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TRANSCRIPTIONAL ADAPTATION OF *Enterococcus faecalis* WITHIN MACROPHAGES AND THE ROLE OF PerA IN VIRULENCE

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TRANSCRIPTIONAL ADAPTATION OF *Enterococcus faecalis* WITHIN MACROPHAGES AND THE ROLE OF PerA IN VIRULENCE

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

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When the end seemed so far away the following people gave me love, guidance and support:

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Abstract

The intestinal ecosystem is comprised of bacteria that are both beneficial and detrimental to human health. The opportunistic pathogens residing within the gastrointestinal tract typically function as commensal members of the human microbiota. However, in certain circumstances these bacteria are able to escape the intestine and cause severe infections throughout the host. This dissertation defines the transcriptional adaptation within macrophages and elucidates the role of transcriptional regulation during pathogenicity in the opportunistic pathogen, *Enterococcus faecalis*.

Chapter 1: Literature review and thesis overview

The work presented in this thesis originates from a question that has intrigued scientists since the discovery of pathogenic bacteria; that is, how are bacteria that predominately function as commensals also able to cause serious infections?

***E. faecalis* as a commensal.** The enterococci are found in nearly every human gastrointestinal (GI) tract and predominately function as commensal members of this ecosystem. The GI tract typically represents a harsh environment for growth, as metabolic substrates are frequently limited yet competition for these resources is often stiff. When presented with nutrient limitation, those bacteria that are able to efficiently metabolize an assortment of substrates may fare better than those who cannot. *E. faecalis* has evolved the ability to metabolize many substrates found in the intestine, including a wide variety of fermentable carbohydrates, amino acids and mucin (16, 21). This metabolic flexibility permits *E. faecalis* to survive in the highly competitive intestinal environment, yet these bacteria represent less than 1 % of the microbiota in an adult GI tract (57). Despite their relatively low abundance, *E. faecalis* is a leading cause of hospital-acquired infections suggesting that the enterococci may be more important medically than as commensals within the intestine.

The initial bacterial inhabitants of the neonatal GI tract are comprised of numerous facultative anaerobes, including staphylococci, enterococci and *Escherichia coli*, which were presumably acquired from the mother during birth. The enterococcal populations that reside within a developing GI tract are not static, but rather they change in size and composition as the intestinal microbiota matures (61). The infant intestinal microbiota is largely devoid of an obligate anaerobic population, as these bacteria do not flourish in the GI tract until milk is supplemented with a solid diet. The absence of

anaerobes provides open niches for colonization, and by approximately 10 days post-birth the enterococci have achieved a large population size (10^8 bacteria per g of intestinal contents) (61). Upon the introduction of solid food, biological succession within the intestine begins, and the anaerobic population increases to numbers approaching 10^{10} bacteria per g of intestinal contents concomitant with a decrease in the facultative anaerobe community. This shift in GI microbiota composition continues until 4-5 years post-birth when the climax community finally develops (61).

The role of the enterococci in the GI tract is poorly understood, however there is some evidence that suggests these bacteria have an impact on immune system function. In a study by Roach et al., conventional mice pre-inoculated with *E. faecalis* were challenged with *Salmonella typhimurium*. Five days post-inoculation, the spleens were removed and homogenized to determine the bacterial load. Spleens collected from mice pre-inoculated with *E. faecalis* contained significantly less *S. typhimurium* than did spleens obtained from control mice (53). Though the mechanisms controlling these observations are currently unknown, the data suggest some enterococci may serve to prevent or mitigate infections caused by some pathogenic bacteria. Furthermore, some enterococcal strains produce bacteriostatic bacteriocins. Bayoub et al. have shown that these bacteriocins inhibit the grown of *Listeria monocytogenes*, the causative agent of listeriosis (4). Having a healthy population of enterococci in the intestine may aid in the proper function of the immune system and inhibit the growth of human pathogens.

For bacteria that have dual roles, such as *E. faecalis*, the distinction between commensal and pathogen is often blurred: the young, elderly and immune-compromised may develop infections from commensal strains. However, the virulent enterococci are

frequently distinguished from commensal strains by their ability to cause infections in multiple individuals during hospital ward outbreaks (33, 46). These pathogenic isolates are genetically different from commensal strains in that they usually harbor mobile genetic elements comprised of genes that act to interfere with the host/bacteria commensal relationship and cause infection (30).

***E. faecalis* as a pathogen.** In addition to being successful colonizers of the GI tract, the enterococci are opportunistic pathogens capable of causing multiple-site infections. Antibiotic resistance through intrinsic or acquired mechanisms has contributed to the emergence of *E. faecalis* as a leading cause of nosocomial infections. *E. faecalis* is intrinsically resistant to various β-lactam antibiotics (especially resistant to cephalosporins) and exhibits a low-level resistance to aminoglycosides (36). Furthermore, the enterococci have evolved resistance to a broad range of antibiotics by acquiring plasmids or transposons comprised of antibiotic resistant loci or through spontaneous mutations that result in an increased resistance (31, 38). Particularly confounding from a medical standpoint is the relative ease with which *E. faecalis* acquires resistance to antibiotics of "last resort", such as vancomycin (48).

Though the antibiotic resistant enterococci possess a selective advantage in the hospital environment (38), the debate regarding the increase in occurrence of enterococcal infections due to antibiotic resistance continues. For example, the rates of infective endocarditis attributed to enterococcus before the advent of antibiotics is the same as those observed after the introduction of antibiotics (44, 50). However, it is clear that the acquisition and spread of antibiotic resistance make the enterococci particularly difficult to treat; the enterococci are now the first, second and third leading cause of

surgical site, bloodstream and urinary tract infections, respectively (52). It is unclear if antibiotic resistance directly leads to an increase in enterococcal infections, however the use of antibiotics may perturb the intestinal microbiota thereby permitting the colonization of strains capable of nosocomial infections. In this scenario, the intestine would serve as a source of dissemination for pathogenic bacteria.

In addition to acquiring mobile elements that confer antibiotic resistance, many enterococcal strains possess loci arranged on a pathogenicity island (PAI) with the potential to disrupt the commensal/host relationship. A 153 kb PAI consisting of 129 open reading frames was discovered in *E. faecalis* MMH594 (58). This PAI contains many loci with roles in virulence as well as factors potentially involved in horizontal transfer and gastrointestinal tract colonization (58). Perhaps the most studied PAI virulence traits are *esp* (encodes enterococcal surface protein) and cytolysin toxin. Esp is an adhesin that contributes to the colonization of the urinary tract and mediates biofilm formation in many enterococcal strains. (32, 59, 63). There is a distinct correlation between *esp* and virulence as the *esp* gene is enriched in enterococcal isolates collected from infections of the bloodstream and heart but rarely obtained from stool samples (60). Additionally *esp* is absent in environmental isolates yet this gene is present in the majority of vancomycin sensitive or resistant clinical isolates, suggesting the link between *esp* and virulence is independent of antibiotic resistance (3, 68).

Many virulent enterococcal strains harbor an operon encoding the cytolysin toxin. This toxin is unique among bacterial toxins in that it exhibits both hemolytic (active against erythrocytes) and bacteriocin (antibacterial activity) in a single toxin (8, 28). Cytolysin toxin is an important determinant of virulence in murine lethality models and

contributes to endocarditis in a rabbit model of infection (11, 35). Furthermore this toxin is active against invertebrates, as cytolysin-producing *E. faecalis* is lethal in a *Caenorhabditis elegans* infection model (24). Taken together, these studies establish the role of cytolysin toxin in enterococcal pathogenicity and reveal that this toxin increases virulence in a wide range of hosts (from *C. elegans* to rabbits) and is active against eukaryotic and prokaryotic cells.

The cytolysin operon and *esp* gene are adjacent to each other on the *E. faecalis* PAI (58). This organization (i.e., the close proximity of an adhesion gene to a toxin gene) is common in many PAI, particularly those found in *E. coli* (29, 30). It is thought that the adhesin and toxin act in concert with each other, where Esp would aid in the binding to host cells and expression of the cytolysin toxin would damage host tissues (27). The synergistic activity between Esp and cytolysin toxin has yet to be studied. However, the expression of another adhesin (aggregation substance) concomitant with the production of cytolysin toxin results in a greater lethality than the production of either trait alone (11).

The increase in lethality afforded by producing an adhesin and a toxin might drive evolution within the *E. faecalis* PAI. Overtly toxic strains may face increased stresses from the host immune response; likewise, surface proteins (such as Esp) could be targets of the immune response (27). It appears that some strains modify these regions of the PAI by eliminating *esp*, the cytolysin toxin or both in order to maintain infections within the host (58). The evolution of the *E. faecalis* PAI can be observed by comparing strains MMH594, V586 and V583. MMH594 was isolated from a hospital ward outbreak, carries an increased risk of death, and expresses both *esp* and the cytolysin toxin (34).

V586 was obtained from a chronically infected patient and has been shown to express *esp* but not cytolysin; the cytolysin operon is insertionally inactivated in V586 (55, 58). V583 was isolated from the same patient as V586 yet does not express either *esp* or cytolysin toxin due to a 17 kb deletion in this region of the PAI (55, 58). These findings highlight the dynamic nature of the *E. faecalis* PAI and provide a mechanism for persistence within a host.

The *E. faecalis* PAI contains a gene that encodes an AraC-type transcriptional regulator designated *perA* (pathogenicity island-encoded regulator) (12). PerA is enriched among clinical enterococcal isolates and influences biofilm formation in the urinary tract-isolate, *E. faecalis* E99 (12, 58). Additionally, PerA contributes to pathogenesis in a mouse peritonitis model and survival within macrophages (12). Given that PerA is important for virulence, it was of interest to determine the full set of genes controlled by this transcriptional regulator. Using microarrays, I determined the PerA regulon in *E. faecalis* E99 and highlight the findings in chapter 3.

The macrophage. Bacteria that are able to evade the immune system may be more successful at causing infections and persisting within a host. Components of the enterococcal gram-positive cell wall elicit the monocyte-derived inflammatory response (7). Despite the activation of monocytes and macrophages, *E. faecalis* fail to be eliminated from host tissues, which suggests the bacteria subjugate immune clearance functions (66). Using mouse peritoneal macrophages, Gentry-Weeks et al. showed that *E. faecalis* is able to survive phagocytosis and subsequently persists within immune cells (26). The ramifications of intracellular survival are two fold. First, survival within the macrophage effectively eliminates an important component of the innate immune

response. Though the macrophages are still capable of activating other components of the immune response through antigen presentation, further recruitment of macrophages would prove futile. Second, during intracellular survival *E. faecalis* could use the macrophage as a vehicle to facilitate entrance into extra-intestinal sites, such as mesenteric lymph nodes or the blood. Indeed, it is thought that survival within macrophages is the primary way in which *E. faecalis* that have translocated across the intestinal epithelial barrier subsequently spread to other sites (67).

