TRANSCRIPTIONAL REGULATION OF THE *DLT* OPERON IN *ENTEROCOCCUS FAECALIS* AND FURTHER CHARACTERIZATION OF A *DLTA* MUTANT

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Abstract

Enterococcus faecalis, a gram-positive member of the mammalian gastrointestinal flora, emerged as an important contributor to nosocomial infections and antibiotic resistance gene transfer. Lipoteichoic acid (LTA), a vital component of gram-positive cell walls, has been reported to function in numerous cellular processes, ranging from maintenance of cation homeostasis and virulence to modulating function and presentation of wall proteins such as adhesins and autolysins. Interestingly, LTA can be covalently modified by the addition of D-alanyl ester residues, which appear to help regulate its function by altering surface charge. In *E. faecalis* the process of esterification is catalyzed by four proteins encoded by the *dlt* operon. Mutants lacking a functional *dlt* operon display the inability to incorporate D-alanyl residues on LTA and are thus deficient in their ability to regulate the anionic charge of the outer envelope in response to extracellular cues. Recent evidence suggests that two-component systems are responsible for sensing environmental conditions and regulating *dlt* operon expression. Utilizing a reporter construct with the upstream promoter region of *dlt* fused to *lacZ*, we were able to determine how extracellular stimuli affect transcription of this operon by measuring β-galactosidase activity. Furthermore, we were able to identify specific response regulators important for bile salt, magnesium and polymyxin B signaling.

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Introduction

The first known use of antibiotics occurred in ancient China some 2,500 years ago when pre-historic pharmacists would prescribe the moldy curd of soybeans as a therapeutic treatment for wound infection. This crude form of medicine was utilized without the knowledge of the active antimicrobial compounds contained in the curd or the biochemical mechanisms of efficacy. It was not until the turn of the 20th century when pioneers like Louis Pasteur, Alexander Fleming, Ernst Chain, and Howard Florey began to revolutionize the knowledge of antibiotics by elucidating the underlying factors of their antimicrobial properties. Fleming, Chain, and Florey shared the Nobel Prize in Medicine in 1945 for their contributions to the field.

Prior to the advent of modern antibiotics, attempts to control microbial infections and disease were largely centered on chemical compounds including strychnine and arsenic that, in addition to their antimicrobial properties, were highly toxic to mammalian cells. The discovery of penicillin, attributed to Alexander Fleming in 1928, had a profound impact on the treatment of microbial infections by identifying naturally produced compounds that were not only capable of treating infectious disease, but were also specific for the cell biology of microorganisms. Penicillin, a diffusible β-lactam that prevents transpeptidation of peptidoglygan, was isolated from the mold *Penicillium notatum* when Fleming observed that the mold inhibited the growth of other microorganisms on an agar plate (36).

The onset of World War II was another critical step in speeding the development of mass produced antibiotics for human usage. A major contributor to war fatalities was wound infection due to the lack of effective treatment options on the battlefields and in the barracks. Advances in fermentation procedures allowed scientists such as Chain and Florey to mass produce penicillin in a laboratory setting, which saved countless lives in the latter stages of the war (128). During this time, scientists were also discovering new antibiotics possessing different modes of action that were isolated from other species of

microorganisms, including chloramphenicol, streptomycin, tetracycline, and cephalosporins. This era clearly marked one of the crowning achievements in medicine.

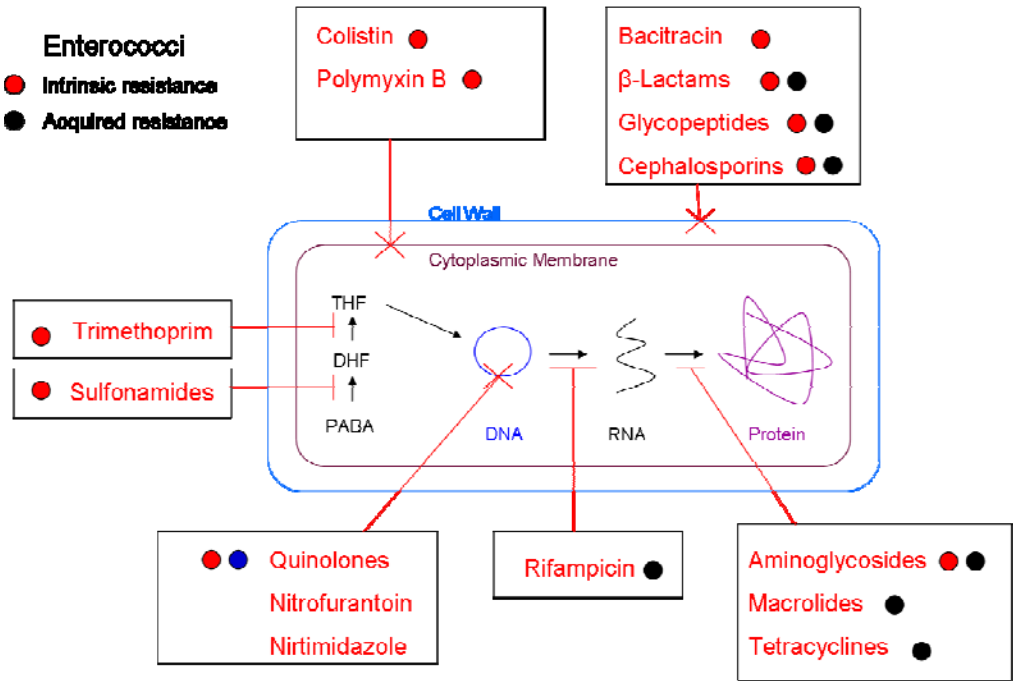
Unbeknownst to researchers and medical personnel at the time, the use of penicillin in mass quantities was effectively providing a selective pressure for bacteria that could resist the antibiotic challenge. Indeed, the first recorded instance of penicillin resistance emerged nearly four years after widespread use in a strain of *Staphylococcus aureus* (44). Resistance to any antimicrobial is usually conferred by either intrinsic properties of the bacterium, by point mutations, or by the acquisition of resistance genes on mobile genetic elements such as plasmids or transposons. Penicillin resistance arose from the transfer of genes encoding a β-lactamase (penicillinase) that is able to disrupt the β-lactam ring needed for activity (130). At first, other antibiotics were successful in treating penicillin resistant infections; however, overuse and misuse of these antimicrobial agents rapidly selected for other resistant strains.

In the late 1950's researchers again gained the upper hand in the battle against bacterial infection with the introduction of semi-synthetic and synthetic antibiotic derivatives. Unlike naturally occurring antibiotics which are produced by microorganisms as an attempt to control the growth of competing species and as a form of nutrient acquisition, the man-made antibiotics are chemically altered analogs designed to overcome resistance mechanisms. An early example was the creation of methicillin, a penicillin derivative containing an *ortho*-dimethoxyphenyl modification to a side chain carbonyl group, by Beecham in 1959 (16). The modification maintained the efficacy of the antibiotic and also rendered it insensitive to β-lactamase. The success was short lived as methicillin-resistant *Staphylococcus aureus* (MRSA) strains began to arise almost immediately (12).

Antibiotics have similar modes of action on microbes in that they interrupt vital cellular functions, usually by attacking cell wall and cell membrane integrity or by interfering with either DNA or protein synthesis (Fig. 1). They can be either bacteriocidal or bacteriostatic depending on their mechanisms of action with bacteriocidals destroying the cellular encasement and bacteriostatics effectively halting cell growth and replication. The interplay between researchers and microbes in an apparent microbial arms race continues to this day with resistance among bacteria being

observed for nearly every antibiotic on the market. Pharmaceutical companies seem reluctant to fund projects aimed at producing novel antibiotics due to the high monetary and time costs associated with developing a marketable drug that could become obsolete within a few short years. Much of today's current research emphasizes the need to find novel cellular processes that are essential for survival and that can be exploited as therapeutic targets.

Figure 1. Mechanisms of action for common antimicrobials.
Antimicrobials mainly target either the cellular encasement or affect DNA structure or proteins synthesis. Colistin and polymyxin B target the cytoplasmic membrane, while the antibiotics bacitracin, β-lactams, glycopeptides, and cephalosporins disrupt cell wall components. Folate synthesis, which is required for construction of DNA base pairs, is a process that includes the conversion of para-aminobenzoic acid (PABA) into dihydrofolate (DHT) and then finally into trihydrofolate (THF). Trimethoprim and sulfanomides block these conversions and effectively halt DNA synthesis. Other antibiotics function by interrupting DNA replication, RNA sythesis, and protein synthesis. Enterococci have been shown to possess a wide range of either intrinsic or acquired resistance to nearly all of the selected antimicrobials.



Enterococci

Enterococci were long believed to be members of the genus *Streptococcus* despite a wide variety of phenotypic differences. Even as early as the late 19th and early 20th centuries, observers began to note the identification of unique streptococci isolated from mammalian fecal products (123, 77, 44). These fecal isolates were quickly divided into the species *Streptococcus faecalis, Streptococcus faecium,* and *Streptococcus durans* based on fermentation patterns observed in mannitol, lactose, and raffinose containing media (3, 94, 118).

The first breakthrough in the creation of the genus *Enterococcus* occurred in the late 1930's when Sherman coined the term enterococcal group, which was designed to differentiate streptococci species that were isolated from fecal origins, grew at 10 and 45°C, grew in media containing 6.5% NaCl, grew in media adjusted to a pH of 9.6, and could withstand heating to 60°C for 30 minutes (118). All of the known enterococcal species at that time were included in this grouping. Around the same time, Lancefield began devising her serological classification of streptococcal bacteria based on antigenic reactivity (70). Again, all of the known enterococcal species were included in her Group D streptococci, a classification that became a mainstay until the establishment of a new genus.

The first suggestion of an *Enterococcus* genus occurred in 1970 when Kalina proposed that the enterococcal streptococci *S. faecalis* and *S. faecium* should be separated from classical streptococci based on their unique cellular arrangement (pairs and short chains) and phenotypic characteristics (62). However, this separation was not universally accepted until researchers published proof that there were significant genetic differences based on DNA-DNA reassociation experiments between the bacterium to warrant an independent genus classification (76). The results of these studies led to today's accepted nomenclature of *Enterococcus faecalis* and *Enterococcus faecium*.

Since 1984, 25 additional species have been added to the *Enterococcus* genus, bringing the total to 27 members (www.atcc.org). These additions were based largely on DNA hybridization experiments, 16S rRNA sequencing, and whole cell protein analysis. All members are gram-positive cocci that possess cellular grouping of singles, pairs, or short chains. They are also mesophilic, facultative anaerobes which make them well

suited to reside in the gastrointestinal tracts of mammals. However, they can reside in fecal excretions, in soil, in water, and, in some cases, on several different species of vegetation. Other defining traits include the production of lactic acid as the end product of glucose fermentation and a wide range of intrinsic resistance to numerous antimicrobial agents (salinity, desiccation, antibiotics, bile salts, etc) (118, 96).

Enterococcus faecalis

E. faecalis is currently the most intensively studied enterococcal species due to its prominence in the nosocomial setting. The only publicly sequenced genome is available at The Institute for Genomic Research (TIGR, www.tigr.org) for stain V583, the first reported vancomycin resistance clinical isolate discovered in the United States (110). The genome consists of a 3.2 Mb circular chromosome encoding for 3182 functional genes and three plasmids encoding an additional 155 genes. Of the 3337 predicted open reading frames, 2115 (66.5%) have been assigned a role category while the other 1222 remain hypothetical in nature (TIGR). The three plasmids present are pTEF1, pTEF2, and pTEF3 (96). Plasmids pTEF1 and pTEF2 are structurally similar to the pheromone-responsive plasmids pAD1 and pCF10 in that they contain the *prgABC* genes belonging to this distinct family of plasmids (96). The plasmid pTEF3 closely resembles pAMβ1 and is a broad host range plasmid. Reports have indicated that approximately one quarter of the genome was acquired by either mobile genetic elements or other exogenous methods (96). Indeed, partial sequencing of other *E. faecalis* isolates has revealed relatively high sequence variance among strains, furthering the notion that there is an abundance of gene acquisitions and dissemination taking place in these organisms (39).

Importance of Enterococci

Enterococci have emerged as nosocomial pathogens due to increasing antimicrobial resistance and their ability to readily transfer those resistance genes through horizontal gene transfer (56, 83). The increased isolation of vancomycin resistant enterococci (VRE) over the last few decades in Europe and the United States has alarmed the medical community with reports of VRE accounting for one-third of infections

occurring in hospital intensive care units (CDC 2005, www.cdc.gov). There is strong evidence that strains of *S. aureus*, including MRSA, have acquired vancomycin resistance genes from *E. faecalis,* rendering common treatment options for these infections obsolete (135, 138).

Enterococci find their importance in the duality of their lifestyle. They exist mainly as commensals of the gastrointestinal tract in a symbiotic relationship with the host, but are also facultative parasites (3). Healthy hosts rarely succumb to enterococcal infections, which usually manifest into commonly classified medical diseases such as urinary tract infections (UTI), bacteremia, and endocarditis. Infection mainly occurs when the bacteria disseminate from the GI tract to other host systems via wounds, internal and external, or medically important abiotic devices, including indwelling catheters and artificial replacements. It has also been speculated that enterococci may translocate across the intestinal epithelium in order to begin the infectious process (99).

Enterococci currently rank third among the leading causes of nosocomial infection in the United States, trailing only the highly publicized staphylococcal and streptococcal infections (77). Interestingly, *E. faecalis* and *E. faecium* account for approximately 90% of all enterococcal infections, with *E. faecalis* constituting the majority (65-80%) of infections (56). *E. faecalis* is by far the more virulent of the two species and possesses many virulence factors not found in *E. faecium* (83). However, vancomycin resistance genes are more frequently harbored in *E. faecium* (107).

Treatment

The treatment of enterococcal infections varies widely from patient to patient, but usually involves a combination of broad spectrum antibiotics. Common cocktails include the use of penicillin and vancomycin, novobiocin and doxycycline, and ciprofloxacin and ampicillin (10). Drug regimens mainly utilize a cell-wall acting antibiotic coupled with an antibiotic that affects protein synthesis. This approach can lead to nephrotoxicity if the antibiotics are used in large quantities (32). In many cases, utilizing broad spectrum antibiotics fails to specifically target the infectious strain and can intensify problems by removing beneficial commensal organisms (10). Furthermore, by exposing the entire

bacterial community of the host to these antibiotics, the medical community is creating an environment with extreme selective pressure for antibiotic resistance through either spontaneous mutation or genetic transfer.

For several years vancomycin has been the choice of last resort when dealing with gram-positive infections due to its efficacy and the medical community wanting to limit its use to reduce the selective pressure for developing resistance (79). However, VRE are now becoming more common in the nosocomial setting and strains have recently been isolated that even have a dependence on vancomycin for growth (23). Again, this underscores the need to discover new therapeutic targets to treat infection.

