. 1): S81-S88.
51. **Jorasch, P., F. P. Woler, U. Zahringer, and E. Heinz.** 1998. A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose

residues to 1,2-diacylglycerol: expression of *ypfP* in *Escherichia coli* and structural analysis of its reaction products. Mol. Microbiol. **29:** 419-430.

52. **Kalafatis, M., Y. Takahashi, J. Girma, and D. Meyer.** 1987. Localization of a collagen-interactive domain of human von Willebrand factor between amino acid residues Gly 911 and Glu 1,365. Blood. **70:** 1577-1583.
53. **Kiriukhin, M. Y., D. V. Debabov, D. L. Shinabarger, and F. C. Neuhaus.** 2001. Biosynthesis of the glycolipid anchor in lipoteichoic acid of *Staphylococcus aureus* RN4220: role of YpfP, the diglucosyldiacylglycerol synthase. J. Bacteriol. **183:** 3506-3514.
54. **Koprivnjak, T., V. Malkar, L. Swanson, B. Fournier, A. Peschel, and J. P. Weiss.** 2006. Cation-induced transcriptional regulation of the *dlt* operon of *Staphylococcus aureus*. J. Bacteriol. **188:** 3622-3630.
55. **Kovacs, M., A. Halfmann, I. Fedtke, M. Heintz, A. Peschel, W. Vollmer, R. Hakenbeck, and R. Bruckner.** 2006. A functional *dlt* operon, encoding proteins required for incorporation of D-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in *Streptococcus pneumoniae*. J. Bacteriol. **188:** 5797-5805.
56. **Kreft, B., R. Marre, U. Schramm, and R. Wirth.** 1992. Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. Infect. Immun. **60:** 25-30.
57. **Kuhn, D. M., T. George, J. Chandra, P. K. Mukherjee, and M. A. Ghannoum.** 2002. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob. Agents Chemother. **46:** 1773-1780.
58. **Kusser, W., and F. Fiedler.** 1984. A novel glycerophosphodiesterase from *Bacillus pumilus*. FEBS Letters. **166:** 301-306.
59. **Kuypers, J. M., and R. A. Proctor.** 1989. Reduced adherence to traumatized rat heart valves by a low-fibronectin-binding mutant of *Staphylococcus aureus*. Infect. Immun. **57:** 2306-2312.

60. Lambert, P. A., I. C. Hancock, and J. Baddiley. 1975. Influence of alanyl ester residues on the binding of magnesium ions to teichoic acids. Biochem. J. **151:** 671-676.

61. Leenhouts, K., G. Buist, A. Bolhuis, A. ten Berge, J. Kiel, I. Mierau, M. Dabrowska, G. Venema, and J. Kok. 2004. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol. Gen. Genet. **253:** 217-224.

62. Leopold, K., and W. Fischer. 1991. Separation of the poly(glycerophosphate) lipoteichoic acids of *Enterococcus faecalis* Kiel 27738, *Enterococcus hirae* ATCC9790, and *Leuconostoc mesenteroides* DSM 20343 into molecular species by affinity chromatography on concanavalin A. Eur. J. Biochem. **196:** 475-482.

63. Loos, M., F. Clas, and W. Fischer. 1986. Interaction of purified lipoteichoic acid with the classical complement pathway. Infect. Immun. **53:** 595-599.

64. Mäkinen, P. L., D. B. Clewell, F. An, and K. K. Mäkinen. 1989. Purificaiton and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase ('gelatinase') from *Streptococcus faecalis* (strain OG1-10). J. Biol. Chem. **264:** 3325-3334.

65. Mantovani, H. C., and J. B. Russell. 2001. Nisin resistance of *Streptococcus bovis*. Appl. Environ. Microbiol. **67:** 808-813.

66. Mildvan, A. S., Z. Xia, H. F. Azurmedi, V. Saraswat, P. M. Legler, M. A. Massiah, S. B. Gabelli, M. A. Bianchet, L. W. Kang, and L. M. Amzel. 2005. Structures and mechanisms of Nudix hydrolases. Arch. Biochem. Biophys. **433:** 129-143.

67. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

68. Morath, S., A. Geyer, I. Spreitzer, C. Hermann, and T. Hartung. 2002. Structural decomposition and heterogeneity of commercial lipoteichoic acid preparations. Infect. Immun. **70:** 938-944.

69. Mori, M., Y. Sakagami, Y. Ishii, A. Isogai, C. Kitada, M. Fujino, J. C. Adsit, G. M. Dunny, and A. Suzuki. 1988. Structure of cCF10, a peptide sex pheromone

which induces conjugative transfer of the *Streptococcus faecalis* tetracycline resistance plasmid, pCF10. J. Biol. Chem. **263:** 14574-14578.

70. Murray, B. E., K. V. Singh, R. P. Ross, J. D. Heath, G. M. Dunny, and G. M. Weinstock. 1993. Generation of a restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic functions. J. Bacteriol. **175:** 5216-5223.