Prior to phagocytosis, the macrophage must first recognize the presence of a pathogen. Macrophages determine the presence of pathogens by detecting conserved microbial patterns called PAMPs (pathogen-associated molecular patterns) present on microbial pathogens yet absent in eukaryotes (42, 43). Microbial PAMPs are recognized by Toll-like receptors (TLRs) displayed on the surface of the macrophages (42). These pattern recognition receptors not only aid in the detection of pathogens, but in coordination with the intracellular adaptor MyD88, also serve to activate the cytokine and chemokine-dependent proinflammatory response (1, 6). Among the 10 human TLRs identified, TLR2 is responsible for identifying gram-positive bacteria by recognizing components of the gram-positive cell wall including peptidoglycan, lipoteichoic acid, and lipoproteins (56, 69). Accordingly, the TLR2-dependent signaling pathway is critical to the host immune response to enterococcal infections (39).

After recognition has occurred, the pathogen is internalized within the macrophage in a modified phagosomal vacuole. Through a series of fusion and fission events, the phagosome combines with endosomes and lysosomes to form a mature phagolysosome. The rate at which the phagosome matures into the phagolysosome

varies, and in the case of *Mycobacteria* maturation is inhibited (47, 49, 65). Concomitant with phagosomal maturation the macrophage produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) as part of the respiratory burst. The ROS are produced by phagocyte NADPH oxidase, an enzyme that reduces molecular oxygen to superoxide ($O_2^{\bullet-}$). The resulting $O_2^{\bullet-}$ can subsequently act as precursors for hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and peroxynitrite (20, 45, 64). The RNS are produced by the inducible nitric oxide synthase (iNOS) and includes nitric oxide, nitrogen dioxide, dinitrogen trioxide, and nitrosothiols (20, 45, 64). ROS and RNS exert cytotoxicity by oxidizing nucleotides, nitrosylating proteins and damaging membranes (45). Additionally, the macrophage acidifies the phagosome in an attempt to destroy the phagocytosed bacteria (41). The mechanisms used by the macrophage to destroy pathogens are similar regardless of bacterial species ingested, however it is clear that many intracellular pathogens persist within the macrophage by circumventing these mechanisms (17, 40, 65).

Macrophages infected with *Salmonella typhimurium* form a modified phagolysosome, called the *Salmonella*-containing vacuole (SCV). The SCVs are deficient in their antibacterial activity, as is evidenced by the replication of *Salmonella* within these modified vacuoles (2, 10, 23). The formation of the SCV is mediated through the production of bacterial virulence factors during intracellular survival. For example, components of the NADPH oxidase are excluded or removed from the SCV, an occurrence that is dependent on *Salmonella* genes located on the SPI-2 pathogenicity island (23). The *Salmonella*-directed exclusion of NADPH oxidase on the SCV membrane inhibits ROS production, thus permitting bacterial replication within the

phagolysosome (23). In a similar manner, SPI-2 genes prevent iNOS from localizing to the SCV resulting in a vacuole lacking RNS (10). Although *Salmonella*-infected macrophages are incapable of fumigating the SCV with ROS or RNS these phagocytic cells should still have other bactericidal strategies at their disposal, namely the acidification of the phagolysosome. Indeed, acidification does occur, however the drop in pH in the SCV is delayed by up to 4 hours (2). This attenuation in SCV acidification permits *Salmonella* survival within the macrophage (2). These results provide an example of how some intracellular pathogens can modify the macrophage from a hostile environment into one supportive of bacterial survival and growth.

Another strategy used by bacteria to survive within the macrophage is to escape the phagosome, a tactic used by *Listeria monocytogenes*. During infection of macrophage, *L. monocytogenes* must transit from the phagosome into the macrophage cytoplasm in order to grow. In fact, *L. monocytogenes* that are unable to reach the cytoplasm are nonviolent (14, 37). To destroy the phagosome, *Listeria* produce a hemolysin called Listeriolysin O (LLO). LLO works in concert with two phospholipases (PI-PLC and PC-PLC) to hydrolyze the vacuole membrane, thus providing *L. monocytogenes* access to nutrients contained within the host cell (9, 25). However it is important that the macrophage remain intact, as *L. monocytogenes* polymerize actin filaments within the host cell to facilitate cell-to-cell spread (13, 62). Therefore the production of LLO must be tightly regulated, such that LLO is produced in the phagosome to degrade the vacuole membrane yet inhibited once the bacteria have been released into the cytoplasm to avoid lysing the host cell. One environmental signal that triggers the production of LLO is pH. The optimal pore-forming activity of LLO occurs

in slightly acidic environments (pH 5.5 – 6.0), such as those found in the early phagosome (5). Once the bacteria have been released from the phagosome, enzymes produced by the macrophage degrade LLO released into the cytoplasm (15). Interestingly, this shows how *L. monocytogenes* uses phagocytosis as a signal to produce proteins that enable the bacterium to escape the phagosome, gain access to host nutrients and subsequently spread to other macrophages.

Although many pathogens are able to survive within the macrophage perhaps the most successful intracellular pathogen is *Mycobacterium tuberculosis*. Despite activating an immune response, *M. tuberculosis* persists for the lifetime of the host within modified phagosomes (22). This accounts for the estimate that nearly a third of humans are latently infected with this bacterium (18). To cause an infection, *M. tuberculosis* must be inhaled into the lung and subsequently phagocytosed into alveolar macrophages. During the early stages of phagocytosis, the bacteria modify the phagosome (much like *Salmonella*) to prevent the formation of a mature phagolysosome (54). Furthermore, *M. tuberculosis*-containing phagosomes exclude proton-ATPase pumps thereby preventing acidification of this vacuole (65). The conditions within the naïve phagosome permit *M. tuberculosis* replication until the onset of cell-mediated immunity. Cytokine production finally stimulates the maturation of the phagosome through phagosome-lysosome fusion, which results in the production of antimicrobial ROS and RNS (22, 65). Bacteria that survive phagosomal maturation enter a dormant (non-replicating) stage until a decline in the host immune response permits bacterial replication. This provides an example of how bacteria can adapt to the maturation of the phagosome and latently persist within a host indefinitely.

A review of published literature regarding facultative intracellular pathogens reveals many studies pertaining to the aforementioned bacteria (in addition to many others not mentioned here). However, relatively little is known about the intracellular survival strategy of *E. faecalis*. This was surprising to me as 1) this opportunistic pathogen is capable of prolonged survival within a macrophage (26), and 2) *E. faecalis* is the leading cause of surgical site infections, the second leading cause of bloodstream infections and the third leading cause of nosocomial urinary tract infections (51). A better understanding of how *E. faecalis* coordinately regulates genes during macrophage survival and subsequent infection of host tissues could be useful in treating infections caused by this pathogen.

Since my first microbiology course taught by Dr. David Elmendorf, I have been fascinated by how 'simple' bacteria regulate the expression of virulent traits during the process of infection. Even more intriguing to me are the opportunistic pathogens that live a Dr. Jekyll and Mr. Hyde existence; functioning at times as a commensal or harmless member of the human microbiota, yet capable of causing severe or life-threating infections in certain circumstances. My interest in the commensal vs. pathogen relationship shaped the questions addressed in the work presented here. Specifically, how does *E. faecalis*, a commensal colonizer of the human GI tract, cause multiple-site infections throughout the body? To achieve this, *E. faecalis* must perform two related tasks; escape the intestine and coordinately regulate virulence traits. At the outset, the first task seems relatively straightforward. *E. faecalis* occupies a niche within the GI tract as a small portion of the intestinal microbiota. However if the intestine is perturbed, the enterococci can expand to newly unoccupied niches thereby causing inflammation of

the epithelial lining of the GI tract. The immune inflammatory response includes macrophages that phagocytose bacteria and subsequently translocate to the lymph system, liver or spleen. This provides a mechanism that *E. faecalis* can use to escape the intestine. Although this scenario seems clear, many unanswered questions remain. The most interesting to me involved survival within the macrophage, a topic highlighted in chapter two.

Preamble to Chapter two: The *Enterococcus faecalis* V583 Transcriptional Profile During Survival Within Macrophages. I entered Dr. Conway's lab in the fall semester of 2005. Soon after I began a collaborative research project, including Dr. Nathan Shankar and Dr. Phillip Coburn, with the goal of determining the *E. faecalis* intracellular survival strategy. To do this, I relied heavily on the work previously published by Dr. Jay Hinton, specifically the seminal intracellular transcriptome paper entitled 'Unraveling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*' (19). This paper provides a robust technique for extracting bacterial RNA out of a cell that is inside of another cell, the audacity of which still fascinates me today. This technique, combined with improved microarray platforms, allowed me to determine the *E. faecalis* V583 transcriptional profile during intracellular survival. We found that the V583 response to phagocytosis includes nearly half of the genome, with a large portion of these genes encoding proteins of unknown function. Furthermore, numerous genes involved in the oxidative stress, heat shock and SOS responses were up-regulated during intracellular survival. Finally the V583-containing phagosome was limited for glycolytic substrates, nucleotides, amino acids and numerous ions necessary for growth and protein function. This work illuminates not only the

environment encountered within the macrophage but also the *E. faecalis* genes important for intracellular survival.

Preamble to Chapter three: Transcriptional Regulator PerA Responds to Bicarbonate and Regulates Biofilm-Associated, Platelet Binding and Metabolic Genes in *Enterococcus faecalis*. The impetus for this chapter is rooted in previous work by Coburn et al (12). Dr. Coburn identified and characterized an AraC-type transcriptional regulator (designated PerA) residing on the *E. faecalis* PAI. PerA influences pathogenesis and the ability of *E. faecalis* to survive within a macrophage. Additionally, PerA contributes to biofilm formation in a medium-specific manner (12). It was our goal to determine the PerA regulon; that is, the total set of genes directly or indirectly controlled by this transcriptional regulator. I determined the PerA regulon by comparing the transcriptional profile of an *E. faecalis* wild-type strain to an isogenic strain lacking *perA*. Our findings reveal that PerA coordinately regulates genes important for metabolism, amino acid degradation, and pathogenicity. Further transcriptional analysis revealed that PerA is influenced by bicarbonate. Additionally, PerA influences the ability of *E. faecalis* to bind to human platelets. Our results suggest that PerA is a global transcriptional regulator that coordinately regulates genes responsible for enterococcal pathogenicity.

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Chapter 2: The *Enterococcus faecalis* V583 Transcriptional Profile During Survival Within Macrophages.