Virulence Factors

E. faecalis possess a plethora of virulence factors designed to help establish infection and then persist in the presence of the host immune response. There are four main stages in the pathogenesis of enterococcal infections. The first is persistence on inanimate objects due to intrinsic properties of bacteria. Resistance to desiccation, heat and other common conditions that impair bacterial endurance outside of the host contribute largely to enterococci's prevalence as a nosocomial pathogen by providing a means of survival until an opportunity for infection presents itself. Next, entry into the bloodstream or other non-native areas of the host is a critical step in the transformation of enterococci from commensals to damaging pathogens. This phase is usually facilitated by damage to host tissues and the presence of bacterial factors such as bile acid hydrolases, adhesins like aggregation substance (AS) and enterococcal surface protein (Esp), and antibiotic resistance that give enterococci an edge in surviving these newly discovered niches.

Once outside of their normal setting, enterococci have been shown to utilize several classified virulence factors for adherence and colonization in the establishment of an infection site. In this step adhesins play a vital role in attachment to host tissues, as well as in the formations cell aggregates such as biofilms. Also, factors that possess immune evasion qualities are vital to cell survival. Many of these characterized virulence factors, including cytolysin, AS, Esp, and LTA, are describe in detail below. The final

stage is the clinical manifestation of infection resulting in damage of vital tissues. This is the result of proteins that carry direct activity against host tissues and include cytolysin, gelatinase, and serine protease.

Cytolysin

The functional cytolysin is encoded on the *cyl* operon which contains eight genes that are involved in the regulation, post-translational activation, and export of the secreted enzyme. (18, 42, 41, 47, 58). The active toxin contains two subunits termed CylLl and CylLs that are post-translationally modified by CylM in the cytosol before they are exported and cleaved at the C-terminus by CylB, which possesses cysteine protease activity (42, 41). Extracellulary, the serine protease CylA completes the final proteolytic cleavage to form the active cytolysin (7, 42). CylI is predicted to be a membrane bound protein that confers self-resistance to cytolytic activity through an unknown mechanism (7). The final two proteins, R1 and R2, are predicted to control operon expression through a quorum sensing mechanism whose exact details remain to be elucidated as they show little sequence homology to other two-component signal transduction systems.

Cytolysin displays both hemolytic and bacteriocidal characteristics making it responsible for both host damage by lysis of native tissues, and colonization by removing competition from other commensal organisms. Its effects on *E. faecalis* pathogenicity have been well characterized, with strains expressing this secreted protein being greater than 10 fold more toxic to host systems in a murine intraperitoneal challenge model (29). Furthermore, a rabbit endocarditis model displayed a 55% mortality rate when infected with a cytolytic strain containing AS as compared to a 7% mortality rate in a strain that only contained AS (13). Interestingly, removing AS greatly reduced the lethality of the cytolytic strain, indicating that both proteins work together in pathogenesis. It is speculated that adhesins such as AS and Esp help facilitate bacterial cell aggregates, leading to increased quorum sensing signals and, hence, production of the cytolysin. This is supported by the increase virulence of strains containing both the cytolysin and AS on a plasmid or the cytolysin in close proximity to Esp on an area of the genome known as pathogenicity island.

It appears to be no coincidence that nearly 60% of clinical isolates from hospital wards possess the cytolysin as compared to 17% of isolates from healthy human stool samples (59). In the infectious sequelae, cytolysin has been shown to a certain extent, to promote entry into and proliferation in the bloodstream via translocation across the intestinal epithelium (136, 57). Little is know about both mechanisms, but it has been hypothesized that proliferation can be contributed to increased host immune evasion due to cytolysins documented activity against macrophages and polymorphonuclear leukocytes (PMNs) in a mouse model (80). Another hypothesis for advanced proliferation of cytolytic strains in the bloodstreams involves increased nutrient acquisition, namely heme, from the lysis of erythrocytes (60).

Enterococcal Surface Protein (Esp)

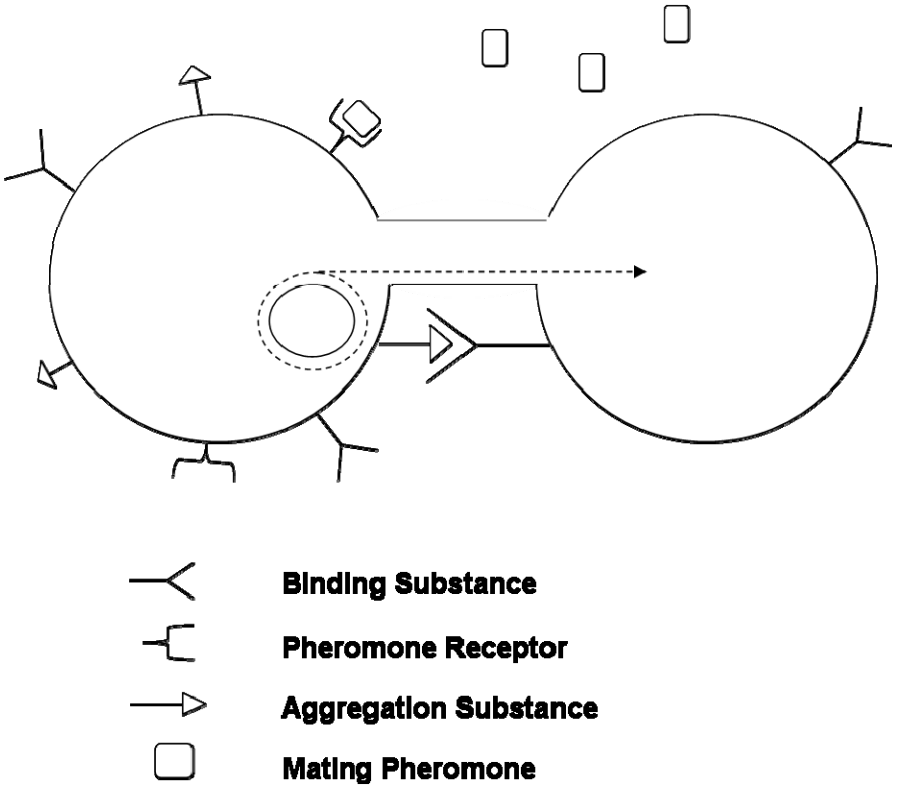
Esp is a large, well characterized, 1,872-amino-acid protein that is encoded by a single gene *esp* . Similar to the cytolysin, Esp is more abundant in enterococci isolated from the bloodstream (41%) than from stool isolates (3%) (115). It is anchored to the cell wall and functions as an adhesin, promoting both colonization of the urinary tract and biofilm formation (114, 124). A mouse transurethral infection model demonstrated that strains expressing Esp could be isolated at a significantly higher frequency from the bladder and urine than strains lacking this surface adhesin, which correlates with the high frequency of *esp* being observed in strains isolated from human UTIs (114). In vitro biofilm assays on polystyrene found that 93.5% of *E. faecalis* strains possessing *esp* could form micro-colonies known as biofilms, while not a single strain lacking *esp* could produce this result. The biofilm-deficient strains could be complemented with the addition of exogenous *esp* (124). Biofilm formation is not solely dependent on the presence of *esp* since strains such as OG1RF are capable of biofilm formation. Together these results demonstrate the importance of Esp in the formation of biofilms on abiotic surfaces including polystyrene.

Pheromone-Responsive Plasmids and Aggregation Substance

Enterococci possess a unique system for conjugative genetic exchange that utilizes secreted pheromones to induce mating and transfer of specific pheromone-responsive plasmids (Fig. 2). These plasmids exhibit a narrow host range and contain many conserved genes that are distinct to this family of plasmids. Transfer of these plasmids between *E. faecalis* strains occurs at a high frequency (10^{-3} to 10^{-1} transconjugates per donor cell) within a few hours during mating in liquid media (28). Moreover, exchange can take place at a frequency of 10^{-1} transconjugates/donor in 10 min when the donor strain is exposed to 10 ng/ml of exogenous pheromone for 2 hours prior to mating (82). Interestingly, cells deficient in AS or enterococcal binding substance (Ebs) display more efficient transfer on solid surfaces and a reduced transfer in liquid culture, indicating these two proteins do not participate in the formation of a functional mating channel (5, 17). The high frequency of transfer is alarming since many of these plasmids encode determinants for hemolysin, bacteriocins, and resistance to UV light and antibiotics, including VanA-type resistance (49, 26).

Genetic exchange of these plasmids is dependent on a sex pheromone, a small peptide which is enzymatically cleaved into a 7-8 amino acid signal inducer, secreted by potential recipient cells (122). This mating signal is only produced by cells that do not possess the plasmid and facilitates the production of several mating factors, including AS on the surface of donor cells harboring the related pheromone-responsive plasmid. AS is a surface adhesin that binds Ebs to form high density mating aggregates to aid in the formation of a stable mating channel, and ultimately plasmid transfer (26). Once a recipient acquires a pheromone responsive plasmid, it begins production of a related pheromone inhibitor and a regulatory protein (PrgY/TraB), both of which are encoded on the plasmid. The secreted pheromone inhibitor neutralizes endogenously produced pheromone that has been secreted, while still allowing the cell to sense exogenous pheromone. In addition, the regulatory protein PrgY functions in binding endogenous pheromone also prevent auto-induction of mating proteins (26).

Figure 2. Model for pheromone-responsive plasmid transfer.
The donor cell is stimulated by the binding of the mating pheromone to the pheromone receptor and begins production or several plasmid encoded mating factors including AS. AS then binds to binding substance in order to form mating aggregates. The plasmid is then transferred to the recipient, which then becomes and viable donor cell. Adapted from Olmstead et al (93).



There are numerous members of the pheromone-responsive plasmid family with the two best characterized members being pAD1 and pCF10. Both of these plasmids carry virulence factors that are transferred upon mating. pAD1 contains genes responsible for the specific AS Asa1 and the cytolysin (25), while pCF10 harbors genes for the specific AS Asc10 and a Tn*925* transposon containing tetracycline resistance (52). The sex pheromones for pAD1 and pCF10 mating induction are cAD1 and cCF10, respectively. Also of importance are the plasmids pHKK702 and pHKK703 which have been show to carry high-level vancomycin resistance in *E. faecium* (50). These plasmids

were discovered in the clinical isolates R7 found in a hospitalized patient and further the concern of growing resistance to vancomycin in these nosocomial pathogens.

AS has been the subject of intense studies that have found additional roles in enterococcal virulence. In addition to its participation as an adhesin in pheromone-responsive mating, AS has also been implicated in host cell attachment and immune evasion. Although the genes for AS are ubiquitously expressed on pheromone-responsive plasmids, there is, in some cases, high sequence diversity among plasmids such as in Asa373 (84). Mostly, genes encoding AS share 75-85% sequence homology, but that is enough to warrant unique gene naming assignments between plasmids and raise questions as to how these differences affect function among versions of the protein.

Bioinformatic analysis of AS proteins have revealed two Arg-Gly-Asp (RGD) motifs sharing high homology to those found in eukaryotic fibronectin that are involved in integrin binding on host cell surfaces (37). This discovery raised speculation that AS may have a role in adherence to host cells and initial colonization of host tissues. Indeed, it has been demonstrated that *E. faecalis* possessing AS displays an increased ability in-vitro to adhere to porcine renal epithelial cells and human macrophages when compared to strains devoid of the adhesin (67, 120). Additional reports have shown Asa1 and Asc10 to help facilitate binding to other extracellular matrix components, including fibrin, collagen type I, vitronectin, and thrombospondin (53, 109). These finding are in support of AS being involved in host cell attachment.

Another potential role for AS is speculated to be increased resistance to the host immune response either through reducing immune cell efficacy or by immune cell evasion. AS has been shown to initiate opsonin-independent binding of enterococcal isolates to human PMN's and human macrophages (129, 120). The attachment to PMN's is dependent on the interaction between the RGD motif in AS and PMN surface β2 integrins (CD11b and CD18) (129). AS also contributes to bacterial survival following phagocytosis by PMNs even when they are activated (106). PMNs that had internalized *E. faecalis* expressing AS were characterized as possessing abnormally high phagosomal pH, which was thought to interfere with the activation of enzymes with bacteriocidal properties (106). Another possible form of immune evasion is thought to be AS mediated internalization of the bacterial cells by the GI epithelium (92). Association to enterocytes

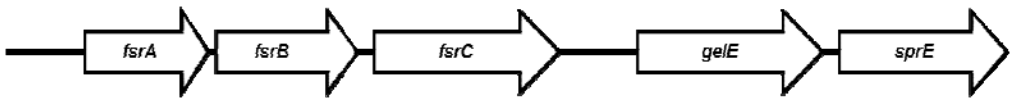
is mediated by the aggregation domain and not the RGD motif, suggesting a distinctive mechanism of binding when compared to PMNs (133). This internalization is not coupled with translocation across the epithelium, so it does not directly lead to dissemination from the GI tract (111).

Gelatinase and Serine Protease

Bacterial proteases are commonly thought to aid the microorganisms in nutrient acquisition and activation of proteins that need to be cleaved in order to facilitate function. However, they may also provide a means of inflicting host cell damage, either directly or indirectly, by exuding proteolytic activity on host proteins. This activity can alter host biochemistry in many ways, including activation of host matrix metalloproteases, and degradation of connective tissue and components of the immune response (8, 104, 71).

Two secreted proteases implicated in enterococcal pathogenesis, gelatinase and serine protease, are encoded in the same operon by *gelE* and *sprE*, respectively (Fig. 3). GelE is a 509 amino acid proenzyme that is truncated to its active form extracellularly (40). It is a zinc-metalloprotease that is able to hydrolyze numerous host proteins, but its main identifying phenotype is the ability to liquefy gelatin. SprE is encoded by a 284 amino acid ORF also displays proteolytic activity to a wide range of proteins. Both proteases are co-transcribed, indicating they may function jointly in biological activities (105). Disruption of either gene is enough to significantly delay mortality in a mouse peritonitis model (105).

Figure 3. Organization of the operon containing *fsrABC, gelE, and sprE*. Diagram of operon containing the spatial arrangement of the *fsr* two component regulatory system loci and the gelatinase and serine protease genes.



The operon also contains the *fsr* (RR15) two component regulatory system, which is directly involved in the regulation of the protease genes (105). The *fsr* locus shows significant similarity to the *S. aureus agr* locus and contains *fsrA* (response regulator), *fsrB* (signal peptide) and *fsrC* (histidine kinase) (90). These three proteins function in a quorum sensing mechanism and may be biologically relevant in pathogenesis after the initial establishment of infection. Increased signal peptide concentrations in biofilms and other cell aggregates would lead to an induction of protease expression and amplify damage to host tissues.