71. Murray, B. E. 1998. Diversity among multidrug-resistant enterococci. Emerg. Infect. Dis. **4:** 37-47.

72. Muscholl-Silberhorn, A. 1998. Analysis of the clumping-mediating domain(s) of sex pheromone plasmid pAD1-encoded aggregation substance. Eur. J. Biochem. **258:** 515-520.

73. Muscholl-Silberhorn, A. 1999. Cloning and functional analysis of Asa373, a novel adhesin unrelated to the other sex pheromone plasmid-encoded aggregation substances of *Enterococcus faecalis*. Mol. Microbiol. **34:** 620-630.

74. National Nosocomial Infections Surveillance Syst. 1999. National Nosocomial Infections Surveillance (NNIS) System report, data summary from January 1990-May 1999, issued June 1999. Am. J. Infect. Control. **27:** 520-532.

75. Neuhaus, F. C., and J. Baddiley. 2003. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol. Mol. Biol. Rev. **67:** 686-723.

76. Olmsted, S. B., S. Kao, L. Van Putte, J. C. Gallo, and G. M. Dunny. 1991. Role of the pheromone-inducible surface protein Asc10 in mating aggregate formation and conjugal transfer of the *Enterococcus faecalis* plasmid pCF10. J. Bacteriol. **173:** 7665-7672.

77. Paulsen, I., L. Banerjei, G. Myers, K. Nelson, R. Seshadri, T. Read, D. Fouts, J. Eisen, S. Gill, J. Heidelberg, H. Tettelin, R. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. Ketchum, B. Dougherty, and C. M. Fraser. 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science. **299:** 2071-2074.

78. Peschel, A., M. Otto, R. W. Jack, H. Kalbacher, G. Jung, and F. Gotz. 1999. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J. Biol. Chem. **274:** 8405-8410.

79. Plow, E. F., T. K. Haas, L. Zhang, J. Loftus, and J. W. Smith. 2000. Ligand binding to integrins. J. Biol. Chem. **275:** 21785-21788.

80. Poyart, C., and P. Trieu-Cuot. 1997. A broad-host-range mobilizable shuttle vector for the construction of transcriptional fusions to β-galactosidase in Gram-positive bacteria. FEMS Microbiol. Lett. **156:** 193-198.

81. Qin, X., K. V. Singh, Y. Xu, G. M. Weinstock, and B. E. Murray. 1998. Effect of disruption of a gene encoding an autolysin of *Enterococcus faecalis* OG1RF. Antimicrob. Agents Chemother. **42:** 2883-2888.

82. Raad, I. I., H. A. Hanna, M. Boktour, G. Chaiban, R. Y. Hachem, T. Dvorak, R. Lewis, and B. E. Murray. 2005. Vancomycin-resistant *Enterococcus faecium*: Catheter colonization, *esp* gene, and decreased susceptibility to antibiotics in biofilm. Antimicrob. Agents Chemother. **49:** 5046-5050.

83. Rakita, R. M., N. N. Vanek, K. Jacques-Palaz, M. Mee, M. M. Mariscalco, G. M. Dunny, M. Snuggs, W. B. Van Winkle, and S. I. Simon. 1999. *Enterococcus faecalis* bearing aggregation substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. Infect. Immun. **67:** 6067-6075.

84. Rolinson, G. N. 1998. Forty years of β-lactam research. J. Antimicrob. Chemother. **41:** 589-603.

85. Rose, R. K., S. D. Hogg, and R. P. Shellis. 1995. Competitive binding of calcium and magnesium to streptococcal lipoteichoic acid. Biochem. Biophys. Acta **1245:** 94-98.

86. Rozdzinski, E., R. Marre, M. Susa, R. Wirth, and A. Muscholl-Silberhorn. 2001. Aggregation substance-mediated adherence of *Enterococcus faecalis* to immobilized extracellular matrix proteins. Microb. Pathog. **30:** 211-220.

87. Sahm, D. F., J. Kissinger, M. S. Gilmore, P. R. Murray, R. Mulder, J. Solliday, and B. Clarke. 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. **33:** 1588-1591.

88. Scheld, W. M., R. W. Strunk, G. Balian, and R. A. Calderone. 1985. Microbial adhesion to fibronectin in vitro correlates with production of endocarditis in rabbits. Proc. Soc. Exp. Biol. Med. **180:** 474-482.

89. Schleifer, K. H., and R. Kilpper-Baelz. 1984. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int. J. Sys. Bacteriol. **34:** 31-34.

90. Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J. Biol. Chem. **274:** 17406-17409.

91. Shankar, N., C. V. Lockatell, A. S. Baghdayan, C. Drachenberg, M. S. Gilmore, and D. E. Johnson. 2001. Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. Infect. Immun. **69:** 4366-4372.