Abstract

Enterococcus faecalis is an opportunistic pathogen capable of surviving within macrophages for extended periods. The bacterial survival strategy during phagocytosis is thought to involve the restructuring of gene expression in response to the harsh intracellular environment. However, the *E. faecalis* transcriptional profile during intracellular survival has, until now, not been investigated. In this study we report the complete intracellular *E. faecalis* V583 transcriptome following infection of RAW264.7 macrophages. During intracellular survival, approximately 45% of the V583 genome was differentially regulated including numerous genes involved in the oxidative stress, heat shock and SOS responses. We observed that the *E. faecalis*-containing phagosome was limited for glycolytic substrates, nucleotides, amino acids and numerous ions necessary for growth and protein function. Approximately 35% of the genes differentially regulated during survival within macrophages were of hypothetical/unknown function, suggesting that the V583 response to phagocytosis involves many previously unstudied loci. Here, we provide the first comprehensive study elucidating the transcriptional response of *E. faecalis* to phagocytosis, which may provide new targets for future studies.

Introduction

Enterococcus faecalis is a member of the human gastrointestinal (GI) tract and is relatively harmless in healthy individuals. However in patients subjected to frequent antibiotic treatment, and in individuals with compromised immune systems, this opportunistic pathogen is capable of causing severe infections of the bloodstream, urinary tract, liver and spleen (33). Furthermore *E. faecalis* is quickly becoming a leading cause of infective endocarditis, an accumulation of bacteria on damaged cardiac tissue that often leads to heart failure or death (20, 33). The intrinsic or acquired antibiotic resistance found within the enterococci frequently complicates the treatment of these infections. Likewise, the use of antibiotics to which indigenous members of the intestinal microflora are sensitive could open intestinal niches and cause a proliferation of resistant *E. faecalis*. Therefore, alternative antimicrobial strategies are continually sought in an effort to treat, and ultimately prevent, infections from pathogenic bacteria.

Macrophages are important components of the innate immune response, often participating in the first line of host defense by scavenging foreign particles. Additionally, these cells stimulate the adaptive immune response through antigen presentation and cytokine production. Macrophages exert antimicrobial activity first by phagocytizing infective bacteria, then by delivering toxic reactive oxygen species (ROS), reactive nitrogen species (RNS) and antimicrobial peptides to the bacteria-containing phagosome (24, 25, 54). Furthermore, macrophages acidify the phagosome in an effort to destroy the phagocytosed bacteria (54). Though these compounds provide the macrophage with an impressive arsenal, many pathogens, including *E. faecalis*, are able to survive and even flourish in this harsh environment (11, 23, 31, 80). The ability of *E.*

faecalis to persist within the macrophage is thought to contribute to the translocation of this opportunistic pathogen from the intestine to extra-intestinal sites (98). Once free from the intestine the bacteria are capable of infecting the blood, liver and spleen (12). Therefore, discovering the strategy used to survive within the macrophage would not only aid in our understanding of host-pathogen interaction, but it could be used to develop therapeutic treatments aimed at preventing enterococcal infections. Since the discovery that *E. faecalis* survives within host cells researchers have sought to elucidate intracellular survival mechanisms (12, 97).

As the oxidative burst is one of the primary tools used by a macrophage to kill phagocytosed bacteria, studies elucidating the *E. faecalis* intracellular survival strategies predominately concentrate on the response to macrophage-induced oxidative stress. La Carbona et al. have shown that three loci (*npr*, NADH peroxidase; *ahpC*, alkyl hydroperoxide reductase; *tpx*, thiol peroxidase) protect *E. faecalis* against exogenous sources of oxidative stress, and that *tpx* is critical for persistence within the macrophage (45). Superoxide dismutase (SodA), an enzyme that detoxifies superoxide radicals ($O_2^{\bullet -}$) by converting them to hydrogen peroxide (H_2O_2) and oxygen, is important for persistence within the macrophage as a Δ*sodA* mutant strain is attenuated for intracellular survival (95). Finally, the oxidative stress response is comprised of loci that repair damaged proteins and include the methionine sulfoxide reductases (*msr*). In *E. faecalis*, *msrA* and *msrB* aid in survival within phagocytes stimulated with gamma interferon (106). However, the role of these loci in enterococcal virulence is unclear as Δ*msrA* and Δ*msrB* strains persist within naïve phagocytes (106). While these studies further our

understanding of the mechanisms necessary for intracellular survival, a complete *E. faecalis* transcriptional profile during infection of macrophage is missing.

Advances in global transcriptional profiling and new methods of obtaining bacterial RNA from infected cells now permit the interrogation of pathogenesis during intracellular survival at the whole-genome scale (23). Using bacterial RNA extracted during macrophage infection and Affymetrix GeneChip microarrays, we obtained the *E. faecalis* V583 global transcriptional profile in response to phagocytosis and intracellular persistence. Our results reveal that throughout intracellular survival the *E. faecalis* transcriptome is drastically restructured, and that a large portion of this response (~ 35%) is comprised of genes of unknown function. Further analysis suggests that despite induction of oxidative stress response and repair mechanisms, important bacterial cell components are damaged during survival in the macrophage as is evidenced by induction of the bacterial SOS response. The down-regulation of genes involved in transcription, translation and cell division suggest the V583 stringent response is activated upon phagocytosis. Finally our data suggest that the *E. faecalis*-containing phagosome lacks glycolytic carbon sources and is devoid of the amino acids and nucleotides required for growth. With these data we now have new insights into the *E. faecalis* strategy utilized to persist inside the macrophage. This information should not only further our understanding of host-pathogen interactions but also illuminate mechanisms of enterococcal pathogenicity.

Materials and Methods

Bacterial strains, media, and reagents. The strain used in this study was *Enterococcus faecalis* V583 (78). *E. faecalis* V583 was routinely cultured in Todd-Hewitt broth (THB) containing 1% glucose and gentamicin (500 μg/ml) (Sigma Chemical, St. Louis, MO). Growth was monitored as absorbance at 600 nm using a Beckman-Coulter DU800 spectrophotometer.

Macrophage survival assay. Macrophages were infected as previously described with modifications (12, 23). Briefly, RAW264.7 (ATCC TIB-71) macrophage were grown in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, glucose (4.5 g/L), L-glutamine (4.0 mM), and sodium pyruvate (1.0 mM) (Mediatech, Manassas, VA.). For each V583 RNA extraction, 6-well cell culture plates (120 wells total; Becton Dickinson, Franklin Lakes, NJ.) were seeded with 10^8 RAW264.7 cells per well and incubated for 16 h at 37°C under 5% CO_2. Following incubation the cells were washed twice with phosphate buffered saline (PBS) infected with *E. faecalis* V583 at a multiplicity of infection (MOI) of 100:1 (bacteria:cells), after which the plates were centrifuged at 1000 g for 5 min, which defined time 0 h. After 1 h incubation, extracellular bacteria were killed by changing the medium to DMEM supplemented with streptomycin (100 μg/ml) and ampicillin (10 μg/ml). Incubations were continued to desired time points (4 h, 8 h, 12 h post-infection).

RNA extraction and microarray analysis. At 4 h, 8 h and 12 h post-infection, infected RAW264.7 cells were lysed on ice for 30 min in 0.1% SDS, 1% acidic phenol and 19% ethanol in water as previously described (23). Macrophage lysates were separated from bacteria by centrifugation at 1000 g for 10 min. The remaining bacterial pellets were

collected following further centrifugation at 10000 g for 15 min. RNA was prepared using the Qiagen RNeasy Minikit (Valencia, CA) with optional on-column DNase treatment according to the manufacturer's specifications. RNA integrity was checked by gel electrophoresis and stored in 2 volumes of ethanol at -80°C. RNA from control samples was obtained from *E. faecalis* V583 grown to mid-log phase (OD 600nm = 0.05) in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, glucose (4.5 g/L), L-glutamine (4.0 mM), and sodium pyruvate (1.0 mM) while incubated at 37°C under 5% CO_2. cDNA was generated by first strand synthesis using Superscript II (Invitrogen, Carlsbad, CA) and random hexamers according to the manufacturer's specifications. Fragmentation and biotinylation of cDNA proceeded according to the Affymetrix prokaryotic labeling protocol using the ENZO Kit from Roche Diagnostics (Indianapolis, IN). Biotinylated cDNA was hybridized to custom *E. faecalis* V583 Affymetrix GeneChips (GEO Accession number: GPL6702) for 16 h at 45°C. Affymetrix protocol ProkGE_WS2v2-450 was used to stain the hybridized arrays. Following scanning, raw data files (.cel) were analyzed using RMA processing with quartile normalization (40). Each macrophage infection assay was performed twice. Biological replicates were averaged, and genes were considered to be significantly induced or repressed if the intracellular:control expression ratio was greater than twofold (103). Heatmaps were generated using DecisionSite for Functional Genomics (Spotfire; Somerville, MA).

Results and Discussion

The intracellular transcriptional profile. The *E. faecalis* V583 intracellular transcriptome was defined by comparing the transcriptional profiles from bacteria during infection of macrophages to that of bacteria grown in culture medium. Total bacterial RNA was extracted from infected macrophages at 4 h, 8 h, or 12 h post-infection and from control samples during mid-log phase (OD 600nm = 0.05). The RNA was reverse transcribed and hybrized to Affymetrix *E. faecalis* V583 whole-genome microarrays. All array data shown are expressed at ratios (intracellular : control) and are considered to be significant if gene expression was induced or repressed greater than twofold. The V583 genetic response to phagocytosis and subsequent persistence in the intracellular environment included a total of 1405 genes representing ~ 45% of the genome (Fig. 1). Strikingly, 35% (492) of the 1405 genes differentially regulated during any intracellular time point tested encoded hypothetical proteins or proteins of unknown function. This suggests the intra-phagosomal conditions sensed by V583 are profoundly different than that in the control sample, eliciting a drastic change and restructuring of the transcriptional profile. Additionally, these data provide evidence that the *E. faecalis* response to phagocytosis involves numerous previously unstudied loci. Furthermore, we observed variations in the intracellular transcriptome when comparing the 4 h, 8 h, and 12 h samples (Fig. 2). At 4 h post-infection 715 genes (~ 23% of the genome) were differentially regulated, while 1065 genes (~ 35% of the genome) and 1005 genes (~ 33% of the genome) were significantly up or down-regulated at 8 h and 12 h post-infection, respectively. Comparisons of the intracellular transcriptomes revealed that 458 genes (~ 15% of the genome) were similarly regulated in all time points (Fig. 2A). Taken together

these results suggest that while a core set of genes are induced and required for persistence in all time points tested, the V583 transcriptional landscape fluctuates from the initial response to phagocytosis to prolonged intracellular persistence.

Oxidative Stress Response. In an effort to destroy phagocytosed bacteria macrophage produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) as part of the respiratory burst. The ROS are produced by phagocyte NADPH oxidase, an enzyme that reduces molecular oxygen to superoxide ($O_2^{\bullet-}$). The resulting $O_2^{\bullet-}$ can subsequently act as precursors for hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and peroxynitrite (25, 57, 94). The RNS are produced by the inducible nitric oxide synthase (iNOS) and includes nitric oxide, nitrogen dioxide, dinitrogen trioxide, and nitrosothiols (25, 57, 94). ROS and RNS exert cytotoxicity by oxidizing nucleotides, nitrosylating proteins and damaging membranes (57).