Pathogenicity Island

Clinical isolates of enterococci, most notably MMH594, have been shown to possess pathogenicity islands that contain many of the previously described virulence factors, including cytolysin, AS, Esp, and a bile acid hydrolase in a 150 kb region of the genome (100). The pathogenicity island of MMH594 contains 129 ORFs and is littered with recombination factors that include transposases, integrases and insertion elements that are believed to have been used in initial recombination events (113). Numerous other strains have been isolated that contain pathogenicity islands with high identity to the one present in MMH594. It is believed that this feature contributes greatly to the pathogenesis of a particularly virulent sub-population of enterococci.

LTA - Function

One candidate that demands consideration as a potential therapeutic target is the cell wall constituent lipoteichoic acid (LTA). It has been demonstrated that functional LTA is essential for cell growth and survival (45), but the inability to create a mutant devoid of LTA has raised question as to its exact roles in bacterial cell biochemistry. This unique polymer has been implicated in a wide range of cellular processes including virulence, biofilm formation, maintenance of cation homeostasis, regulation of autolytic activity, ultraviolet radiation sensitivity, acid tolerance, and low to intermediate antibiotic resistance (88). Elucidating compounds that either block synthesis or interfere with normal LTA functions is a practical avenue of research that may provide novel treatment

options for bacterial infections as well as a more comprehensive understanding of biochemical processes in bacteria.

There has been much speculation on the relevance of LTA to pathogenicity as a virulence factor due to its ability to act as an adhesin and elicit a robust immune response in host models. Some of the roles of LTA in bacterial biochemistry and pathogenesis have been well documented and include adherence to host tissues (19), the post infectious sequelae (22), resistance to anitmicrobial compounds (99), maintenance of cation homeostatis (54), regulation and presentation of autolysins (6), and – in *Enterococcus faecalis* – even bacterial conjugation (133, 126).

The ability of LTA to act as an adhesin to host uroepithelial, mucosal, and mesoepithelial cells has been documented in strains of staphylococci (2, 11). Furthermore, LTA has been show to facilitate the attachment of *S. mutans* to hydroxylapatite of tooth surfaces (15), of *S. epidermidis* to fibrin-platelet clots in the establishment of endocarditis (14) and of other streptococci to erythrocyte membranes (24, 87). These results demonstrate the importance of LTA in the establishment of host colonization. Its role as an adhesin is not limited to host binding, but it also important for bacterial coaggregation in mating and biofilm formation (20, 27). Dunny et al. showed that LTA could interact with AS to help brings cells into close proximity to facilitate mating (27).

In addition to its adhesin characteristics, LTA can also induce proinflammatory mediators and activate complement. LTA has been shown to mediate the release of IL-1, IL-6, TNFα, and nitric oxide by monocytes and macrophages in-vitro (127). It is also specifically recognized by the pattern-recognition receptors CD14 and Toll-Like Receptor 2 (TLR2), resulting in an inflammatory response (16, 112). Activation of the complement cascade is achieved by direct binding of LTA to the C1q subcomponent of C1 (75). This activation can produce local tissue damage in the area of activation.

LTA, and specifically D-alanylation of LTA, is also shown to help regulate many biochemical processes of bacteria including cation regulation, activity of autolysins, and resistance to antimicrobial compounds. D-alanylation of LTA plays a role in autolysin presentation at the cell surface by altering the anionic charge, and hence, the number of binding sites required for autolysin attachment (6). Furthermore, it seems to play a role

in facilitating autolytic activity since reducing LTA content alters autolytic activity without changing the surface protein profile (33). Support for LTA to regulate cation homeostasis is largely centered on its ability to sequester Mg^{2+} around the cell surface and findings of cation-dependent regulation of the *dlt* operon (66). The anionic properties of LTA seem to provide a unique place to accumulate cationic metals for biochemical usage and/or to help regulate the electrochemical and concentration gradients of the cell. Finally, mutants deficient in the ability to produce D-alanyl-LTA are almost universally described as possessing a decreased survivability when used in mouse host models and when exposed to cationic antimicrobial peptides or defensins in vitro (99, 1) Vancomycin resistance in a strain of *E. faecium* was also accompanied by a two fold increase in D-alanine content on LTA (46). This demonstrates a crucial role for LTA in bacterial survival against host innate immunity.

LTA- Structure and Synthesis

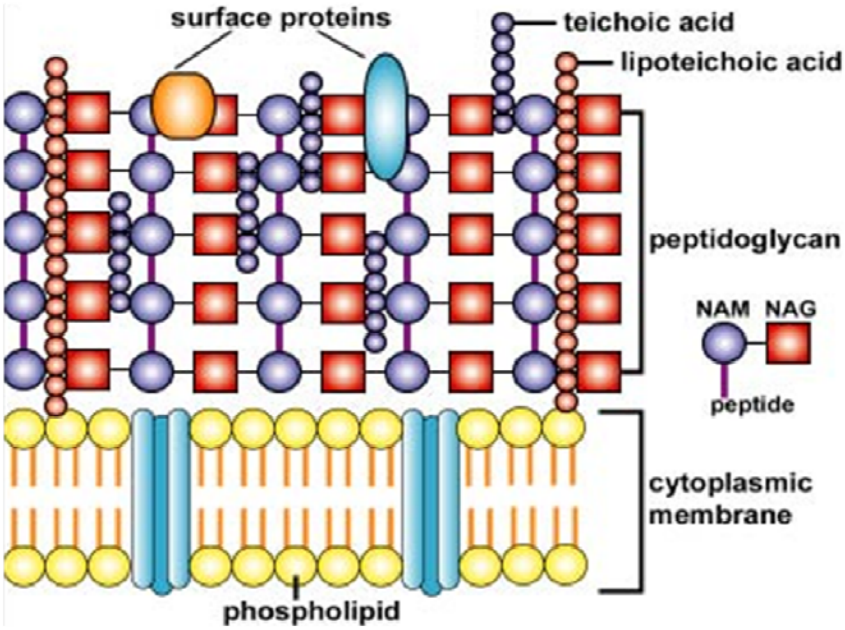
The cell envelope of gram-positive bacteria is a multi-faceted structure that not only serves as semi-permeable encasement responsible for maintaining cellular shape and integrity, but also as a scaffold for the presentation of proteins and polysaccharides (Fig. 4). A major constituent of the cellular envelope are teichoic acids (TA), which can be divided into two subcategories: the amphipathic, membrane anchored lipoteichoic acids (LTA) and peptidoglycan attached wall teichoic acids (WTA). TA's contribute largely to the anionic properties of the gram-positive cell and constitute an integral part of cell wall structure and function (4, 34).

LTA's necessity is underscored by the inability to create mutants completely devoid of functional LTA. Recently, the *ltaS* gene was discovered to encode an essential membrane bound protein responsible for the elongation of the hydrophilic chains. Complete knockout of *ltaS* results in a null phenotype, while quelling expression during log phase leads to cell growth arrest (45). Additionally, a strain of *S. aureus* containing an 87% reduction in LTA displayed reduced autolytic activity, increase hydrophobicity, and an complete absence in biofilm formation, while showing no differences in patterns of cell wall proteins and autolytic enzymes (33). Gram-positive bacteria that do not

synthesize LTA compensate by producing structurally similar compounds that also impart an anionic charge to the outer membrane. These atypical derivatives of LTA have been termed macroamphiphiles, lipoglycans, or surface glycolipids (121).

Figure 4. Gram-positive cell wall.

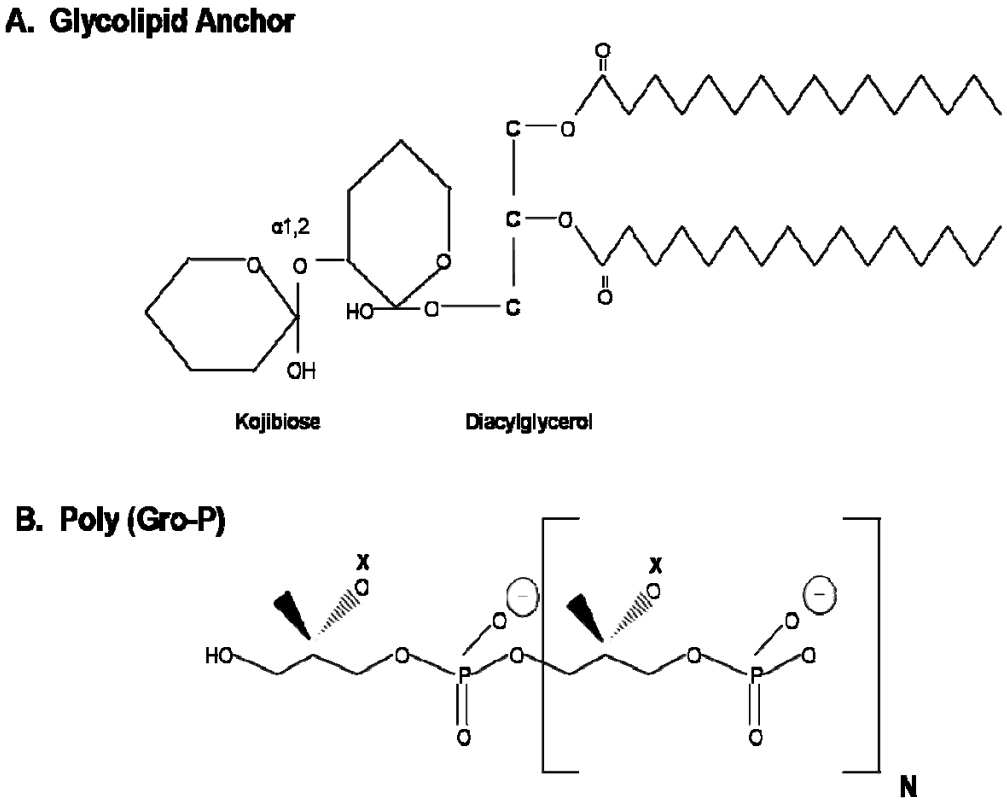
Diagram of a representative gram-positive cell wall showing spatial arrangement of its components. Adapted from http://www.kcom.edu/faculty/chamberlain /LECTS/Bacteria.htm.



Most LTA consists of repeating chains of either glycerol phosphate or ribotol phosphate. However, some bacteria utilize arabitol-P (*Agromyces cerinu*) and erythrol-P (*Glycomyces tenuis*) (116, 101). Enterococci possess Type I LTA which is comprised of a repeating glycerol-phosphate (Gro-P) moiety that is embedded into the cell membrane through a glycolipid anchor (Fig. 5) (125). The synthesis of fully functional LTA proceeds in a three step process that includes synthesis of the glycolipid anchor, creation and elongation of the Gro-P chain, and decoration of the poly Gro-P backbone through glycosylation and esterification.

Figure 5. Model of Type I LTA found in enterococci.

The glycolipid anchor (A) show the attachment of kojibiose to diacylglyerol. The poly (GroP) chain (B) consist of repeating negatively charged glycerol phosphate moieties that can be substituted at the **X** positions by either kojibiose, D-alanine, or H^+. Adapted from Neuhaus and Baddiley (88).



The glycolipid anchor consists of a glycosylated diacylglyerol compound in the lipid bilayer. In *B. subtilis* and *S. aureus* the diglucosyldiacylglycerol anchor is synthesized by the gene product of *ypfP* which encodes for a diglucosyldiacylglycerol synthase (65). The *E. faecalis* genome does not possess a *ypfP* homolog, but this is not surprising due to the differing sugars and sugar linkages between species. Instead, the lipid anchor in enterococci is though to be a phospatidylkojibiosyldiacylglycerol compound that is constructed in part by LtaA (38, 45). Interestingly, disruption of the anchor synthesis proteins does not result in non-functional LTA. Instead, the poly Gro-P

moieties are placed directly on diacylglycerol, again underscoring the importance of producing the Gro-P chains despite how they are anchored to the membrane (45).

The poly Gro-P chains of LTA and wall teichoic acid (WTA) are synthesized in differing ways. LTA utilizes glycerol-1-phosphate at the membrane surface, while WTA chains are created in the cytosol with glyercol-3-phophate before being exported through the membrane to the outer wall. The poly Gro-P chain length of LTA is highly variable among species with *E. faecalis* LTA containing anywhere from 14-33 subunits (74). LTA chains are constructed one sub-unit at a time and it has recently been demonstrated that the LTA Gro-P chain of *S. aureus* is constructed by an intermembrane polyglycerol phosphate synthase termed LtaS (45). Prior to this research it was unknown how this step in the LTA creation process was facilitated because the synthase is an essential gene. By placing LtaS on a plasmid under an IPTG inducible promoter in an *ltaS* null mutant, it was shown that the removal of IPTG resulted in severe growth and cell division defects (45). This is the only step in LTA synthesis that results in non-viable cells when disrupted.

The final step in LTA synthesis is the decoration of the poly Gro-P chain. The backbone of both TA's in *E. faecalis* can be modified through glycosylation or D-alanylation. In *E. faecalis g*lycosylation occurs with the addition of a kojibiose sugar moiety (α-D-Glc-(1→2)-D-Glc) and alanylation occurs when the amino acid D-alanine is added in an esterification process (64). Covalent modification of LTA with D-alanyl residues provides counter-ions to the negatively charged Gro-P subunits, thereby potentially allowing the cell to adjust its surface charge properties. Reports have suggested D-alanylation of LTA plays significant roles pertaining to regulation of cation binding, virulence, and activity of surface proteins such as autolysins (88). Across genera, mutants deficient in the ability to modify LTA with D-alanyl residues have been characterized as possessing increased susceptibility to host innate immune factors such as defensins and cationic peptides, increased autolytic activity and reduced virulence (98).