92. Shepard, G. S., and M. S. Gilmore. 1995. Electroporation and efficient transformation of *Enterococcus faecalis* grown in high concentrations of glycine. Methods in Mol. Biol. **47:** 217-226.

93. Silver, L. L., and K. A. Bostian. 1993. Discovery and development of new antibiotics: the problem of antibiotic resistance. Antimicrob. Agents Chemother. **37:** 377-383.

94. Sussmuth, S. D., A. Muscholl-Silberhorn, R. Wirth, M. Susa, and R. Marre. 2000. Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. Infect. Immun. **68:** 4900-4906.

95. Sutcliffe, I. C., and N. Shaw. 1991. Atypical lipoteichoic acids of Gram-positive bacteria. J. Bacteriol. **173:** 7065-7069.

96. Sutcliffe, I. C. 1995. Identification of a lipoarabinomannan-like lipoglycan in *Corynebacterium matruchotii.* Arch. Oral. Biol. **40:** 1119-1124.

97. Suzuki, A., M. Mori, Y. Sakagami, A. Isogai, M. Fujino, C. Kitada, R. A. Craig, and D. B. Clewell. 1984. Isolation and structure of bacterial sex pheromone, cPD1. Science. **226:** 849-850.

98. Teng, F., L. Wang, K. V. Singh, B. E. Murray, and G. M. Weinstock. 2002. Involvement of PhoP-PhoS homologs in *Enterococcus faecalis* virulence. Infect. Immun. **70:** 1991-1996.

99. Tomich, P. K., F. Y. An, S. P. Damle, and D. B. Clewell. 1979. Plasmid related transmissibility and multiple drug resistance in *Streptococcus faecalis* subspecies zymogenes strain DS16. Antimicrob. Agents Chemother. **15:** 828-830.

100. Trotter, K. M., and G. M. Dunny. 1990. Mutants of *Enterococcus faecalis* deficient as recipients in mating with donors carrying pheromone-inducible plasmids. **24:** 57-67.

101. Tul'skaya, E. M., K. S. Vylegzhanina, G. M. Streshinskaya, A. S. Shashkov, and I. B. Naumova. 1991. 1,3-poly(glycerol phosphate) chains in the cell wall of *Streptomyces rutgersensis* var. castelarense VKM Ac-238. Biochim. Biophys. Acta. **1074:** 237-242.

102. Uttley, A. H. C., C. H. Collins, J. Naidoo, and R. C. George. 1988. Vancomycin-resistant enterococci. Lancet. **i:** 57-58.

103. Van Amersfoort, E. S., T. J. C. Van Berkel, and J. Kuiper. 2003. Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. Clin. Microbiol. Rev. **16:** 379-414.

104. Vanek, N., N., S. I. Simon, K. Jacques-Palaz, M. M. Mariscalco, G. M. Dunny, and R. M. Rakita. 1999. *Enterococcus faecalis* aggregation substance promotes opsonin-independent binding to human neutrophils via a complement receptor type 3-mediated mechanism. FEMS Immunol. Med. Microbiol. **26:** 49-60.

105. Walsh, P. S., D. A. Metzger, and R. Higuchi. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques. **10:** 506-513.

106. Waters, C. M., and G. M. Dunny. 2001. Analysis of Functional Domains of the *Enterococcus faecalis* pheromone-induced surface protein aggregation substance. J. Bacteriol. **183:** 5659-5667.

107. Waters, C. M., C. L. Wells, and G. M. Dunny. 2003. The aggregation domain of aggregation substance, not the RGD motifs, is critical for efficient internalization by HT-29 enterocytes. Infect. Immun. **71:** 5682-5689.

108. Waters, C. M., H. Hirt, J. K. McCormick, P. M. Schlievert, C. L. Wells, and G. M. Dunny. 2004. An amino-terminal domain of *Enterococcus faecalis* aggregation substance is required for aggregation, bacterial internalization by epithelial cells and binding to lipoteichoic acid. Mol. Microbiol. **52:** 1159-1171.

109. Wicken, A. J., S. D. Elliott, and J. Baddiley. 1963. The identity of streptococcal group D antigen with teichoic acid. J. Gen. Microbiol. **31:** 231-239.

110. Wirth, R. 1994. The sex pheromone system of *Enterococcus faecalis*. More than just a plasmid-collection mechanism? Eur. J. Biochem. **222:** 235-246.

111. Yagi, Y., R. E. Kessler, J. H. Shaw, D. E. Lopatin, F. An, and D. B. Clewell. 1983. Plasmid content of *Streptococcus faecalis* strain 39-5 and identification of a pheromone (cPD1)-induced surface antigen. J. Gen. Microbiol. **129:** 1207-1215.

112. Zervos, M. J., A. E. Bacon, J. E. Patterson, D. R. Schaberg, and C. A. Kauffman. 1988. Enterococcal superinfection in patients treated with ciprofloxacin. J. Antimicrob. Chemother. **21:** 113-115.