E. faecalis is equipped with many antioxidant enzyme systems that respond to oxidative stress. These include alkyl hydroperoxide reductase (*ahpC*), NADH peroxidase (*npr*), hydrogen peroxide regulator (*hypR*), superoxide dismutase (*sodA*), catalase (*katA*), thiol peroxidase (*tpx*), methionine sulfoxide reductase system (*msrABC*) and thioredoxin (*trx*). Among these, *sodA*, *tpx*, *npr*, *ahpC, hypR* and *msrAB* have all been shown to contribute to survival inside the macrophage (45, 95, 96, 106). To determine the expression of each of these oxidative stress genes during intracellular survival we compared the transcriptome of V583 inside the macrophage to that of control bacteria. During initial stages of infection (4 h post-infection) *npr*, *ahpC*, *hypR* and *sodA* transcript levels are similar to that of the control samples (Fig. 3). Upon intracellular persistence (8-12 h post-infection) the expression of these genes is down-regulated (Fig. 3). This

suggests that *npr*, *ahpC*, *hypR* and *sodA* expression is most important during early stages of phagocytosis. Although *npr*, *ahpC*, *hypR* and *sodA* transcription is the same as the control samples 4 h post-infection, we do not assume this means the transcript is in low abundance. *E. faecalis* produces $O_2^{\bullet-}$ during growth and presumably expresses genes with antioxidant properties to prevent cellular damage during growth (39). Furthermore RAW264.7 cells produce high levels of $O_2^{\bullet-}$ from 0-4 h post-infection, yet at 10 h post-infection $O_2^{\bullet-}$ levels are undetectable (56). The decrease in *npr*, *ahpC*, *hypR* and *sodA* expression reflects this decrease in $O_2^{\bullet-}$ produced by the macrophage.

Approximately 8 h post-infection, macrophages produce nitric oxide (NO) that inhibits bacterial DNA replication and respiration (21, 63, 79). When present with H_2O_2 NO participates in the Fenton reaction, which increases oxidative stress by producing OH^- (102). One way to prevent an increase in oxidative stress through the Fenton reaction would be to remove H_2O_2 from the environment. Catalase reduces H_2O_2 to H_2O and at 8 h post-infection the *E. faecalis katA* gene is highly induced (Fig. 3).

Another consequence of oxidative stress is the induction of systems that repair cellular damage. During oxidative stress, proteins containing methionine residues are especially vulnerable to damage. When oxidized, the methionine residues form methionine sulfoxides that render the protein useless. In an effort to restore protein function, repair systems are induced to reduce the oxidized residues (4). We found that the *E. faecalis* methionine sulfoxide reductase system (*msrABC*) is highly induced throughout all intracellular time points tested (Fig. 3). The *msr* system uses electrons derived from thioredoxin to reduce methionine sulfoxide residues to methionine (5, 6). Concomitant with *msrABC* induction during intracellular survival is induction of the *E.*

faecalis thioredoxin (*trx*) (Fig. 3). The *msr* system was recently shown to contribute to the *E. faecalis* oxidative stress response, and therefore is important for macrophage survival and virulence (106). Additionally, our results suggest that *E. faecalis* uses the methionine sulfoxide reductase and thioredoxin systems to repair proteins damaged during intracellular survival. Taken together, these data suggest that though the majority of the oxidative stress response is not highly induced, V583 experiences oxidative stress inside the macrophage and uses the methionine sulfoxide reductase and thioredoxin pathways to repair damage from ROS and RNS.

The Fsr system. The *E. faecalis fsr* system is similar to the *argABCD* operon found in *Staphylococcus aureus (60)*. *fsr* is a quorum-sensing system that mediates the production of a cyclic peptide termed gelatinase-biosynthesis activating pheromone (GBAP) (60, 61). Through the production of GBAP, *fsr* activates two genes encoding a gelatinase (*gelE*) and a serine protease (*sprE*) resulting in biofilm formation (38, 72, 73). Furthermore, GelE has been shown to contribute to pathogenesis in endocarditis (90). Upon phagocytosis and throughout intracellular persistence, *fsr*, *gelE* and *sprE* expression is significantly induced (Fig. 3). SprE contributes to pathogenesis during infection and GelE is capable of inhibiting the immune complement system (64, 65, 73, 90). Though the functions of these proteins during macrophage survival is poorly understood, each could enhance survival inside the macrophage or aid in persistence inside host tissues once the bacteria have escaped the intracellular environment. In the former scenario GelE released by V583 could interrupt the immune complement cascade, thereby modulating the host response to intracellular *E. faecalis*. In the latter scenario

intracellular bacteria released from the macrophage into the liver, spleen, and lymph system could persist through SprE production.

Lysin production. *E. faecalis* reside within a phagocytic vacuole during early stages of macrophage survival, yet by 24 h post-infection the vacuole membrane degrades followed by release of the bacterial cells into the cytoplasm (31). Approximately 48 h post-infection the macrophage are destroyed and bacteria are released from their intracellular compartment into the surrounding environment (31). The mechanisms employed by *E. faecalis* to escape the intracellular vacuole and destroy the macrophage are poorly understood. During intracellular survival, *Listeria monocytogenes* produces cytolysins that degrade the macrophage phagosomal compartment (30, 69). Prompted by these observations we mined the V583 transcriptome for induction of annotated lysin genes. We found the majority of lysins were either down-regulated or showed no significant difference in gene expression while inside the macrophage (Fig. 3). A notable exception was *hly-3*, a gene encoding a putative hemolysin in V583. This gene was significantly induced throughout intracellular persistence (Fig. 3). Intriguingly *E. faecalis* hemolytic culture supernatant has been shown to lyse neutrophils and macrophages (58). Hly-3 could be produced by V583 inside the macrophage as a means of escaping the phagocytic vacuole and subsequently destroying the macrophage. A BLASTN search revealed >97% identity among all sequenced *E. faecalis* genomes, suggesting that *hly-3* is a highly conserved gene found in strains of various origins.

SOS and heat-shock stress response. The SOS response is a highly conserved DNA repair system induced during conditions that cause DNA damage or stalled DNA replication (101). At the apex of SOS regulation is RecA and LexA. During DNA

damage, the formation of single stranded DNA (ssDNA) stimulates the production of RecA. This protein binds to ssDNA forming the RecA nucleoprotein filament (RecA*), the active form of the RecA enzyme (43). RecA* promotes self-cleavage of the repressor of the SOS response, LexA, causing an induction of the SOS response (42). Since free radicals produced during phagocytosis are known to induce the bacterial SOS response (8, 41), we sought to determine which genes within the *E. faecalis* SOS regulon were induced during macrophage survival. Our microarray analyses revealed a significant induction of *lexA* and *recA*, as well as the DNA damage repair genes *dinJ, dinP, uvrA* and *uvrB* (Fig. 3). The induction of the SOS response is common among intracellular pathogens. *Listeria monocytogenes* induces genes of the SOS regulon during growth within the host cell cytosol (11). *Mycobacterium tuberculosis* induces the SOS response inside the phagosome, yet this response appears to be independent of NO production as the SOS regulon was also upregulated in NOS2-deficient macrophages (80). Finally, the *Salmonella enterica* SOS response is induced during macrophage survival at a level that permits both bacterial replication and DNA repair (23). Our data suggest that while inside the macrophage *E. faecalis* encounters conditions leading to considerable induction of the SOS response.

The bacterial heat shock response to a variety of environmental insults consists of induced proteins with proteolytic and chaperone functions. Class I heat shock genes encode for the classical molecular chaperones (DnaJ, DnaK, GroEL, GroES, GrpE) and are responsible for folding and refolding of damaged proteins. The class I response is negatively regulated by HrcA. During macrophage survival *E. faecalis* highly upregulated many of the class I heat shock genes, including *dnaJ*, *grpE*, and *hrcA* (Fig.

3). The class III heat shock response is comprised of proteolytic proteins that function to degrade abnormal proteins formed during stress conditions and recycle amino acids during starvation (35, 74). CtsR, a DNA-binding protein, controls the class III heat shock in gram-positive bacteria by regulating *clp* expression (17). Intracellular *E. faecalis* induced many class III heat shock genes including *ctsR, clpP, clpC* and *clpE* (Fig. 3). The importance of *clp* expression during intracellular survival has been elucidated in other pathogens. In *L. monocytogenes*, ClpP is critical for intracellular parasitism and expression of virulent traits (27, 28). Additionally, *clp* is required for intracellular replication of *Staphylococcus aureus* within epithelial cells (26). The high up-regulation of heat shock genes suggests this stress response is critical to repair and recycle damaged proteins in *E. faecalis* during intracellular survival.

Ion transport. Metal ions are needed for many bacterial systems as they act as cofactors critical for protein function. The availability of ions during intracellular survival differs depending on the infective organism and the characteristics of the vacuole (11, 23, 80). We analyzed the expression of ion transport systems to determine ion abundance within the *E. faecalis*-infected macrophage. Three genes involved in copper transport were initially down-regulated 4 h post-infection, yet expression of these genes increased significantly 12 h post-infection (Fig. 3). These genes are *copY* (encodes a transcriptional repressor of *cop* genes), *copZ* (encodes a copper transport protein) and EF0298 (copper-translocating P-type ATPase) and, together, are responsible for maintaining an appropriate copper concentration through copper influx and efflux (86) (Fig. 3). Copper is critical for cellular respiration and protects the cell from free radicals (53, 68). However copper accumulation becomes cytotoxic, hence cytosolic copper concentrations

are tightly controlled (105). In addition to *cop*, *E. faecalis* contains *cutC* to achieve copper homeostasis (47). CutC appears to contribute to copper efflux and is induced upon prolonged exposure to elevated copper levels (47). During intracellular survival *E. faecalis cutC* is not differentially regulated at any time point (data not shown). Taken together we interpret these results to mean that upon phagocytosis V583 does not experience copper starvation, yet during intracellular persistence copper becomes limited which leads to induction of the *cop* system.

Phosphate is another ion critical to bacterial survival, as this ion is essential for nucleotide synthesis and DNA replication. Intracellular *E. faecalis* induced two phosphate-responsive genes, *psiE* and *phoZ,* at all intracellular time points (Fig. 3). *psiE* is a ubiquitous phosphate starvation-inducible gene known to be up-regulated by *S. typhimurium* during macrophage survival (93). *phoZ* encodes an alkaline phosphatase, an exported enzyme induced during phosphate starvation that hydrolyzes various compounds to yield inorganic phosphate (13, 50). Therefore, our data suggests that *E. faecalis* is limited for phosphate while inside the macrophage and induces genes in an attempt to sequester any free phosphate from the surrounding environment. Induction of phosphate acquisition systems is critical during macrophage survival and is a common mechanism amongst many intracellular pathogens such as *S. enterica* and *M. tuberculosis* (23, 75, 76).