D-alanylation of LTA

The esterification of LTA with D-alanine is facilitated by the gene products of the *dlt* operon. The importance of this operon is evident by its presence and high conservation among nearly all gram-positive bacteria (TIGR). In *E. faecalis* the operon encodes four characterized genes (*dltABCD*) and a novel fifth gene (*dltX*) whose function has yet to be elucidated (Fig. 6) (TIGR). The proposed mechanism of action of DltA-D in facilitating the addition of D-alanine to LTA is schematically depicted below (Fig. 7). Dcl (DltA) is proposed to be a ligase that transfers D-alanine to the carrier protein Dcp (DltC) as an D-alanyl residue in an adenosine-triphosphate (ATP) dependent manner (21, 51). DltD is a membrane bound protein that is believe to bring Dcl and Dcp in close proximity to each other to initiate the transfer of the D-alanine residue within the cytosol (21). DltB shows high sequence homology to other transport proteins and is thought to be involved in shuttling the Dcp-D-alanyl compound to the extracellular environment. (89). Once outside on the cell, Dcp can facilitate the esterification of D-alanyl residues to LTA. Collectively, all four are involved in the following two-step reaction:

$$\text{ATP} + \text{D-alanine} + \text{Dcp} \rightarrow \text{AMP} + \text{PP}_i + \text{D-alanyl-Dcp} \qquad (1)$$

$$\text{D-alanyl-Dcp} + \text{LTA} \leftrightarrow \text{D-alanyl LTA} + \text{Dcp} \qquad (2)$$

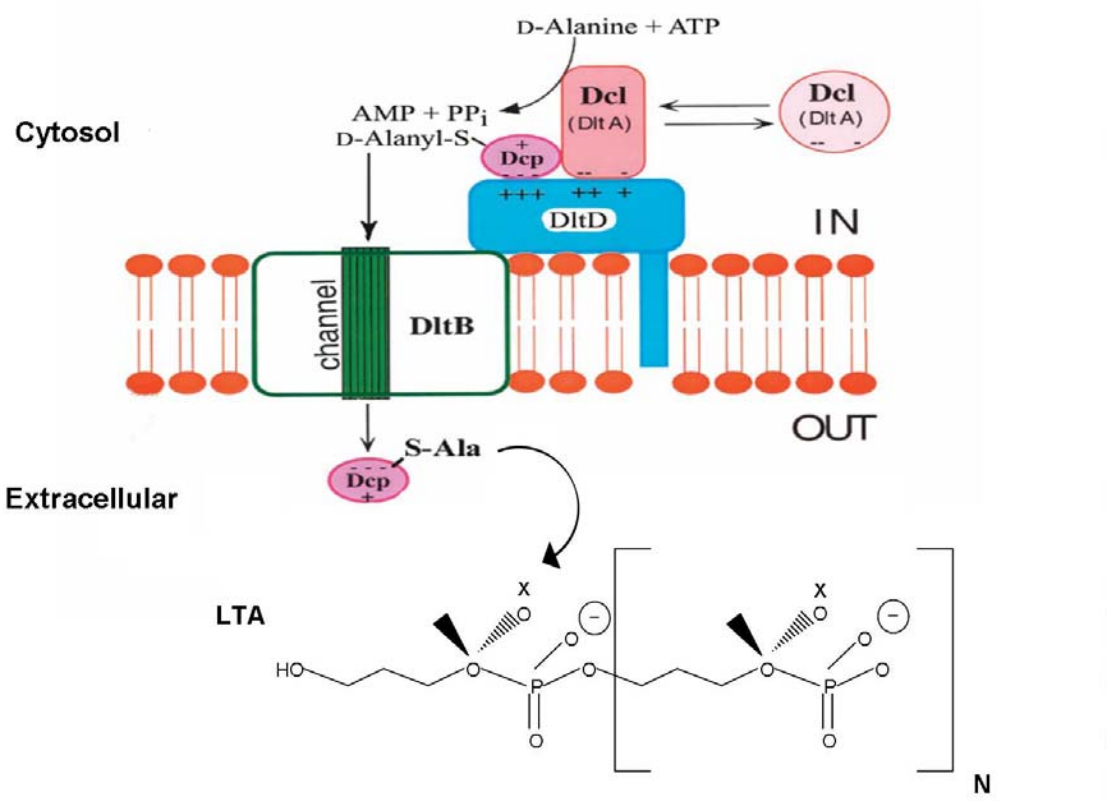
Little is known about the involvement of the gene product of *dltX* in the function of this operon, or if the ORF is even translated into a functional protein. Only recently has this gene even been characterized as belonging to the *dlt* operon in *S. aureus* (66).

Mohamed et al. (81) identified a mutant strain in *E. faecalis* (TX5427) through random transposon mutagenesis that possessed an insertional deactivation of the *dltA* gene and lacked the ability to covalently modify LTA with D-alanyl residues. Inactivation of any single gene (*dltA-D*) is enough to completely abrogate operon function, leaving the cell wall largely anionic (97). The degree of LTA substitution is dependent upon environmental conditions, with high salt concentrations, high temperatures, and basic pH conditions promoting alanine hydrolysis (30, 35, 55, 91). The observed reduction in alanine content of LTA was reversible when salt concentrations

were reduced, indicating that cells can adjust their degree of alanylation depending on environmental conditions (30).

Figure 6. Organization of the *dlt* operon in *E. faecalis* V583.

Figure 7. Proposed mechanism for the incorporation of D-alanine. Adapted from Neuhaus (88).



Little is known about the regulation of the *dlt* operon in general. In *Bacillus subtilis*, operon regulation has been shown to be under control of the global regulation factor σ^x (9), while *Streptococcus agalactiae* possesses two additional genes in the operon, *dltR* and *dltS*, encoding a two component system (TCS) that is believed to

directly regulate operon expression (102). In *Staphylococcus aureus* increased concentrations of cations (Na^{+}, Mg^{2+}, and Ca^{2+}) repressed operon expression, with Mg^{2+} signaling occurring partially through the ArlRS two component system (66).

Objectives of the Study

1.) Further characterize *E. faecalis* strain TX5427, a Δ*dltA* knockout deficient in D-alanylation of LTA, in regards to phenotypes observed in minimal inhibitory concentrations of selected bacteriocins, autolytic activity, pheromone-responsive mating, and survival in host model systems.

2.) Determine if the ORF (*dltX*) is indeed a constituent of the *dlt* operon in *E. faecalis* OG1RF and if it conveys any influences on operon expression.

3.) Identify environmental factors that influence expression of the *dlt* operon.

4.) Assess the involvement of TCSs on operon expression.

5.) Identify TCSs involved in signaling specific environmental stimuli.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. faecalis* strains were routinely grown at 37°C without aeration in brain heart infusion (BHI), Todd-Hewitt broth (THB), M17, or GM17, while *E. coli* strains were cultured at 30°C with aeration in Luria-Bertani broth (LB). All media was supplied by Difco and prepared following manufacturer's instructions.

Table 1. Strains and plasmid used in this study

Strain or Plasmid	Characterization	Reference/Source
Strains		
E. faecalis		
OG1RF	Wild type	(43)
OG1RF:pTCV-*lac*	Wild type with promotorless vector	This study
OG1RF:pMHK100	Wild type with expression vector 1 (V1)	This study
OG1RF:pMHK200	Wild type with expression vector 2 (V2)	This study
OG1RF:pCF10	Wild type with mating vector	(25)
TX5427(*dltA*)	D-ala-LTA deficient strain	(81)
TX5427(*dltA*):pMHK100	D-ala-LTA deficient strain with V1	This study
TX5427(*dltA*):pMHK200	D-ala-LTA deficient strain with V2	This study
TX5427(*dltA*):pCF10	D-ala-LTA deficient strain with pCF10	This study
OG1RFΔRR01	Resposne regulator 01 mutant	Perego (unpub.)
OG1RFΔRR02	Response regulator 02 mutant	Perego (unpub.)
OG1RFΔRR03	Response regulator 03 mutant	Perego (unpub.)
OG1RFΔRR04	Response regulator 04 mutant	Perego (unpub.)
OG1RFΔRR05	Response regulator 05 mutant	Perego (unpub.)
OG1RFΔRR06	Response regulator 06 mutant	Perego (unpub.)

OG1RFΔRR09	Response regulator 09 mutant	Perego (unpub.)
OG1RFΔRR10	Response regulator 10 mutant	Perego (unpub.)
OG1RFΔRR13	Response regulator 13 mutant	Perego (unpub.)
OG1RFΔRR14	Response regulator 14 mutant	Perego (unpub.)
OG1RFΔRR15	Response regulator 14 mutant	Perego (unpub.)
OG1RFΔRR17	Response regulator 17 mutant	Perego (unpub.)
OG1RFΔRR18	Response regulator 18 mutant	Perego (unpub.)
OG1RFΔRR01:pMHK100	Response regulator 01 mutant with V1	This study
OG1RFΔRR02:pMHK100	Response regulator 02 mutant with V1	This study
OG1RFΔRR03:pMHK100	Response regulator 03 mutant with V1	This study
OG1RFΔRR04:pMHK100	Response regulator 04 mutant with V1	This study
OG1RFΔRR05:pMHK100	Response regulator 05 mutant with V1	This study
OG1RFΔRR06:pMHK100	Response regulator 06 mutant with V1	This study
OG1RFΔRR09:pMHK100	Response regulator 09 mutant with V1	This study
OG1RFΔRR10:pMHK100	Response regulator 10 mutant with V1	This study
OG1RFΔRR13:pMHK100	Response regulator 13 mutant with V1	This study
OG1RFΔRR14:pMHK100	Response regulator 14 mutant with V1	This study
OG1RFΔRR15:pMHK100	Response regulator 15 mutant with V1	This study
OG1RFΔRR17:pMHK100	Response regulator 17 mutant with V1	This study
OG1RFΔRR18:pMHK100	Response regulator 18 mutant with V1	This study
OG1SSp:pCF10		(25)
CK111:pCF10-101		(68)
E. coli		
EC1000		(73)
Plasmids		
pTCV-*lac*	Promotorless *LacZ* expression vector	(103)
pMHK100	*LacZ* expression vector	This study
pMHK200	*LacZ* expression vector	This study
pCF10	Conjugative plasmid, Tet^R	(25)
pCJK47	Plasmid for markerless exchange	(68)

Molecular Techniques

Chromosomal DNA was obtained using Chelex® 100 Resin (Bio-Rad Laboratories, Hercules, CA) following the DNA extraction for PCR method (131), Plasmid DNA was extracted and purified using the Mini Prep Spin Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. Custom primers used for PCR and RACE reactions were supplied by IDT Technologies (Coralville, IA) (Table 2). Bioinformatics, including BLAST searches and identification of genome sequences, were carried out on The Institute for Genomic Research website (TIGR). PCR conditions were optimized for individual reactions based on primer T_m and length of amplified sequence using Bio-X-act Short DNA polymerase (Bioline, Tauton, MA).

Electrocompetent enterococci were prepared as described by Shepard and Gilmore (117). Transformations were performed on the BIO-RAD GenePulser Xcell using 2.5 kV current, 200 Ω resistance, 25 μF capacitance, and 2 mm cuvetes. Restriction digests were performed with appropriate restriction enzymes supplied by Promega at 37°C for 4-16 hours depending on cutting efficiency. Primers Vlac1 and Vlac2 are located on the expression vector pTCV-*lac* and encompass the multiple cloning site. They were used in colony PCR for screening transformants following the protocol described on the Oklahoma University Enterococcus Research Site (http://w3.ouhsc.edu/enterococcus/).

Table 2. Custom primers used in this study

Name	Sequence (5'-3')
REP01	TTT AAT GAA TTC GCC GTT GTG AAG CGG
REP02	ATA TAA GGA TCC CAT TAT CAT TCA CCT CCT
REP03	TTA TTA GGA TCC CAT GTA TAG CCG CCT CCT
DA09RACE	GCT TGC GGA ACG ACA TAG GC
DA10RACE	GCT GGT AGC CCA TCT AAT TGG
DA11RACE	GCT TCT GTC GGC CCA TAC G
dltA F	CGG AAG AAT AAC AGA AAT GAT GAT GC
dltA R	ATG AGC CAC CTA ACG CCA ATG
gryB F	CAA GCC AAA ACA GGT CGC C
gyrB R	ACC ACC ACC GTG CAA GCC
*dltX*01	AAT AAA TCT AGA GCC ACA GAA TGG CAA GCG
*dltX*02	TAA AAT CTG CAG TCA CCT CCT AAG GTT AAT CGC
*dltX*03	ATA TAT CTG CAG ATG GAA AAA GTA ATT ATT ATG
*dltX*04	TAT TAT GAA TTC GCG CAG TTG GGA AAC GAG
P1	GCG ATT AAC CTT AGG AGG TG
P2	GAA ATT TCA ATG GAC TTT GC
P3	GCT GAA ATA TAG CAT AAA ATA ATG G

Creation of a *dltX* Knockout

To clone the allele of interest into pCJK47, PCR was used to amplify 800-1000 bp of the DNA regions flanking the site of the planned mutation. The two regions were made using primers *dltX*O1 (XbaI), *dltX*O2 (BamHI), *dltX*03 (BamHI), and *dltX*04 (EcoRI), and then inserted into plasmid pCJK47 at the appropriate restriction sites using restriction digest followed by ligation reactions. The plasmid containing our inserts was transformed into and propagated in *E. coli* EC1000 cells using the previously described conditions for electroporation. EC1000 cells carrying pCJK47, or its derivatives, were cultivated in BHI supplemented with 100 μg/ml erythromycin at 30°C with aeration. The recombinant plasmid was then extracted using a Qiagen mini-prep kit (Qiagen), and

transformed into *E. faecalis* CK111:pCF10-101. Colonies carrying the plasmid were selected by plating on BHI supplemented with 10 μg/ml erythromycin and 250 μg/ml X-gal. Blue transformants were restreaked on similar media 2 times to ensure the retention of the blue color prior to use as a donor in the mating procedure.

For conjugation, the donor strain was grown overnight in BHI containing 10 μg/ml erythromycin at 37°C. Donor and recipient were then washed 2 time with BHI and resuspended in the original volume of BHI. Both cultures were then diluted 10-fold in BHI and incubated at 37°C for one hour. Conjugation was initiated by mixing 1 part donor to 9 parts recipient and 500 ml of the mixture was spread over the surface of a BHI agar plate. Mating proceeded for 6 hours at 37°C before the cells were collected from the surface by adding 2 ml of PBS and scraping the cells from the agar surface. The cells were then collected and serially diluted in PBS before selection of transformants on BHI containing 1000 μg/ml spectinomycin, 10 μg/ml tetracycline, and 10 μg/ml erythromycin. Blue colonies from the transconjugant plates were restreaked on the same media at least two times to ensure the retention of the blue color. Colony PCR was performed on several colonies to ensure recombination occurred at the desired locus.

Appropriate colonies were then cultured in BHI without selection until stationary phase. The culture was then used to inoculate fresh media at a rate of ~100 cfu/ml. The new culture was allowed to reach stationary phase prior to serial dilutions in PBS. 100 ml of MM9YEG with 10 nM p-CL-Phe was prepared using 10 ml of 10x M9 salts, 0.25 g yeast extract, 199.6 mg p-Cl-Phe, 1.5 g agar, 1 ml of 50% glucose and 250 μg/ml X-gal solution, and filled up to a total volume of 100 ml with water. Dilutions were plated on MM9YEG agar to select for white colonies representing an excise of the plasmid. Primers Xcom1 and Xcom2 were used to amplify DNA which included the planned mutation site in potential mutants. PCR products were sequenced using Applied Biosystems 3730 DNA analyzer.

Transmission Electron Microscopy (TEM)

Strains OG1RF and TX5427 were grown to either mid-log phase (O.D. 0.6-0.8 at 600 nm) or stationary phase in BHI media at 37°C prior to fixation. 1 ml of culture was

pelleted in a 1.5 ml microcentrofuge tube at 8,000 x g for 10 min. The supernatant was decanted and the pellet washed three time in PBS buffer. Primary fixation was carried out by resuspending the washed pellet in 1 ml of 0.1 M sodium cacodylate buffer (pH 7.2-7.4) buffer, containing 2% paraformaldehyde, 2% glutaraldehyde and placing at room temperature overnight with constant rotation. Samples were then washed 3 times in 0.1 M sodium cacodylate buffer, and a secondary staining was performed by incubating samples in 1 ml of 0.1 ml sodium cacodylate buffer supplemented with 2% osmium tetroxide for 1-2 hours until the pellet turn amber or black in color. The sample was again washed three time in 0.1 ml sodium cacodylate buffer, followed by three washes 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 in the absent of light. Samples were washed a final three times in 0.2 M sodium acetate buffer before dehydration. Dehydration was performed by exposing samples to increasing concentrations of alcohol, 50%, 60%, 70%, 80%, 90%, 95% and 100%, until the samples were devoid of water. Polymerization was carried out at 60°C in EMBED 812/Araldite resin.

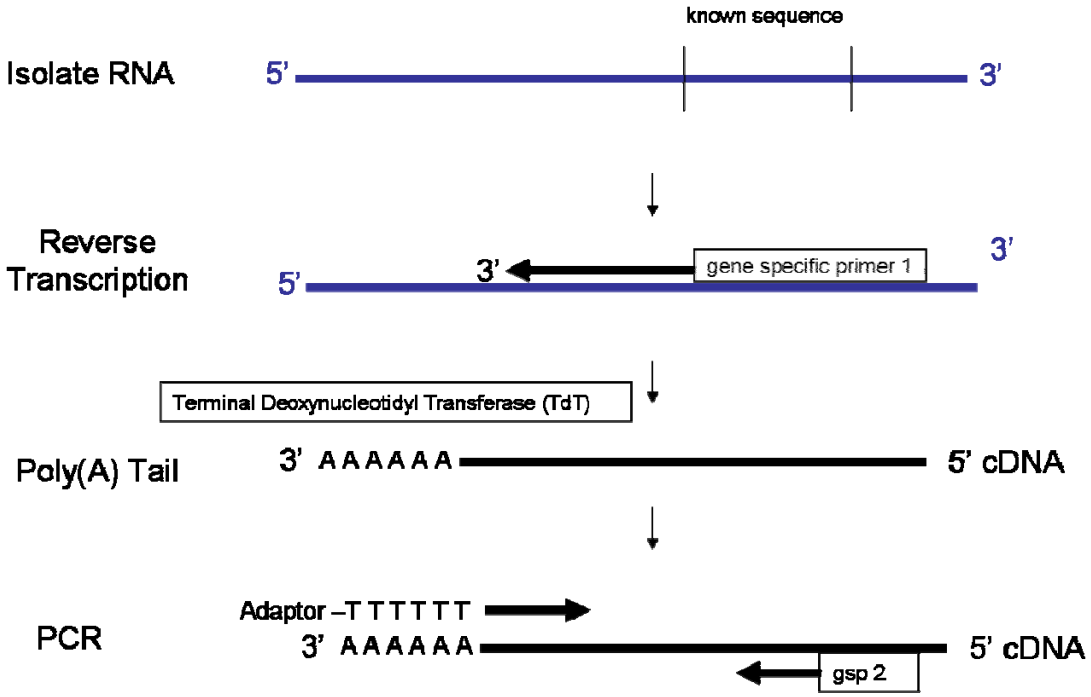
Sectioning of the samples was performed using a Reichert Ultracut S ultramicrotome (Leica). Sectioned samples were placed on 200 mesh copper grids and visualized on a CM100 (FEI Company) transmission electron microscope at 100 kV. Images were captured using a Hamamatsu digital camera (Advanced Microscopy Techniques Corp.) equipped with capture engine software version 5.4.2.22.B.

Rapid Amplification of cDNA Ends (RACE)

E. faecalis OG1RF was grown to mid-log phase (O.D. 0.3 at 600 nm). Two volumes of Bacterial RNAprotect (Qiagen, Valencia, CA) was added to the culture and incubated at 25°C for 5 min. prior to isolation of total RNA using the RNeasy Kit according to manufacturer's protocol (Qiagen, Valencia, CA). All glassware, dispensing materials, and solutions were treated with either sodium dodecyl sulfate (SDS) or DPEC to neutralize RNase activity. Identification of the 5' transcript start site was carried out using the 3'/5' RACE Kit, 2nd Generation (Roche, Indianapolis, IN) (fig 8). Sequencing was completed on the Applied Biosystems 3730 DNA analyzer.

Figure 8. Methods for RACE.

Schematic diagram of the steps in RACE. Adapted from Roche 3'/5' RACE Kit, 2nd Generation product insert.

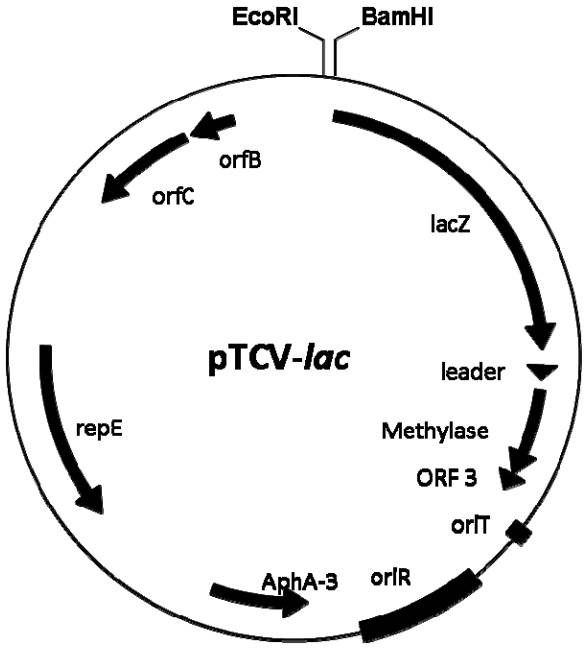


Construction of Reporter Plasmids

Expression vectors pMHK100 and pMHK200 (Fig. 14) were made by inserting cloned sections of the upstream promoter region of the *dlt* operon into pTCV-*lac* at the BamHI and EcoRI restriction sites (Fig. 9) (103). Expression vectors were propagated in *E. coli* EC1000 cells before transformation into appropriate *E. faecalis* strains. Sequencing of reporter constructs was performed using the Applied Biosystems 3730 DNA analyzer to ensure proper promoter sequence insertion.

Figure 9. A diagram of the reporter vector pTCV-*lac*.

Shows the gene organization and the restriction sites used to introduce the upstream sequence for the reporter constructs pMHK100 and pMHK200 (103).



Minimal inhibitory concentrations (MICs)

Sensitivities to vancomycin (Sigma; Vancomycin Chloride), polymyxin B (Sigma; Polymyxin B sulfate), nisin (Sigma), and bile salts (Sigma; Bile Salt mixture) were determined following the protocol provided by the Clinical and Laboratory Standards Institute (CLSI) for Antimicrobial Susceptibility Testing (86). All chemicals were provided by Sigma (Sigma, St. Louis, MO). Appropriate starting concentrations of the antimicrobials were serially diluted in a 96-well microtiter plate using BHI media. Inoculation was performed by the addition of 50 µl of overnight culture diluted to ~$1x10^6$ cfu/ml to each well. MICs were identified as the last well that exhibited growth inhibition after 24 hours of incubation at 37°C.

Autolysis Assay

Overnight cultures were prepared using freezer stocks in fresh BHI media. Determination of autolysis in stationary phase was carried out using the overnight cultures. Otherwise, cultures were inoculated at a 1:100 ratio in fresh BHI and grown to and O.D. of 0.600 at 600 nm for determination of autolysis in log phase. Cultures were then placed on ice for 10 min. before 1.5 ml was removed for centrifugation at 16,000 x g for 5 min at 4°C. The supernatant was removed and the pellet resuspended in 1.5 ml of ice cold water before centrifugation at 16,000 x g for 5 min at 4°C. The pellet was washed twice more with ice cold water and resuspended in 1ml ice cold 10 mM $NaPO_4$ (pH6.8) supplemented with 0.5 µg/ml trypsin. 200 µl of the suspensions were placed in triplicate in the center of a 96-well flatbottom microtiter plate and the remaining wells were filled with 200 µl ddH_2O. Samples were incubated at 37°C and O.D. readings were taken every 30 min for 9 hours at 600 nm in a spectrophotomer. Autolysis was expressed as percent of initial turbidity at 600 nm.

Plasmid Transfer

Overnight cultures were used to inoculate fresh BHI media at a 1:10 ratio. Media used for donor strains carrying the pCF10 plasmid was supplemented with either 10 ng/ml, 1 ng/ml or 100 pg/ml of cCF10 in order to initiate conjugation. Cultures were grown at 37°C for 2 hours before mating. Donors and recipients were co-cultured at a 1:10 ratio for 10 min at 37°C. Serial dilutions in 0.9% NaCl were plated on BHI plates containing 1 mg/ml streptomycin for selection and enumeration of donors and BHI containing 1 mg/ml rifampicin and 10 µg/ml tetracycline for selection and enumeration of transconjugates. Mating efficiency was assessed as the number of transconjugates recovered per donor.

Plate Expression Assay

Overnight cultures containing the *lac*Z expression vector pMHK100 were used to swab BHI agar plates containing 10 µg/ml erythromycin and 150 µg/ml X-gal. Effects of

antibiotics were tested using pre-made 6 mm paper disks (BD, Franklin Lakes, NJ). Blank 6 mm paper disks (BD Franklin Lakes, NJ) were used to test other environmental stimuli by placing the disks on agar plates and saturating with 10 µl of tested environmental stimuli. Plates were incubated for 24 - 48 h at 37°C before analysis of reporter expression.

Modified Miller Assay for Quantifying β-galactosidase Activity

Miller Assays were performed following the protocol described on the Oklahoma University Enterococcal Research Site (http://w3.ouhsc.edu/enterococcus/) with modifications. Overnight cultures were used to inoculate fresh BHI, at a 1:100 ratio, containing 1 mg/ml kanamycin and appropriate concentrations of environmental stimuli. Cultures were incubated at 37°C until late log phase (O.D. 0.6-0.8 at 600 nm) and chilled on ice. Cells were pelleted at 8,000 x g at 4°C for 10 min in a Beckman countertop centrifuge. The supernatant was removed the cells were washed in 3 ml of ice cold Z-buffer (0.06 M $Na_2HPO_47H_2O$, 0.04 M NaH_2PO4H_2O, 0.01 M KCl, 0.05 M $MgSO_47H_2O$) and centrifuged at 8,000 x g at 4°C for 10 min. Cultures were resuspended in 1.5 ml of ice cold Z-buffer and placed in 2 ml screw cap tubes containing 0.5 ml (by volume) of 0.1 mm glass beads (BioSpec, Bartlesville, OK). Cells were lysed for 60 sec at 5000 rpm in a Mini-bead beater (BioSpec, Bartlesville, OK) and placed on ice. Samples were pulse spun to settle glass beads. The supernatant was removed and centrifuged for 10 min at 16,100 x g at 4°C to pellet cellular debris. Cell extracts were then used to quantify total protein using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) according to manufacturer's recommendation. To measure β-galactosidase activity, 100 µl of a 4 µg/ml solution of ONPG was added to 500 µl of cell extract, vortexed briefly, and incubated in a water bath at 37°C for 30 min. The reaction was stopped by the addition of 250 µl sodium carbonate (Na_2CO_3). 200 µl of sample was placed in triplicate on a 96-well flatbottom microtiter plate (Corning Incorporated, Corning, NY) and O.D. was measured at 405 nm. Miller activity was calculated as O.D. 405 nm divided by mg/ml total protein. Statistical analyses were

performed on the Microsoft Graph Pad program using a one-way ANOVA followed by a Neuman-Kuhls post-test. Significant differences were determined by a value of $P<0.05$.

Real-Time PCR

Enterococci samples were either grown to O.D. 0.3-0.5 at 600 nm and processed or grown to O.D. 0.3-0.5 at 600 nm, exposed to stimuli for an additional 15 min at 37°C, and then processed. For RNA extraction, cultures were treated with 2x volumes of bacterial RNAprotect (Qiagen, Valencia, CA) for 5 min and processed using the RNeasy kit (Qiagen, Valencia, CA). Total RNA was used for quantification using the two-step Iscript Reverse Transcriptase and IQ-SYBR Green procedure (Bio-Rad, Taunton, MA). Quantification reactions were carried out on the IQ5 Real-Time PCR Detection System (Bio-Rad) using 35 cycles of 94°C denaturation for 1 min, 72°C annealing temp. for 30 sec, and 57° elongation for 1 min. The primers used were *dltA* F and *dltA* R for the gene of interest (GOI) and gyrB F and *gyrB* R for the housekeeping gene (HK) *gyrB*.

Results

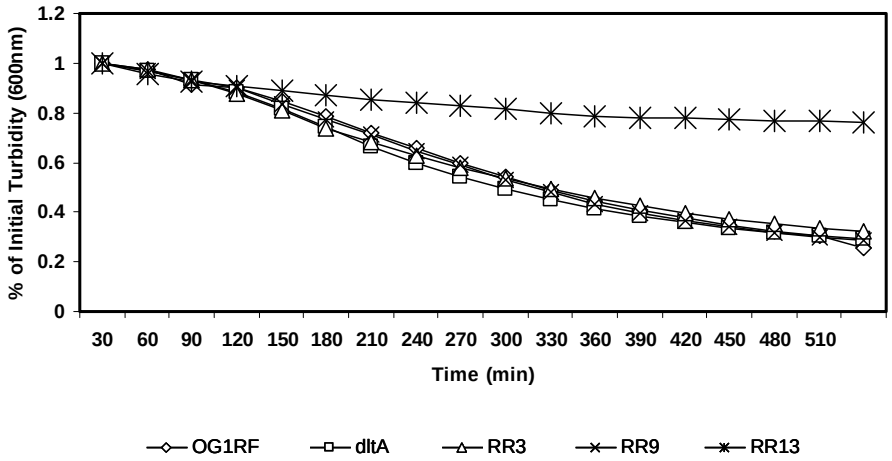
Autolytic Activity of *E. faecalis* Strains

A commonly described phenotype related to the loss of alanylation is changes in the autolytic behavior of the mutants (95, 98, 134). We investigated if the common laboratory strain OG1RF showed any differences in autolytic activity between wild type and a *dlt* mutant (Fig. 10a). We found no difference between the two strains. However, when Response Regulator (RR) mutants were tested, RR13 was found to possess dramatically reduced autolytic activity. None of the other RR mutants presented an autolysis phenotype. A RR13 knockout in the vancomycin resistant clinical isolate V583 showed the same phenotype (Fig. 10b).