Further analysis of the V583 intracellular transcriptome revealed that a number of genes corresponding to potassium uptake were significantly up-regulated (Fig. 3). Among these are *kdpABC* that encode a primary potassium uptake system and *kdpD*-EF0571 (EF0571 is orthologous to *E. coli kdpE*) that encode a potassium-responsive two-

component system (TCS) in *E. faecalis* (36, 67) (Fig. 3). When an appropriate stimulus is received KdpD initiates a signaling cascade resulting in the KdpE-dependent activation of *kdpABC* (36). KdpABC then facilitates the transport of potassium into the bacterial cell (36). Our data suggests *E. faecalis* encounters significant potassium starvation upon phagocytosis, since *kdpABC* is only expressed under severe potassium limitation (46). Further data suggesting potassium is limiting in the *E. faecalis* intracellular environment is the observation that V583 also induced EF2910 during intracellular survival, a gene encoding a putative potassium uptake protein (Fig. 3).

Interestingly the *kdp* potassium uptake and TCS system is among the few V583 pathogenicity island (PAI)-encoded genes differentially regulated within the macrophage (Fig. 3). Since the PAI is enriched among virulent *E. faecalis* yet absent in commensal strains, *kdp* may aid in *E. faecalis* pathogenicity in certain environments while being dispensable for commensal *E. faecalis* (49, 81, 89). However *kdp* does not appear to be critical for intracellular persistence, as this locus is absent from other *E. faecalis* strains capable of macrophage survival.

Iron is critical for both the macrophage and bacteria during phagocytosis. For the macrophage, iron is an indispensible ion that contributes to the production of both the ROS and RNS (2, 19). For an intracellular pathogen, iron is essential for the proper function of peroxidases and cytochromes. If the intracellular iron concentration is too low, ROS and RNS production is impaired, yet if the intracellular iron concentration is too high, excess iron could be used for bacterial persistence. Therefore the macrophage must tightly control the iron concentration within the phagosome; a feat accomplished using two iron translocation proteins, Nramp1 and Nramp2. Nramp2 transports iron from

the phagosome into the cytoplasm during the early stages of phagocytosis. Nramp1 is capable of transporting iron into or out of the phagosome during the latter stages of macrophage infection (34). At 8 h and 12 h post-infection intracellular V583 induced EF0188, EF0191, EF0192 and *fhuG*, genes that show similarity to the ferric hydroxymate siderophore (Fhu) system in *Staphylococcus aureus* (87) (Fig. 3). As the cell only produces siderophores during iron limitation, our results suggest the *E. faecalis* intracellular environment is iron deprived. RAW264.7 macrophage cell lines are derived from BALB/c mice, which carry a mutation in Nramp1, and have been shown to recycle iron less efficiently than macrophage containing functional Nramp1 (85). The iron starvation response observed during intracellular survival could reflect this phenomenon. However based on the oxidative stress response generated by phagocytosed *E. faecalis* (Fig. 3), there appears to be sufficient concentrations of iron to elicit ROS and RNS.

Magnesium is an ion important for the replication of many pathogens during intracellular survival (7, 55). Accordingly, bacteria residing within macrophages have evolved mechanisms to acquire this essential ion. For example, *S. typhimurium* induces genes involved in magnesium transport (*mgtABC*) in magnesium limiting conditions and within macrophages (22, 83, 84). These observations suggest the SCV is limited for magnesium. *E. faecalis* V583 has 4 loci putatively involved in magnesium transport (*mgtE, mgtC*, EF1304 and EF1352) (67). To determine the availability of magnesium in the *E. faecalis*-containing phagosome, we mined the intracellular transcriptome for expression of these magnesium-transporting genes. We found that none of these genes were differentially regulated at any intracellular time point tested (data not shown). These data could suggest that V583 is not limited for magnesium during macrophage

survival. Unfortunately little is currently known about the regulation of any of these magnesium-transporting genes in *E. faecalis*. It is possible that basal levels are sufficient for intracellular survival, or it is possible that these genes are constitutively expressed. In regards to this latter possibility, *mgtC* is thought to be constitutively expressed *M. tuberculosis* (3, 92).

Two-component systems. Bacteria use two-component regulatory systems (TCS) to modulate gene expression in response to environmental stimuli (88). Frequently pathogenic bacteria use TCS to control virulence, thereby accurately timing the expression of virulence traits only when in the correct environment (14, 62). The V583 genome contains 18 TCS elements that mediate a genetic response to heat shock, bile and pH (37, 49). Furthermore Muller et al. screened strains containing deletions within TCS loci and found numerous *E. faecalis* TCS contribute to macrophage survival (59). We mined the V583 intracellular transcriptome and found many TCS systems were up-regulated (Fig. 3). Interestingly the majority of the TCS induced in the macrophage showed the greatest induction at the 4 h time point (Fig. 3). Corroborating Muller et al., we found EF1260-EF1261 (err06-ehk06) and EF3289-EF3290 (*croSR*) to be induced during macrophage survival (Fig. 3). While *croSR* was up-regulated at all time points, EF1260-EF1261 showed differential regulation only at the 4 h time point. EF1260-EF1261 has been shown to protect the cell from H_2O_2-mediated oxidative stress and is induced in the presence of H_2O_2 (59). Our results suggest V583 uses EF1260-EF1261 to sense the oxidative landscape upon phagocytosis, a finding that is in accordance with the timing of RAW264.7 production of oxidative radicals (56).

Other *E. faecalis* TCS induced during intracellular survival include EF0372-EF0373 (ehk13-err13) and EF2911-EF2912 (Fig. 3). Le Breton et al. have shown that EF0372-EF0373 is induced during heat shock, yet the role of this TCS in macrophage survival remains to be studied (48). EF2911-EF2912 is a poorly studied TCS that showed the highest induction through all intracellular time points (Fig. 3) suggesting this locus is important during intracellular survival, yet serves an unknown role inside the macrophage. The EF1050-EF1051 TCS (ehk10-err10) is known to respond to acidic conditions (48). Interestingly, this TCS was not differentially regulated during intracellular survival (data not shown), indicating that the *E. faecalis* phagosomal environment is perhaps not acidified. The data presented here corroborates previous results of TCS systems known to function during intracellular survival (59) while expanding the list of TCS differentially regulated during *E. faecalis* persistence within macrophages.

Transcription/translation apparatus. Intracellular survival assays reveal that *E. faecalis* is able to persist within the macrophage yet does not appear to grow appreciably (12, 31). These observations lead us to examine the expression of transcriptional and translational genes during intracellular survival. Strikingly, many aminoacyl tRNA synthetase genes, including *serS, hisS, thrS, aspS, glyS, pheS, tryS, csyS, ileS, leuS, valS, alaS, and argS*, were significantly down-regulated at all time points tested (Fig. 2). These enzymes use free amino acids to charge the tRNA during translation. Concomitant with the down-regulation of the tRNA synthetase genes was the induction of peptide and amino acid transport systems (Fig. 3). These transporters provide amino acids to the cell and are essential for the growth of the auxotrophic lactic acid bacteria (18). Furthermore,

ATP-binding cassette (ABC) amino acid transporters are repressed by amino acids and maximally induced during nutrient limitation (82). Taken together our data suggests an amino acid limiting environment in the *E. faecalis*-containing phagosome.

A consequence of amino acid starvation would be the binding of uncharged tRNAs to the ribosomal A site, a condition that induces the stringent response (99). The stringent response reprograms the cellular genetic response from that of growth to survival and typically involves the down-regulation of the translational apparatus (66, 91). Accordingly, the stringent response involves the cessation of ribosome and macromolecule synthesis, and DNA replication (10, 91). Coordination of the stringent response occurs through the alarmone ppGpp (guanosine 5`,3` bispyrophosphate) (9). In *E. faecalis* ppGpp is produced through the activity of RelA and RelQ, where RelQ produces ppGpp at basal levels while the bifunctional synthetase/hydrolase RelA is responsible for both ppGpp accumulation and degradation (1). ppGpp coordinates the adaptation to various environmental stresses, contributes to antibiotic resistance, and is important in *E. faecalis* virulence (1, 104). Though the ppGpp-mediated regulon is currently unknown in *E. faecalis*, we observed that several hallmarks of the bacterial stringent response are differentially regulated during intracellular survival. Down-regulated genes included several involved in the transcription and translation apparatus (39 genes), ribosome synthesis (16 genes), cell wall synthesis (5 genes) and DNA replication (3 genes) (Figs. 2 and 3). The stringent response is critical for intracellular survival of many pathogens, including *Helicobacter pylori*, *Francisella novidica* and *Mycobacterium tuberculosis* (16, 71, 107). Our data suggests the *E. faecalis* stringent

response is activated during macrophage survival, which results in the repression of genes involved transcription, translation and growth (Figs. 2 and 3).

Metabolic gene expression. We analyzed the expression of metabolic genes to elucidate the availability of carbohydrates within the *E. faecalis*-containing phagosome. Throughout all of the intracellular time points tested, genes involved in intermediary metabolism and energy production were down-regulated (Fig. 2). In particular, genes encoding enzymes involved in both the first half (the conversion of glucose to glyceraldehyde-3-phosphate) and second half (the conversion of glyceraldehyde-3-phosphate to pyruvate) of the Embden-Meyerhof-Parnas (EMP) pathway were down-regulated. These included *glcK* (glucokinase), *pfk* (phosphofructokinase), *fba* (fructose bisphosphate aldolase), *tpiA* (triosephosphate isomerase), *gap* (glyceraldehyde-3-phosphate dehydrogenase), *pgk* (phosphoglycerate kinase), *gpm* (phosphoglycerate mutase) and *eno* (enolase) (Fig. 2). These data suggest glycolytic substrates are limiting in the *E. faecalis*-containing phagosome: a common theme amongst intracellular pathogens is down-regulation of EMP pathway in *L. monocytogenes* and *S. enterica* during macrophage survival (11, 23).

Carbon sources used during macrophage infection could be incorporated into *E. faecalis* as phosphorylated compounds via the phosphotransferase (PTS) system. To further determine available growth substrates within the phagosome, we mined the V583 intracellular transcriptional data for differentially regulated genes encoding members of the PTS system. We found 5 genes encoding PTS proteins (EF0019, EF0021, EF0028, EF0717 and EF3307) were down-regulated throughout intracellular survival (Fig. 2). Based on annotated sequences EF0019 appears to encode a generic PTS component,

EF0021 encodes a mannose-specific transporter, EF0028 encodes PTS transport subunits specific to maltose and glucose, while EF0717 and EF3307 participate in the uptake of fructose and sorbitol, respectively. In addition to being under global regulatory control based on ATP and phosphate concentrations, PTS systems are substrate-induced (70). Based on these data, we conclude that the *E. faecalis* intracellular environment is devoid of carbon sources, particularly hexose sugars.

Nucleotide biosynthesis. When mining the V583 intracellular transcriptome for differentially regulated genes, we observed the up-regulation of numerous loci involved in purine and pyrimidine nucleotide biosynthesis (Fig. 2). Throughout all intracellular time points, *purEKCSQLFMNHD* expression was up-regulated, while the gene encoding a putative repressor of purine synthesis, *purR*, was down-regulated (Fig. 2). The pathway and regulatory mechanisms for purine biosynthesis are poorly studied in *E. faecalis*, however the V583 PurR protein shares 79% similarity to PurR in *Bacillus subtilis*. The *B. subtilis* PurR represses *pur* expression in the presence of purine nucleotides, thereby preventing induction of this pathway when exogenous purines are available (100). If the V583 PurR regulates *pur* expression in the same manner, our data suggests the *E. faecalis* intra-phagosomal environment is purine nucleotide limited. Similarly genes involved in pyrimidine nucleotide biosynthesis, *pyrC-pyraA*, *pyrD-2* and *pyrE*, were up-regulated during intracellular survival (Fig. 2). In contrast to purine biosynthesis, the regulation of pyrimidine nucleotides in *E. faecalis* is relatively well studied (32, 52). Ghim et al. have shown the *pyr* genes are repressed in the presence of uracil (32). Thus, our data suggest that V583 encounters pyrimidine starvation conditions within the macrophage.

Conclusions

The quest to discover the mechanisms used by *E. faecalis* to survive within the macrophage has spanned the better part of a decade. During this time numerous studies have begun to elucidate not only the environment sensed within the *E. faecalis*-containing phagosome, but also the coordinated response elicited to harsh conditions. However, these studies do not provide a comprehensive view of the *in vivo* mechanisms used during intracellular survival. Here we determined the *E. faecalis* V583 genome-wide transcriptional profile in the macrophage with the goal of expanding our current understanding of the intracellular survival strategy.

Paramount to survival in a changing environment is the ability to accurately sense conditions and coordinate an appropriate response. The detection of environmental stimuli is often mediated through two-component systems (37, 59). Analysis of the microarray data revealed that 12 members of *E. faecalis* TCS systems respond during intracellular survival (Fig. 3). These data not only corroborate previously published data (59), but also increase the number of TCS systems thought to coordinate intracellular survival. Interestingly, the findings from our study provide clues about the *in vivo* environment sensed during enterococcal infection of the macrophage. Our data suggest that life inside the macrophage is anything but luxuriant, as *E. faecalis* appears to be starved for glycolytic substrates, nucleotides, amino acids and numerous ions necessary for growth and protein function (Figs. 2 and 3). The limiting conditions encountered within the macrophage appear to induce the V583 stringent response, as evidenced by the down-regulation of the transcription and translational apparatus (Fig. 2). Furthermore, *E. faecalis* induces a robust response to the oxidative environment, particularly during the

earliest stages of intracellular persistence, and appears to prefer the methionine sulfoxide reductase and thioredoxin pathways for repairing damaged proteins (Fig. 3). In spite of these repair mechanism it is clear that bacterial cell components are damaged, as V583 strongly induces both the SOS and heat shock responses inside of macrophages (Fig. 3).

Some of the more intriguing findings from our study include the induction within the macrophage of the *fsr* system and hemolysins. To our knowledge this is the first study to show induction of the *fsr* system, including *gelE* and *sprE*, during intracellular survival. The quorum-sensing *fsr* system activates *gelE* and *sprE* expression through the production of gelatinase-biosynthesis activating pheromone (GBAP) in cell-density dependent manner (in late log or early stationary phase) (60, 61). From our data we are not able to determine what causes the activation of *fsr* within the macrophage, as the density of *E. faecalis* in the macrophage is undoubtedly less than that in stationary phase cultures, nor are the roles of *fsr*, *gelE* or *sprE* during intracellular survival understood. However, since SprE contributes to pathogenesis (73) and GelE is capable of destroying the immune compliment system (64, 65), we propose that they contribute to the pathogenicity of *E. faecalis* once this pathogen is released from the macrophage. In this scenario, the intracellular production of SprE may aid in the V583 infection of host tissues while the concomitant production of GelE could participate in immune-avoidance by hydrolyzing components of the complement system.

Many intracellular pathogens, including *Salmonella typhimurium* and *Mycobacterium tuberculosis* reside within a modified vacuole or phagosome during macrophage survival (29, 77). Yet others, such as *Listeria monocytogenes*, are able to exploit the macrophage cytosol by escaping the phagosome through the production of the

cyolysin LLO (30, 69). Though the intracellular trafficking of *E. faecalis* is poorly understood, it is clear that *E. faecalis* can be released from the phagosome into the cytosol (31) and that culture supernatant lyses the macrophage (58). Our finding that V583 expresses a gene encoding a hemolysin (*hly-3*) throughout all intracellular survival time points tested (Fig. 3) suggests a mechanism by which *E. faecalis* escapes the phagosome and/or the macrophage. The regulation of *hly-3* in V583 is virtually unknown. However in *L. monocytogenes*, LLO production is tightly regulated by the transcriptional regulator PfrA and is induced in response to nutrient limitation and heat shock (15, 44, 51). It is possible that *hly-3* is induced under similar conditions as LLO, as nutrient limitation and the heat shock response were observed in the *E. faecalis*-containing phagosome (Figs. 2 and 3).

In conclusion, this study provides the first comprehensive study elucidating the transcriptional response of *E. faecalis* to phagocytosis, a significant proportion of which is comprised of loci with unknown function. These data should provide new targets for future studies.

Figure legends

Figure 1. Comparisons of microarray results during intracellular survival at 4 h, 8 h and 12 h post-infection. Control RNA was used to normalize RNA extracted from bacteria during macrophage survival (intracellular : control). All data presented here are shown as fold change in gene expression. (A) Venn diagram comparing differentially regulated genes (induced or repressed > 2 fold) at 4 h, 8 h and 12 h post-infection. (B) Hierarchically-clustered heat map of all genes differentially regulated during infection.

Figure 2. Heat maps of $\log_2$ expression ratios for intracellular bacteria at 4 h, 8 h and 12 h post-infection. All intracellular array data were normalized to control array data before analysis (intracellular : control). All genes shown were differentially regulated (induced or repressed) > 2 fold. Genes involved in metabolism and nucleotide biosynthesis were up and down-regulated. The transcriptional and translational apparatus were down-regulated.

Figure 3. Intracellular expression profiles of genes with various functions. Intracellular array data were collected at 4 h, 8 h and 12 h post-infection. Expression profiles show genes altered for expression > 2 fold.

Figure 1.

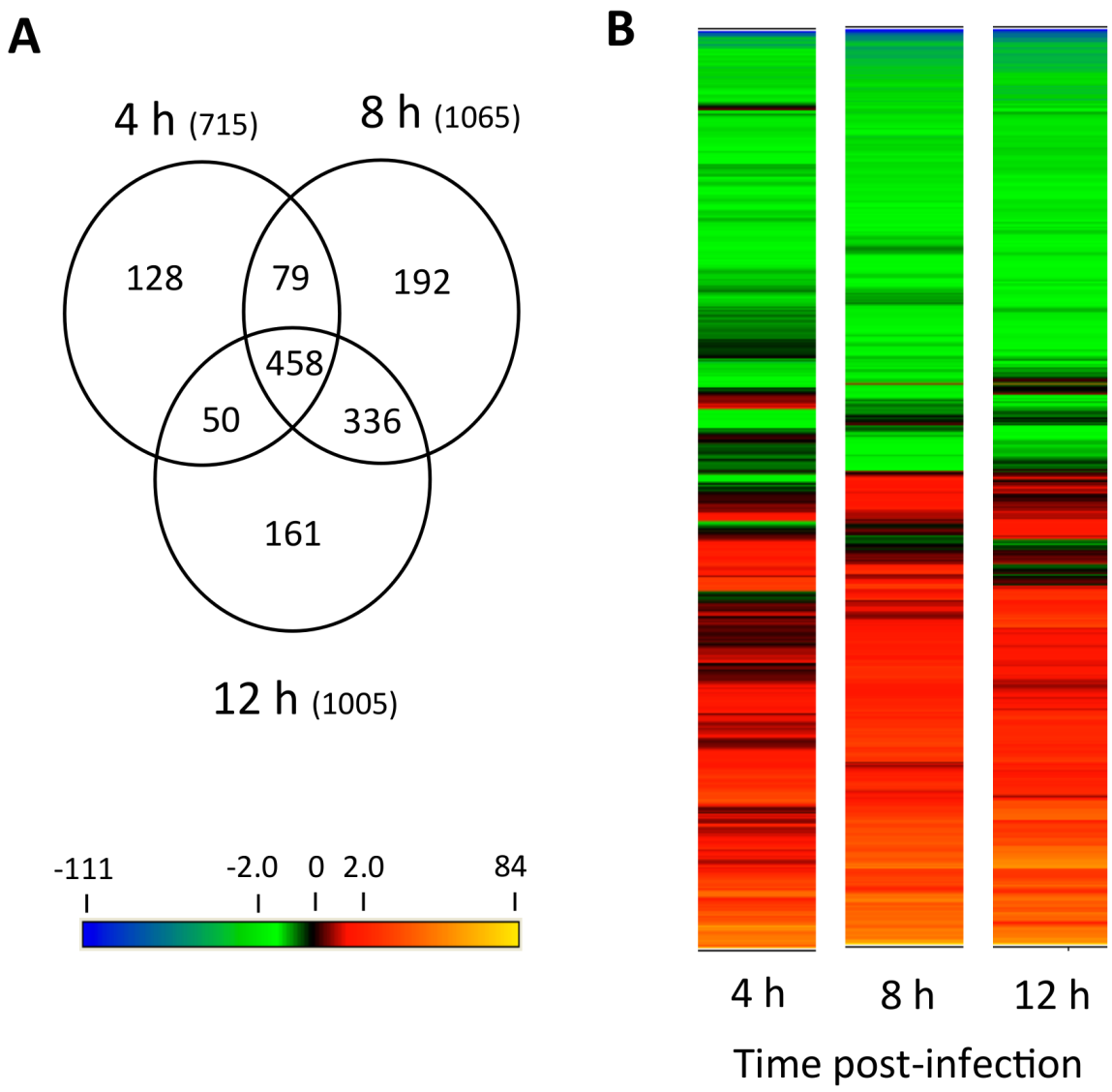

A
4 h (715)
8 h (1065)
128
79
192
458
50
336
161
12 h (1005)
-111
-2.0
0
2.0
84
B
4 h
8 h
12 h
Time post-infection

Figure 2.