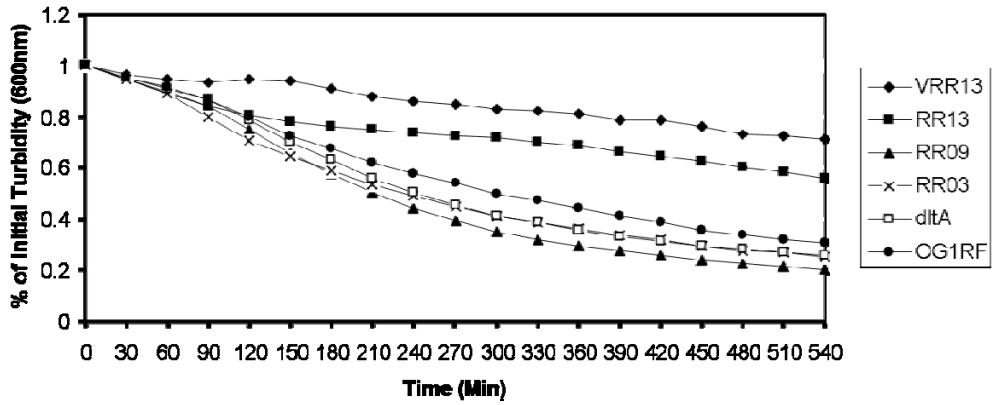
Figure 10. Autolysis assay data.

Values expressed as percent of initial turbidity vs. time. OD. reading were recorded every 30 min. A comparison of OG1RF (wild type), TX5427 (*dltA*) and the response regulators RR03, RR09, and RR13 is shown in A. In addition to those five strain in and OG1RF background, a RR13 knockout in a V583 background was also tested (B). The data is the average of three independent experiments. Error bars were omitted for clarity sake, but both strains of RR13 displayed significantly reduced autolysis.

A.



B.



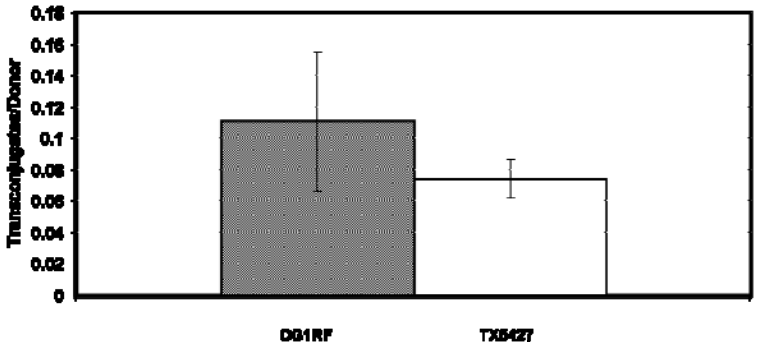
Effects of a *dlt* Knockout on Plasmid Transfer

Recently, Waters et al. showed that Aggregation Substance (AS) binds in vitro to purified LTA in a dose dependent manner (132), providing evidence that LTA may participate in mobile genetic element transfer by promoting the formation of mating aggregates. It has been demonstrated that the binding of AS to LTA was abolished with an in frame deletion of N-terminal amino acids 156-358, while a purified N-terminal fragment consisting of amino acids 44-331 displayed high LTA binding (132). A closer look at the N-terminal amino acid profile of AS reveals a cluster of positively charges amino acids in the stretch were binding occurs. Little is known about the chemical interactions between LTA and AS, but it is feasible that the positively charged N-terminal amino acids could electrostatically interact with the anionic poly-glycerol phosphate backbone of LTA and that D-alanylation of LTA could impact this interaction. Our findings suggest there is no significant difference between strains TX5427 and OG1RF as a recipient during plasmid transfer when the donor media is supplemented with 10 ng/ml of the inducing pheromone cCF10 (Fig. 11A). This concentration of pheromone greatly exceeds naturally occurring pheromone levels. As a result, the cells may be over-expressing mating proteins, namely AS, and thereby masking the significance of the putative LTA-AS interaction. To test this we reduced the concentration of exogenous pheromone in the mating procedure by 10- and 100-fold. There was also no significant difference in mating efficiency when the pheromones concentration was lowered 10-fold to 1 ng/ml (Fig. 11B) or 100-fold lower to 100 pg/ml (Fig. 11C).

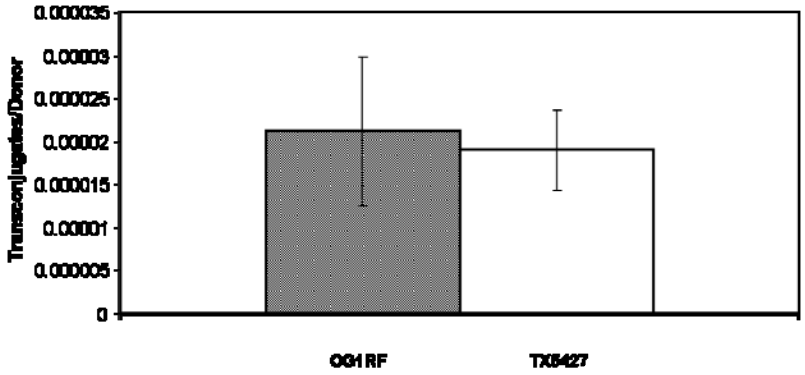
Figure 11. Plasmid transfer data.

Mating assay data expressed as the number of transconjugates observed per donor recovered. The concentrations of pheromone cCF10 used were 10 ng/ml (A), 1 ng/ml (B), and 100 pg/ml. Data shown is the average of three independent experiments.

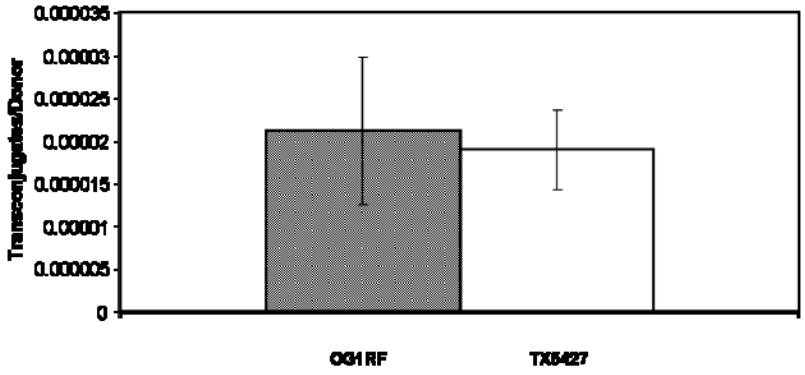
A. 10ng/ml



B. 1ng/ml



C. 100pg/ml



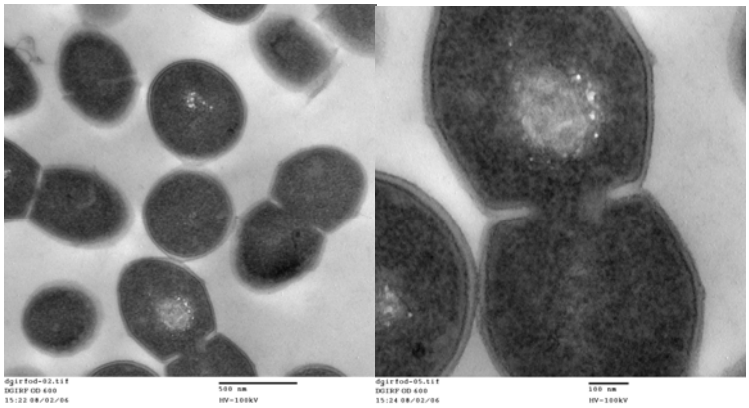
Morphological Characteristics of Strains OG1RF and TX5427

In order to visualize any phenotypical differences that may be present between OG1RF and TX5427, we utilized TEM to get a closer look at fixed cell samples. After analyzing several frames from cells either in mid-log or stationary phase, there was nothing noticeably different between the two strains (Fig. 12). These results are supported by a report published after our findings that found there was no difference between wild type and a *dlt* mutant in the clinical isolate *E. faecalis* 12030 (31).

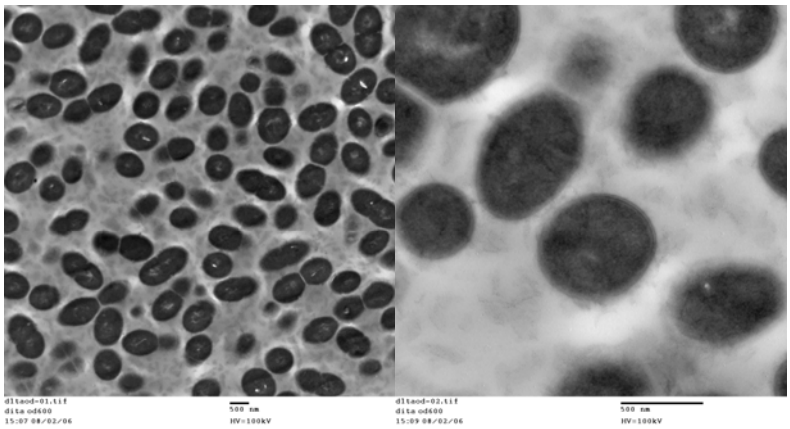
Figure 12. TEM photos.

Pictures of OG1RF in log phase (A), TX5427 in log phase (B), and OG1RF in stationary phase (C).

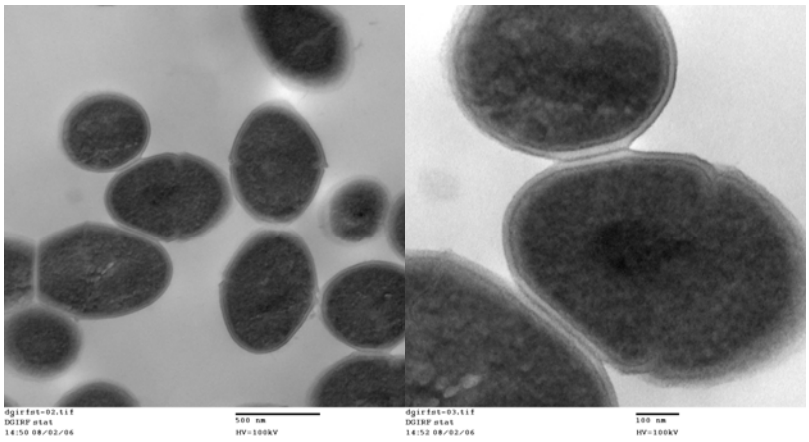
A. OG1RF (Log Phase)



B. TX5427 (Log Phase)



C. OG1RF (Stationary Phase)



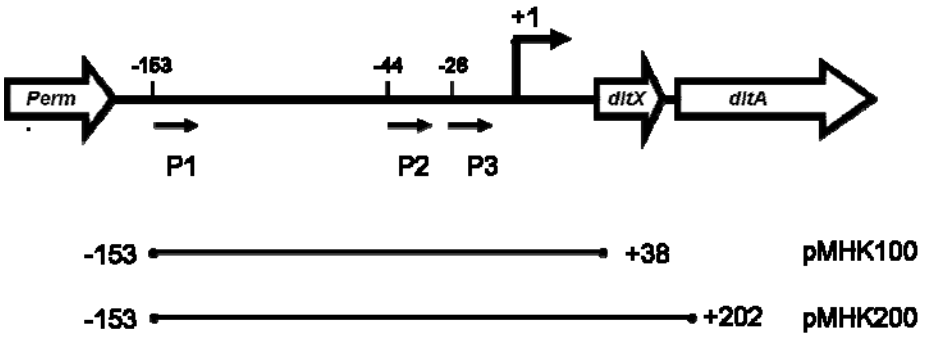
Identification of *dlt* Operon Transcript Start Site

The 150 base pair open reading frame (*dltX*) upstream of *dltA* is present and highly conserved among gram-positive bacteria containing the *dlt* operon. In *S. aureus* the open reading frame was shown through RACE to be contained on the same transcript as *dltA* with the transcript start site approximately 250 base pairs upstream of *dltA* (82 bp upstream of *dltX*). Because of the ORF's high conservation, we hypothesized that *dltX* would also be a member of the operon in *E. faecalis*. Utilizing RACE, we identified the transcript start site of the operon in *E. faecalis* to be 191 bases upstream of *dltA* and 29 bases upstream of *dltX* (Fig. 13A), showing that *dltX* is indeed a constituent of the operon. A promoter region of 29 base pairs appeared to be rather small when compared to the findings in *S. aureus*. In order to test the accuracy of RACE we performed PCR with total cDNA and primers P1, P2, and P3 (Fig. 13B). The presence of a PCR product with P3 coupled with the lack of products with P1 and P2 confirm that there was likely RNA degradation of the RACE product and that the transcriptional start site is several bases upstream of the predicted +29 bases.

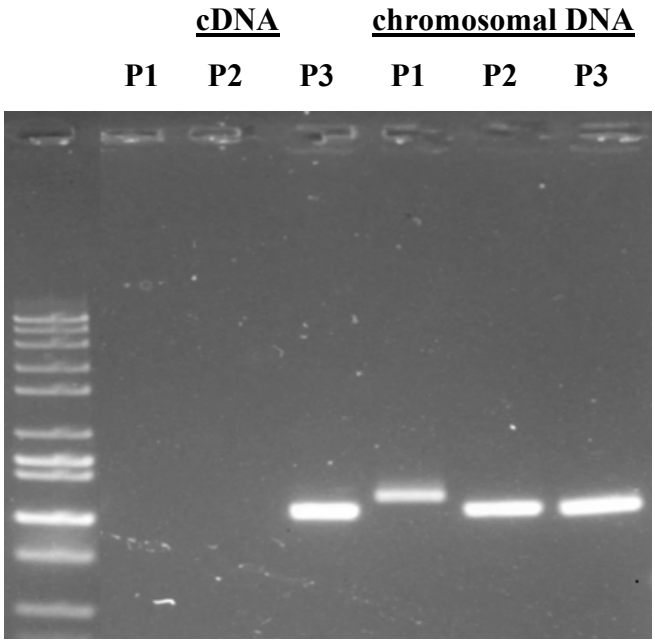
Figure 13. Race and reporter constructs.

A diagram for the identification of the transcription start site through RACE and the sequence of the reporter constructs (A). Determination of the accuracy of RACE was confirmed through PCR using custom primers P1, P2, and P3 to amplify cDNA and chromosomal DNA (B).

A.



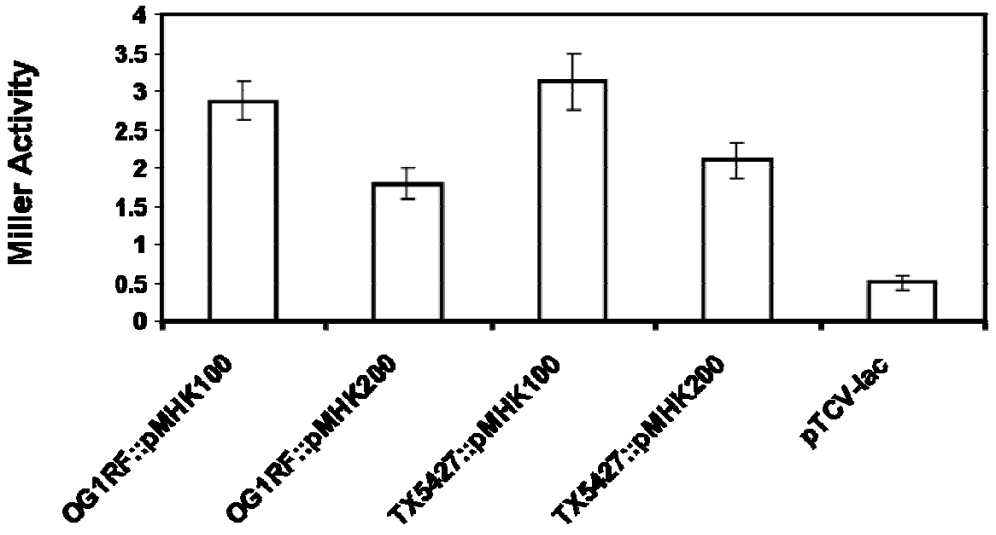
B.



Effects of *dltX* on Operon Expression

Upon discerning that *dltX* was part of the operon, we wanted to see if the gene possessed any effects on transcription levels of the operon. A 150 amino acid protein is quite small, so we hypothesized that *dltX* may be playing a role in operon regulation by either acting as a cis- or trans-element. To test this hypothesis two reporter constructs, one containing *dltX* (pMHK200) and one lacking the gene (pMHK100), were created in order to quantify operon expression. The constructs were introduced into OG1RF and TX5427 and β-galactosidase expression was quantified for mid-log phase cells (Fig. 14). All reporters showed activity over the empty reporter in both strains. Also, in both strains, the construct containing *dltX* showed reduced Miller activity, indicating that *dltX* is interfering with transcription in some capacity.

Figure 14. Miller activity for reporter constructs.
Miller assay of selected strains containing either pMHK100, pMHK200, or the promotorless pTCV-*lac* plasmid. Cells were grown to late log (O.D. 0.6-0.8) prior to quantification of Miller activity. Data is expressed as the average of three independent experiments.

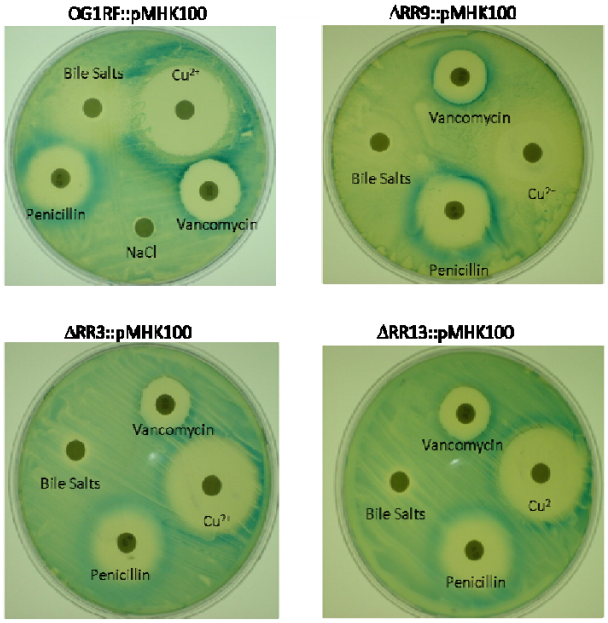


Identification of Stimuli Affecting *dlt* Operon Expression

It has been demonstrated that metal cations, especially divalent cations, possess repressive properties on *dlt* operon expression in *Staphylococcus aureus* (66). These results suggested that gram-positive bacteria could regulate their cell surface chemistry by altering the charge of their teichoic acids via modulation of the D-alanine substitution rate. We utilized a plate expression assay to quickly screen other environment conditions that could impact operon expression in *E. faecalis*. The reporter construct pMHK100 was created using the upstream promoter region of the *dlt* operon fused to the promotorless *lacZ* gene in the plasmid pTCV-*lac*. Up or downregulation of the *lacZ* reporter was observed in the presence of the cationic compounds vancomycin, polymyxin B, Cu^{2+}, as well as penicillin and bile salts (Fig. 15). Strain OG1RF displayed a ring of up-regulated reporter expression around the zone of inhibition for the cell wall acting antibiotics vancomycin and penicillin, as well as the cationic peptide polymyxin B. Copper produced an interesting phenotype by displaying a gradient with no/slightly repressive effects on reporter expression near minimum inhibitory concentrations (MICs) and an inducing effect at lower concentrations. Bile salts produced a distinct phenotype in that there was noticeable down regulation of reporter expression around the zone of inhibition that also seemed to supersede the over-expression induced by other stimuli. The phenotypes observed were consistent with the bacteria being able to sense environmental stimuli and adjust transcription of the *dlt* operon accordingly.

Figure 15. Plate expression assay.

Expression profile of selected strains carrying pMHK100 on BHI containing 10 µg/ml erythomycin and 200 µg/ml X-gal.



Quantification of Operon Expression in RR Knockouts

A common theme among bacteria when responding to environmental stresses is the involvement of two component sensory and regulatory systems to sense the stimulus and transform the signal into gene expression. The only publicly available sequence for *E. faecalis* is for the vancomycin resistant clinical isolate V583 (TIGR). The genome encodes 17 two-component systems comprised of a sensory kinase and cognate response regulator, along with an orphan 18th response regulator. Hancock and Perego systematically inactivated all 18 response regulators in V583 using targeted insertional mutagenesis and characterized the strains according to antibiotic sensitivity, biofilm formation, and environmental stress (48). Of the 18 response regulators in V583, only RR07 was unable to be inactivated. RR07 is an ortholog of the essential YycF for gram-positive bacteria. The same approach was utilized to inactivate homolog response regulators in OG1RF (Hancock and Perego, unpublished). There is relatively high

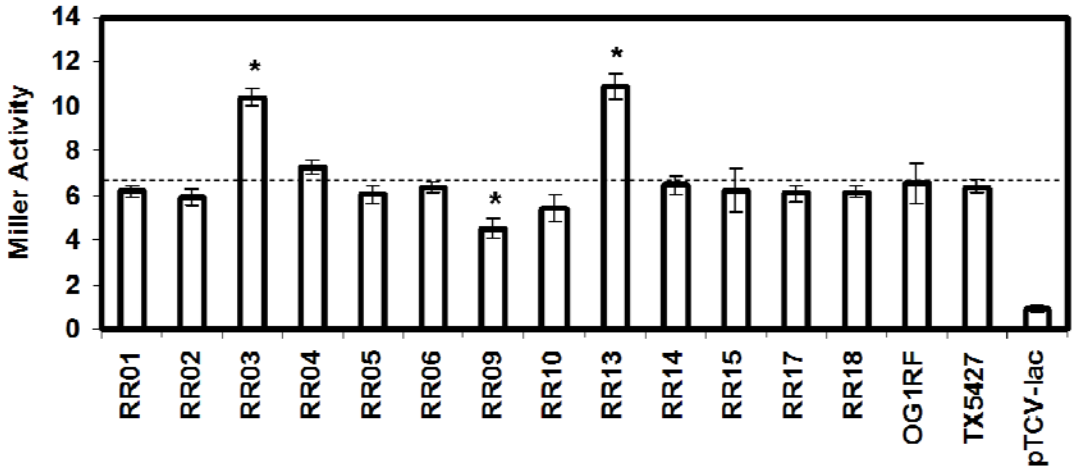
sequence variance among strains of *E. faecalis* (39) and only 14 response regulators are conserved in OG1RF. The OG1RF genome is smaller than V583 (2.8 MB compared to 3.2 MB) and is lacking four strain specific two component systems. Three of these four missing TCS, including VanRS which is involved regulation of vancomycin resistance genes, are linked to mobile DNA elements (96, 114).

In order to assess the TCS involvement in operon expression, the reporter vector pMHK100 was transformed into the 13 response regulator mutants (Table 1) and expression in comparison to wild type was assessed throughout the growth cycle (Fig. 16). Operon expression was maximal during mid to late log phase and substantially subsided after entry into stationary, with none of the strains showing significantly higher expression over the control. Strains OG1RF and TX5427 showed the same expression profile indicating that the cells are not sensing their degree of alanylation, but rather responding to different stimuli by either activating or repressing expression of the genes responsible for this covalent modification. This observation was confirmed with a plate expression assay with both strains again displaying an identical expression profile (data not shown). RR03 and RR13 showed significant overexpression in comparison to wild type, while in RR09 operon expression was significantly reduced.

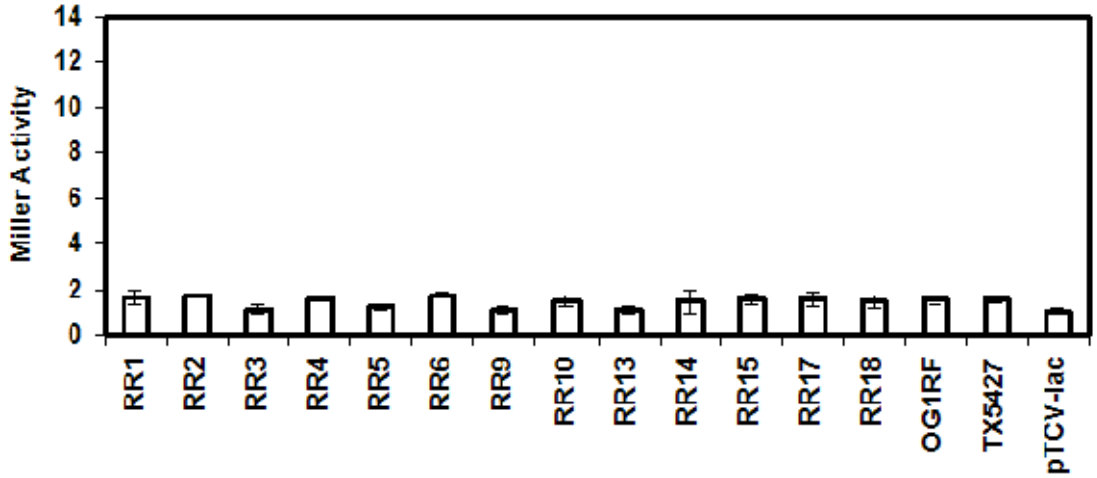
Figure 16. Miller assay for RR's during log and stationary phases.

Miller assay of selected strains containing pMHK100 or the promotorless pTCV-*lac* plasmid. Cells were grown to late log (O.D. 0.6-0.8) (A) or stationary phase (B) prior to quantification of Miller activity. Data is expressed as the average of three independent experiments. Dotted line represents wild type expression levels. P<0.05 when compared to the control is indicated by (*).

A



B.



Analysis of RR Knockouts on Plate Expression Assay

Upon discerning expression variance in the Miller assay, RR03, RR09, and RR13 were compared to OG1RF to visualize changes in expression profiles to environmental stimuli utilizing the plate expression assay (Fig. 16). Response regulator knockouts RR03 and RR13 showed similar phenotypes to the wild type strain except for in response to bile salts. The noticeable ring of repressed reporter expression was absent in these two strain, indicating they may play essentials roles in bile salt induced repression of the *dlt* operon. The RR09 knockout lacked wild type levels of reporter up-regulation in response to Cu^{2+} and polymyxin B, again indicating potential roles in signal recognition and regulation of the operon. None of the tested TCS were indicated in regulation of the *dlt* operon in response to vancomycin or penicillin (data not shown).

Antimicrobial Susceptibility

The MICs of stimuli that produced a distinct phenotype on the plate assay were determined to obtain proper concentrations for use in the Miller Assay to confirm effects on regulation (Table 3). Despite seeing differences in *dlt* gene expression through β-galactosidase activity, there was no discernable difference between strains in bile salt susceptibility. In addition to repressing operon expression, the bile salts may also be producing a microenvironment around the cell that promotes cleavage of the covalently attached D-alanyl residues from TA. If the cells are undergoing D-alanine hydrolysis and the *dlt* operon is being repressed, it would be expected that wild type cells would show the same phenotype as a *dltA* mutant, since both would have significantly reduced D-alanine substitution on the cell wall. As expected, *dltA* showed increased sensitivity to the cationic compounds polymyxin B and nisin, as well as to the cell wall acting vancomycin. RR09, a strain in which the *dlt* operon is under-expressed also showed increased sensitivity to the cationic peptides polymyxin B and nisin, while maintaining wild type levels of resistance to vancomycin.

Table 3. Minimum inhibitory concentration data.

Results are indicative of three independent experiments.

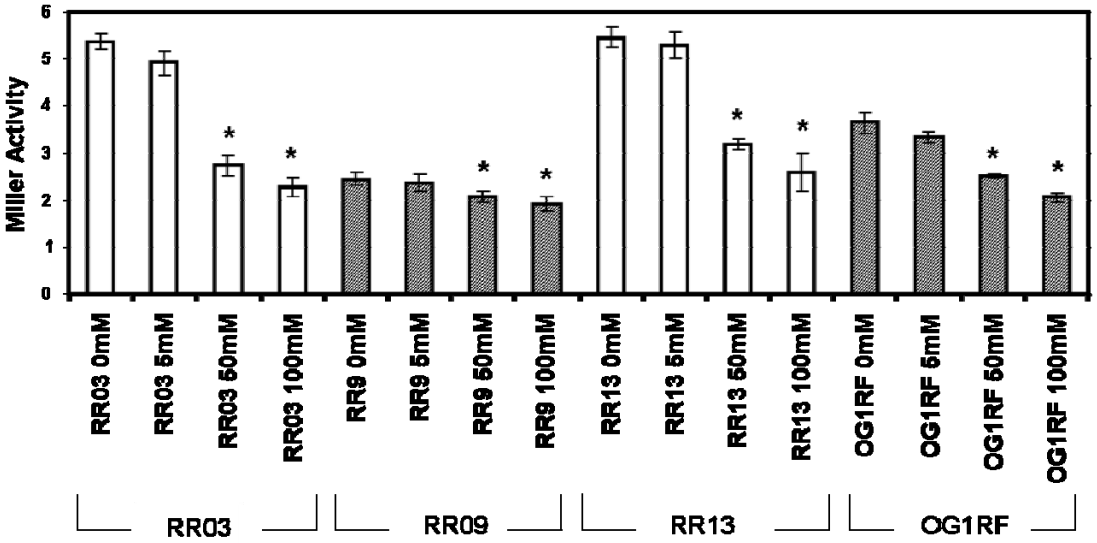
	VAN	POLY B	NISIN	BILE
OG1RF	1.56µg	512µg	6.25µg	.0625%
ΔdltA	0.78µg	256µg	3.12µg	.0625%
RR03	1.56µg	512µg	3.12µg	.0625%
RR09	1.56µg	256µg	3.12µg	.0625%
RR13	1.56µg	512µg	6.25µg	.0625%