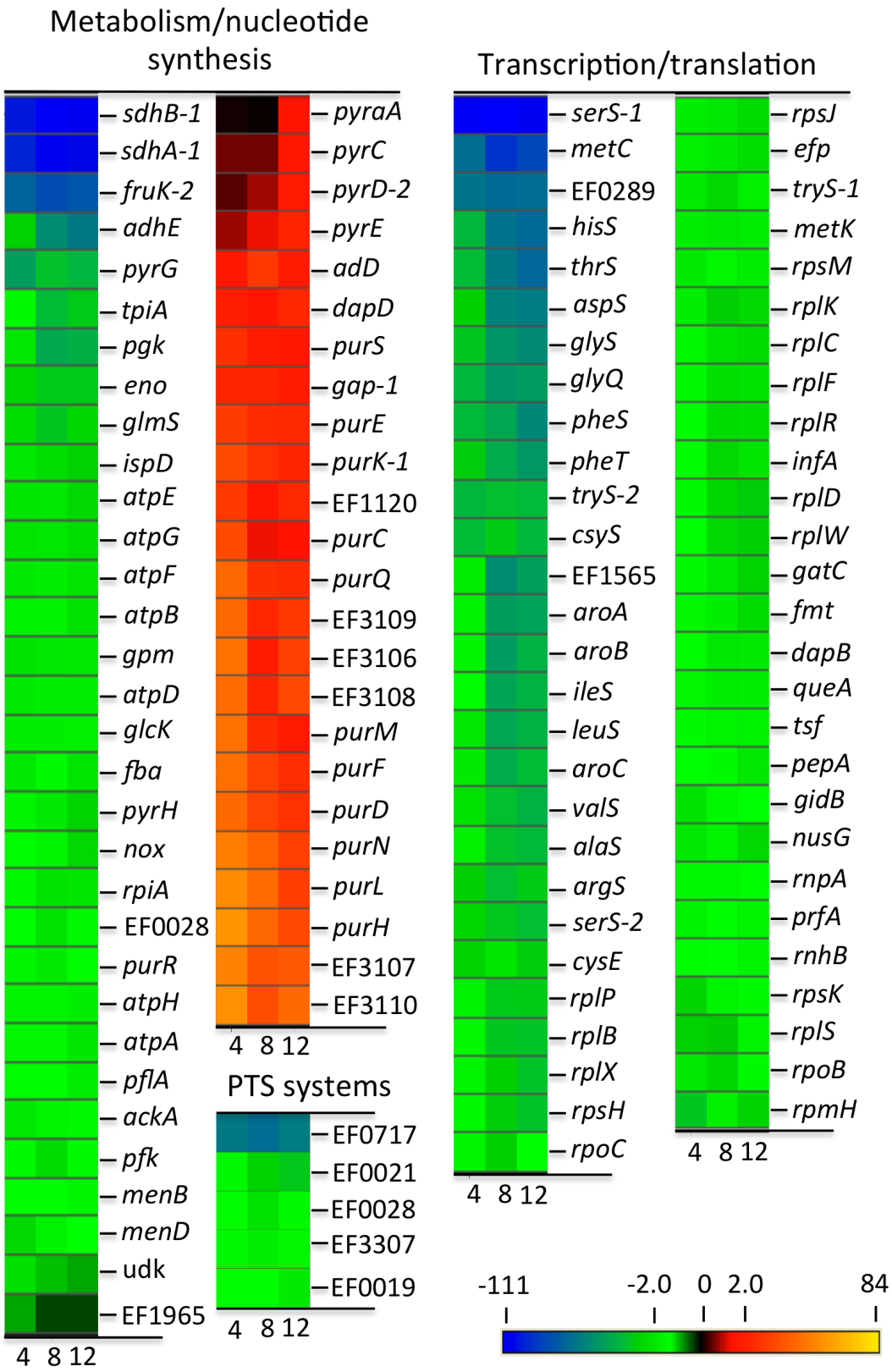
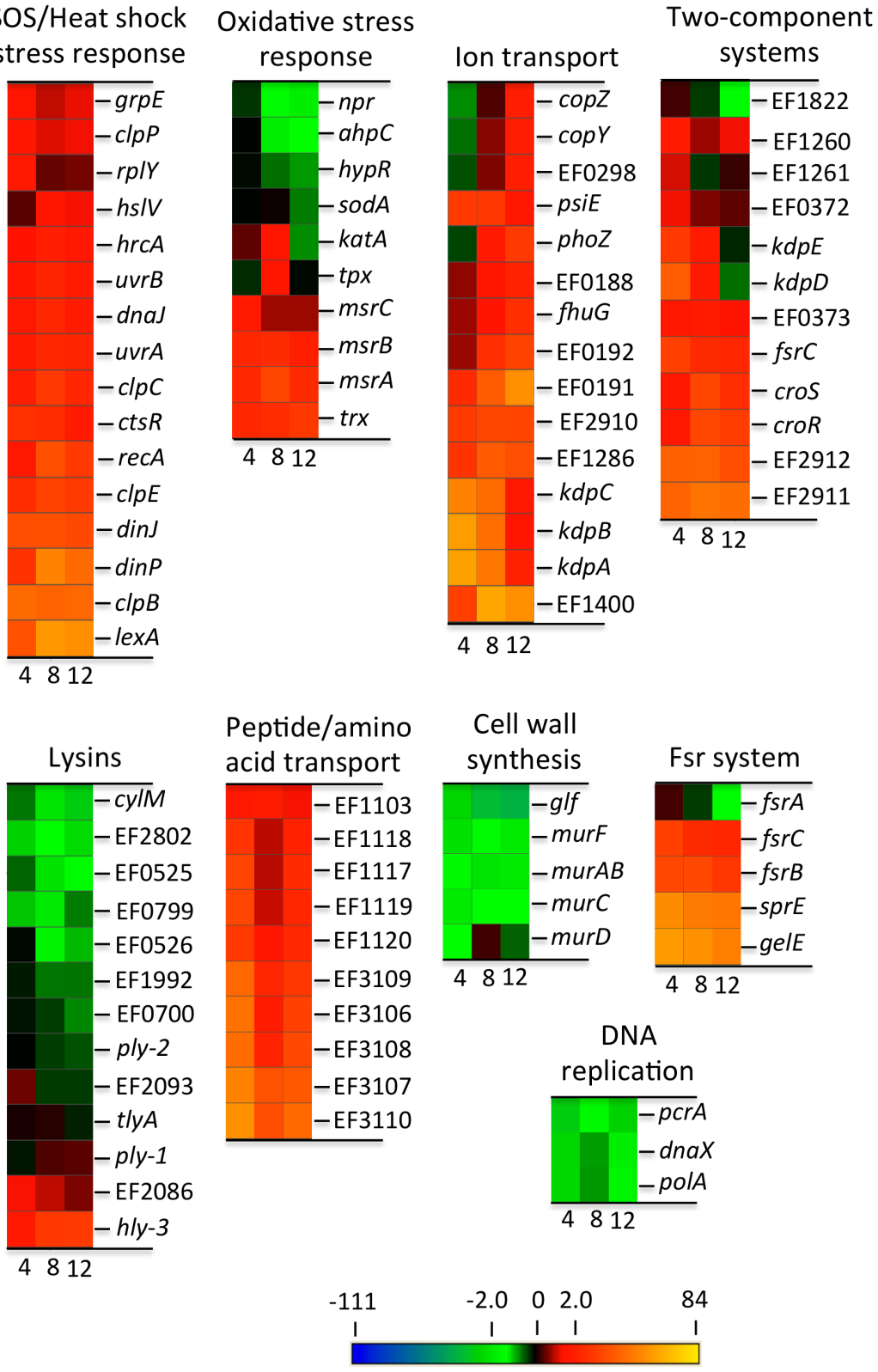

Metabolism/nucleotide synthesis
sdhB-1
sdhA-1
fruK-2
adhE
pyrG
tpiA
pgk
eno
glmS
ispD
atpE
atpG
atpF
atpB
gpm
atpD
glcK
fba
pyrH
nox
rpiA
EF0028
purR
atpH
atpA
pflA
ackA
pfk
menB
menD
udk
EF1965
4 8 12
pyraA
pyrC
pyrD-2
pyrE
adD
dapD
purS
gap-1
purE
purK-1
EF1120
purC
purQ
EF3109
EF3106
EF3108
purM
purF
purD
purN
purL
purH
EF3107
EF3110
4 8 12
PTS systems
EF0717
EF0021
EF0028
EF3307
EF0019
4 8 12
Transcription/translation
serS-1
metC
EF0289
hisS
thrS
aspS
glyS
glyQ
pheS
pheT
tryS-2
csyS
EF1565
aroA
aroB
ileS
leuS
aroC
valS
alaS
argS
serS-2
cysE
rplP
rplB
rplX
rpsH
rpoC
4 8 12
rpsJ
efp
tryS-1
metK
rpsM
rplK
rplC
rplF
rplR
infA
rplD
rplW
gatC
fmt
dapB
queA
tsf
pepA
gidB
nusG
rnpA
prfA
rnhB
rpsK
rplS
rpoB
rpmH
4 8 12
-111
-2.0
0
2.0
84

Figure 3.


SOS/Heat shock stress response
grpE
clpP
rplY
hslV
hrcA
uvrB
dnaJ
uvrA
clpC
ctsR
recA
clpE
dinJ
dinP
clpB
lexA
4 8 12
Oxidative stress response
npr
ahpC
hypR
sodA
katA
tpx
msrC
msrB
msrA
trx
4 8 12
Ion transport
copZ
copY
EF0298
psiE
phoZ
EF0188
fhuG
EF0192
EF0191
EF2910
EF1286
kdpC
kdpB
kdpA
EF1400
4 8 12
Two-component systems
EF1822
EF1260
EF1261
EF0372
kdpE
kdpD
EF0373
fsrC
croS
croR
EF2912
EF2911
4 8 12
Lysins
cylM
EF2802
EF0525
EF0799
EF0526
EF1992
EF0700
ply-2
EF2093
tlyA
ply-1
EF2086
hly-3
4 8 12
Peptide/amino acid transport
EF1103
EF1118
EF1117
EF1119
EF1120
EF3109
EF3106
EF3108
EF3107
EF3110
Cell wall synthesis
glf
murF
murAB
murC
murD
4 8 12
Fsr system
fsrA
fsrC
fsrB
sprE
gelE
4 8 12
DNA replication
pcrA
dnaX
polA
4 8 12
-111
-2.0
0
2.0
84

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Chapter 3: Transcriptional Regulator PerA Responds to Bicarbonate and Regulates Biofilm-Associated, Platelet Binding and Metabolic Genes in *Enterococcus faecalis*

Abstract

Enterococcus faecalis is an opportunistic pathogen and a leading cause of nosocomial infections, traits facilitated by the ability to quickly acquire and transfer virulence determinants. A 150 kb pathogenicity island (PAI) comprised of genes contributing to virulence is found in many enterococcal isolates and is known to undergo horizontal transfer. We have shown that the PAI-encoded transcriptional regulator PerA contributes to pathogenicity in the mouse peritonitis infection model. In this study, we used whole-genome microarrays to determine the PerA regulon. Our findings reveal that PerA coordinately regulates genes important for metabolism, amino acid degradation, and pathogenicity. Further transcriptional analysis revealed that PerA is influenced by bicarbonate. Additionally, PerA influences the ability of *E. faecalis* to bind to human platelets. Our results suggest that PerA is a global transcriptional regulator that coordinately regulates genes responsible for enterococcal pathogenicity.