Quantification of Operon Expression Through Miller Assays and Real-Time PCR

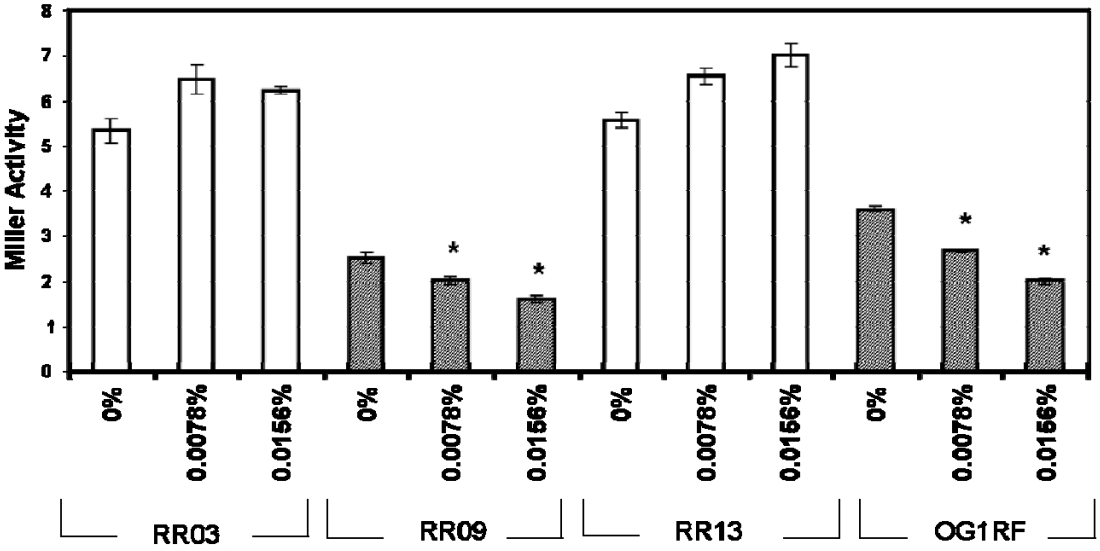
To confirm the results of the plate assay, cultures containing expression vector pMHK100 were grown in media containing sub-inhibitory concentrations of environmental stimuli/stress to assess effects on β-galactosidase expression. In *E. faecalis* the divalent Mg^{2+} ion has a repressive action on *dlt* operon expression for OG1RF, RR03, RR09, and RR13 in a concentration dependent manner (Fig. 17A). Furthermore, none of the 13 response regulator mutants used in our study ceased this down regulation, suggesting that there are other mechanisms for Mg^{2+} induced regulation of the *dlt* operon (data not shown). The strains were also grown in increasing concentrations of bile salts before quantification of reporter expression (Fig. 17B). OG1RF and RR09 display reduced reporter expression in a concentration dependent manner, while RR03 and RR13 failed to show this reduction. To quantify the effects of polymyxin B on reporter expression all strains were grown in BHI or BHI supplemented with 25 µg/ml polymyxin B before processing. All of the strains showed 2-3 fold induction of reporter expression in the presence of polymyxin B except for RR09, which is again consistent with the plate expression assay (Fig. 17C).

Figure 17. Miller assay for Mg^{2+}, bile salts, and polymyxin B.
Strains containing pMHK100 were grown in the presence of (A) increasing concentrations of MgCl, (B) increasing concentrations of bile salts, or (C) 25 µg/ml polymyxin B. Data is expressed as the average of three independent experiments. $P<0.05$ when compared to the control is indicated by (*).

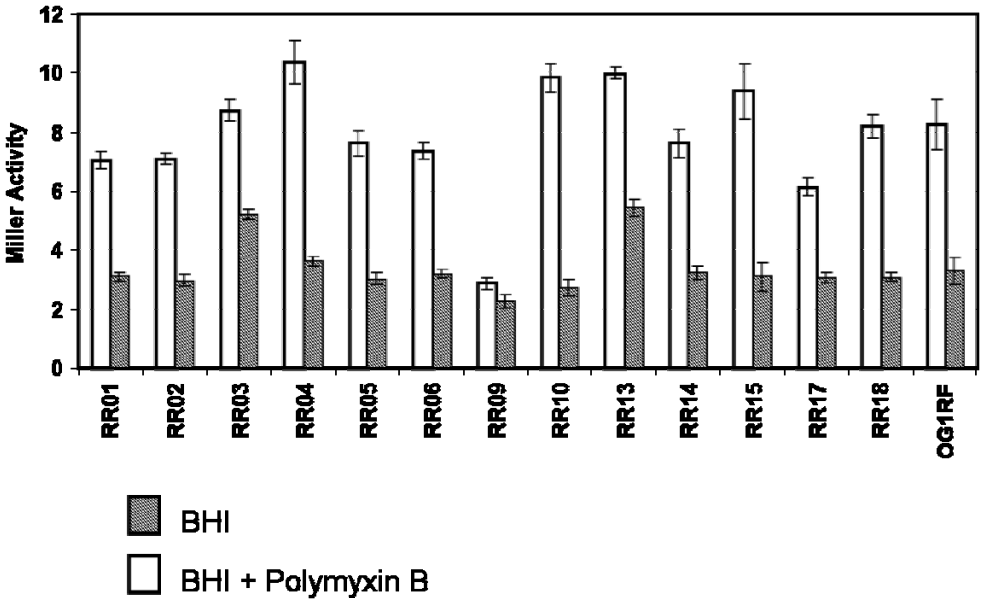
A. MgCl



B. Bile Salts



C. Polymyxin B

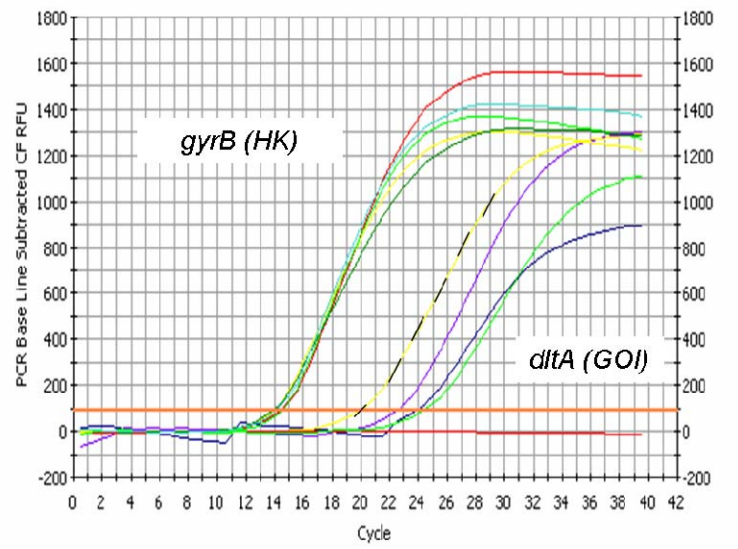


Real-time PCR was then employed to detect changes in transcript levels after acute (15 min) exposure to bile salt and polymyxin B (Table 4). We observed a 13 fold induction in transcript levels after a 15 min. exposure to polymyxin B and a 3 fold reduction after exposure to bile salts.

Figure 18. Real-time PCR.

A representative graph obtained from real-time PCR experiments (A). The four tightly overlapping lines represent the house keeping (HK) gene GyrB that serves as a loading control. The other lines represent DltA expression in the presence of polymyxin B (yellow with black lines), normal BHI media (purple), magnesium (blue), and bile salts (green). Figure B quantifies the fold induction or repression observed for polymyxing B, bile salts, and magnesium during 4 independent experiments.

A.



B.

	Fold Induction/(Repression) (n=4)
PolyB	12.9 ± 2.3
Bile Salts	(3.1) ± 0.99
Mg^{2+}	(1.6) ± .3

Discussion

Autolytic Activity

The effect of D-alanine deprivation on autolytic activity appears to be species dependent. Reports have shown *dlt* mutants to be more susceptible than wild type to autolysins in both *Staphylococcus aureus* (98) and *Bacillus subtilis* (134). Additionally, in *Lactobacillus plantarum* a *dlt* mutant shows cell wall perforations at the divisional septum mediated by the increased activity of the Acm2 autolysin (95). However, reports have shown no difference between wild type and a *dlt* mutant strain in the *Enterococcus faecalis* 12030 clinical isolate (31). We confirmed those finding for the *E. faecalis* strain OG1RF. Therefore, lack of D-alanylation does not seem to significantly influence autolytic activity in *E. faecalis.* Interestingly, a screening of the response regulator mutants indicated greatly diminished autolytic activity in the RR13 mutant in both OG1RF and V583. It is still unclear if there is a correlation between increased *dlt* operon expression and reduced autolysis since RR03, an additional over-expressed *dlt* strain, did not produce the same phenotype. RR13 may be playing a more direct role in regulation of autolysin production or activity. Future studies aimed at determining the effects of RR13 on autolytic activity would be valuable.

Transcript Start Site and *dltX*

Transcript initiation of the *dlt* operon starts at least 29 bases upstream of the *dltA* start codon and includes the open reading frame designated *dltX*. The presence of a PCR product when using a primer just upstream of the start site obtained through RACE provides evidence that the actual start site is more likely 20-40 bp further upstream. This would a more realistic place for the start site when compared to the positioning of other characterized start sites in relation to the translational start site in the genome. No consensus promotor sequences have been documented in *E. faecalis* so analyzing the upstream sequence provides little insight as to the stretch of DNA need for promotor recognition.

The gene product of *dltX* is predicted to be a 50 amino acid transmembrane protein. It is unclear whether the *dltX* ORF is translated into a functional protein that helps facilitate the esterification of LTA or if it plays a role in operon regulation by acting as a cis- or trans-element during transcription. A direct biosynthetic role in D-alanylation seems unlikely since all necessary tasks are performed by the DltA-D proteins (88). The role of *dltX* in the *dlt* operon will be an interesting subject of future research.

Operon Regulation

We observed no difference in the LacZ expression profiles in the OG1RF and TX5427 backgrounds, indicating that the cells are not sensing their degree of D-alanylation. These findings are in agreement with previous reports in *Staphylococcus aureus* that showed no difference in expression performed by both real-time PCR and a chloramphenicol acetyltransferase (CAT) assay in wild type and a *dltA* mutant (66). We also observed a substantial decrease in operon expression after transition into stationary phase. This is not surprising since actively dividing cells would have to supply the continuously synthesized LTA with alanine to avoid unwanted cell surface charge imbalances; a function no longer required in stationary phase.

The results displayed in this report from both real-time PCR and β-gal activity of the reporter construct demonstrate that *dlt* transcription is affected by the presence of vancomycin, polymyxin B, and bile salts at least partially through TCS activity. These stimuli also produced distinct phenotypes of altered reporter expression at the edge of the zone of inhibition on the plate assay. Upregulation of *dlt* transcription with the cell wall acting antibiotic vancomycin and the cationic peptide polymyxin B was not all that surprising since *dlt* mutants are almost universally characterized by increased sensitivity to these compounds (99). There have also been reports of intermediate vancomycin resistant *S. aureus* that in addition to a thickened cell wall also possessed an increased D-alanine content of their LTA (98). Our results also indicated that *E. faecalis* can, up to a certain limit in responding to the presence of cationic peptides and cell wall active antibiotics, defend itself and counteract the damaging effects by adjusting the degree of D-alanylation of its teichoic acids.

Analysis of the Involvement of TCS

RR03 is a member of the NarL family which possesses the highly conserved orthologs *yvqC* in *B. subtilis* and *vraR* in *S. aureus*. Both orthologs, *yvqC* and *vraR*, have been shown to function in bacitracin antibiotic response. Exposure to bacitracin results in an increased expression of *yvqC* in *B. subtilis* (78) and *vraR* plays an important role in resistance to a wide variety of cell wall acting antibiotics, including bacitracin (69). In *E. faecalis* RR03 was also characterized as bacitracin sensitive; however, there was no difference in phenotype compared to wild type in the cell wall acting antibiotic ampicillin (48). RR13 was identified by Le Breton et al. as belonging to the OmpR family based on sequence homology to the *E. coli* OmpR protein (72). It shows significant sequence homology to the *S. aureus* response regulator SrrA that acts in global regulation of virulence factors (137). No phenotype in regards to antibiotic sensitivity, biofilm formation, or environmental stress has been assigned to this mutant in *E. faecalis*. RR09 has also been assigned to the OmpR family, yet it possesses little homology to any known response regulator and there is no assigned phenotype to this mutant (48, 72). Even though RR09 is needed for maximal expression of the *dlt* operon, it is of importance to note that no single RR knockout completely abrogated expression to control values indicating multiple levels of regulation. RR10 (EtaRS) has been characterized as possessing heat and bile-salt resistance (72). However, the bile salt resistance phenotype does not appear to be a direct result of changes in *dlt* operon expression because RR10 produced a similar expression profile to OG1RF (data not shown).

The absence of complete loss of expression due to any single RR knockout raises questions about other mechanisms of environmental sensing. Evidence has shown that a one-component signaling protein named PrkC contributes to inherent resistance to certain antimicrobials and proliferation in the GI tract (55). PrkC is predicted to contain a cytosolic kinase domain and an extracellular domain thought to bind uncross-linked peptidoglycan (55). A PrkC knockout is characterized as possessing wild type growth rates in the absences of environmental stresses, but an increased sensitivity to cell-envelope acting antimicrobials and bile detergents (55). There are 158 one-component systems in the *E. faecalis* V583 genome (55). It is reasonable to believe that there may be a role for one-component systems in the overall biology of the outer membrane in

general by regulating expression of key genes involved in maintaining cell wall and membrane integrity. The *dlt* operon may very well fit into this category due to the nature of its function.

In *Staphylococcus aureus* Mg^{2+} regulation is under the control of the ArlSR two component system (66). We were unable to identify a RR knockout that abrogated Mg^{2+} dependent repression of the *dlt* operon. It is unclear why a RR could not be identified, but OG1RF may contain additional two component systems not found in the V583 sequence that are as yet unidentified. The orphan RR18 could also participate in cross talk between histidine kinases as suggested in *Pseudomonos aeruginosa* TCS regulation (108). It is not impossible that Mg^{2+} signaling is controlled by the essential RR07 since regulation of this metal may be important for the activity of vital metalloproteases and other essential enzymes such as DNA polymerase.

Our study shows that *E. faecalis* is capable of fine-tuning its cell surface charge dependent on environmental conditions by the modification of teichoic acids through D-alanylation. Not surprisingly, two component regulatory systems are involved in the process of *dlt* operon regulation. Signals can be transmitted through one TCS to the *dlt* operon as is the case with polymyxin B or involve more complex patterns such as the involvement of two TCSs in the detection of bile salt signals. These results emphasize the intricate and complex interactions of bacteria with their environment and the capacity to respond and adapt to a variety of challenges. D-alanylation of teichoic acids is without a doubt only one of the many dynamic responses of bacterial cells to ever changing environmental conditions. LTA decoration is certainly a contributor to increased toughness of *E. faecalis* as evident in virulence models and in vitro exposure to antimicrobial agents. It is most likely part of a much larger network that allows the cells to withstand environmental changes, even if only for a limited time, and contribute to the virulence of enterococci.

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