Introduction

As a commensal member of the intestinal microbiota, the enterococci play an important role in establishing a healthy GI tract and typically coexist in the host as a relatively small, yet stable, population. Alternatively if the delicately balanced host/commensal relationship is disrupted, if specific environmental cues are detected, or if virulence traits are acquired, enterococci can act as opportunist pathogens capable of multiple-site infections, including infections of the heart, urinary tract, and bloodstream (25, 44, 64). In an effort to better understand the differences between commensal and pathogenic enterococci, studies of pathogenic enterococci increasingly seek to discover which traits promote virulence, how these traits are inherited and what mechanisms are used to coordinately regulate these traits to achieve pathogenicity.

While the enterococci have been known as infective agents for more than 100 years (41), the majority of information regarding the acquisition and deployment of virulence traits has been gathered in the last few decades (33, 55, 74). As a result of these studies, we have a clearer picture of how the enterococci successfully transition from a commensal to a pathogen. At the heart of this transition is enterococcal promiscuity: the ease and frequency with which many strains acquire and transmit mobile genetic elements harboring loci that contribute to pathogenesis. In addition to being intrinsically resistant to a broad range of antimicrobial agents, enterococci have evolved resistance to many antibiotics by acquiring plasmids or transposons comprised of genes that confer resistance. Developing antibiotic resistance has increased the pathogenic potential of the enterococci, as is evident by these organisms becoming the leading cause of surgical site infections, the second leading cause of bloodstream infections and the

third leading cause of nosocomial urinary tract infections (61). Furthermore, antibiotic resistant strains are more likely to contain mobile genetic elements that may harbor virulence traits (54). Especially problematic are strains that acquire both antibiotic resistance and virulence traits, as the concurrence of these factors is correlated with strains capable of producing infection outbreaks on a global scale (43).

Facilitating the spread of virulence traits in a particularly efficient manner are pathogenicity islands (PAI). PAI's are characterized as clusters of genes encoding proteins with roles involving transfer functions, virulence, stress survival, and transcriptional regulation (30). Furthermore these mobile genetic elements can be distinguished from the native chromosome by a significantly different G + C content (30). While first discovered in pathogenic *Escherichia coli* (31, 40), these mobile genetic elements are disseminated throughout many bacterial genera (30). A 153 kb PAI consisting of 129 open reading frames was discovered in *Enterococcus faecalis* MMH594 and shown to disperse to many *E. faecalis* strains of various origins (15, 43, 63). This PAI contains many loci with roles in virulence, including *esp* (encodes enterococcal surface protein), cytolysin toxin, and aggregation substance, as well as factors potentially involved in horizontal transfer and gastrointestinal tract colonization (63). Esp is enriched among infection-derived isolates and has been shown to increase *in vitro* biofilm formation (65, 70). The eight genes comprising the cytolysin operon (*cylR1, cylR2, cylL$_L$, cylL$_S$, cylMBAI*) form a two-peptide lytic toxin (8, 28). Cytolysin toxin is effective against both prokaryotic and eukaryotic cells (18, 19), and contributes to mortality in various pathogenic models of infection (14, 27, 36). A pheromone-inducible aggregation substance (AS) can also be found in many enterococcal strains.

AS promotes aggregation and conjugation (53, 75), increases enterococcal adherence to and uptake in eukaryotic cells (39, 52) and increases bacterial survival inside the macrophage (69).

Frequently, PAI's contain genes encoding transcriptional regulators with various regulatory schemes, and the *E. faecalis* PAI is no exception (30, 63). The *E. faecalis* PAI encodes an AraC-type regulator, named PerA (for pathogenicity island-encoded regulator) (17, 63). PerA is enriched among clinical *E. faecalis* isolates and lies adjacent to the aforementioned PAI-encoded virulence traits, which suggests PerA-dependent regulation of these genes (63). Through mutational analysis, we have previously shown that PerA influences biofilm formation in a medium-specific manner and contributes to virulence in a mouse peritonitis model (17). Additionally, the PerA-deficient strain was significantly attenuated during macrophage survival, further supporting the role of PerA as an important regulator of *E. faecalis* pathogenesis (17).

Prompted by the observation that PerA coordinates *E. faecalis* virulence in the mouse peritonitis infection model, we sought to identify the genes that are regulated directly or indirectly by PerA. We used Affymetrix GeneChip microarrays to experimentally define the PerA regulon throughout exponential growth, upon transition into stationary phase and during stationary phase persistence. Our results suggest that PerA primarily regulates genes located outside of the PAI in a growth phase-dependent manner. These PerA-regulated genes are located throughout the *E. faecalis* chromosome and include loci responsible for amino acid metabolism, biofilm formation and phage-associated genes putatively involved in platelet binding. Further experimentation revealed that PerA influences the ability of *E. faecalis* to bind human platelets and

respond to the presence of bicarbonate. Taken together with our previous findings (17), we interpret these results to mean that PerA acts as a global transcriptional regulator to coordinately regulate genes responsible for enterococcal pathogenicity.

Material and Methods

Bacterial strains, media, and reagents. The strains used in this study were *E. faecalis* E99 (71) and an isogenic Δ*perA::ermR* mutant (DBS01) (17). The strains were routinely cultured in Todd-Hewitt broth (THB) containing 1% glucose or THB + 1% glucose supplemented with 100mM sodium bicarbonate when appropriate. Antibiotics used for selection included kanamycin (25 μg/ml) and erythromycin (50 μg/ml) (Sigma Chemical, St. Louis, MO). Growth was monitored as absorbance at 600nm using a Beckman-Coulter DU800 spectrophotometer.

RNA isolation and Microarray analysis. RNA extraction and microarray analysis proceeded as previously described (73) with a few modifications. Briefly, strains E99 and DBS01 were grown at 37°C overnight in THB + 1% glucose in appropriate antibiotics. The bacteria were diluted 1:10,000 into fresh, pre-warmed medium and incubated at 37°C. At predetermined optical densities (600 nm; 0.05 for mid-exponential, 0.5 for late-exponential, and 1.0 for stationary phase) cells were sampled directly into ice-cold RNA*later* (Ambion, Foster City, CA). Total RNA was extracted using Qiagen RNeasy Minikits (Valencia, CA) with optional on-column DNase treatment steps according to the manufacturer's specifications. RNA integrity was checked by gel electrophoresis and stored in 2 volumes of ethanol at -80°C. cDNA was generated by first strand synthesis using Superscript II (Invitrogen, Carlsbad, CA) and random

hexamers according to the manufacturer's specifications. Fragmentation and biotinylation of cDNA proceeded according to the Affymetrix prokaryotic labeling protocol using the ENZO Kit from Roche Diagnostics (Indianapolis, IN). Biotinylated cDNA was hybridized to custom Affymetrix GeneChips for 16 h at 45°C. The custom microarrays used in this study contained probes for several prokaryotic genomes including *Enterococcus faecalis* V583 (GEO Accession number: GPL6702). Affymetrix protocol ProkGE_WS2v2-450 was used to stain the hybridized arrays. Following scanning, raw data files (.cel) were analyzed using RMA processing with quartile normalization (37). Biological and technical replicates were averaged, and genes were considered to be significantly induced or repressed if the DBS01 : E99 expression ratio was greater than twofold (76). Heatmaps were generated using DecisionSite for Functional Genomics (Spotfire, Somerville, MA). The microarray data has been deposited at GEO (GEO accession number, GSE31538).

qRT-PCR. Transcript levels were confirmed by qRT-PCR using RNA extracted from cells harvested during mid-exponential, late-exponential, and stationary phase. Primers were designed using Primer Express software provided with the ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). Amplicon lengths were 100 bp. Quantification of 16S rRNA levels was used as an internal control and to normalize RNA. Amplification was detected using SYBR Green PCR Master Mix (Applied Biosystems) with automatic calculation of threshold value. Analysis was repeated in triplicate on two biological replicates for each time point. Replicates were averaged and the results are presented in Table 2.

Assessment of Platelet Binding. The ability of *E. faecalis* cultures to bind human platelets was assessed as previously described (45). Briefly, human platelets were washed, fixed and immobilized on poly-L-lysine-coated 22-mm-diameter tissue culture wells at a concentration of 1 x 10^8 platelets per well. Following 30 min incubation at 37°C, unbound platelets were removed by aspiration. The remaining bound platelets were subsequently incubated in a 1% casein solution for 1 h at 37°C to reduce non-specific adherence. Following removal of the blocking solution, each well was inoculated with 1 x 10^8 of *E. faecalis* E99, DBS01, or DBS01 (pGT101) suspended in PBS and further incubated with gentle rocking. After 1 h unbound bacteria were removed by washing each well twice with PBS and the bound bacteria were collected by scraping and resuspending them in PBS. The number of bacteria bound to platelets was determined by plating suspensions on THB supplemented with appropriate antibiotics. Binding was expressed as a percentage of the inoculum. Platelet binding assays were performed three times, each assay replicated in triplicate (n=9) using blood from multiple, healthy volunteers. Differences in platelet binding efficiencies were determined using an unpaired *t*-test, as shown in Fig. 7.

Ethics Statement. This study was performed under the supervision and approval of the Institutional Review Board at the University of Oklahoma. The platelets used in this study were purchased from Bioreclamation (Long Island, NY) and obtained from a blood bank supplied by healthy volunteers.

Results

Overview of microarray data. PerA is an AraC-type transcriptional regulator that contributes to pathogenesis in *E. faecalis* (17). To define the PerA regulon, transcriptional profiling was performed on *E. faecalis* E99 and an isogenic Δ*perA* mutant strain (designated DBS01) using RNA extracted from both strains at time points corresponding to mid-exponential, late-exponential, and stationary phase (O.D. 600nm ~ 0.05, 0.5, and 1.0, respectively) (Figs. 1A and 1B). The RNA was reverse-transcribed and subsequently hybridized to *E. faecalis* V583 genome microarrays. All array data shown are expressed as ratios (DBS01 : E99) and considered to be significant if gene expression was induced or repressed in the mutant strain greater than twofold. The PerA regulon is extensive, as transcriptional analysis revealed 151 genes differentially regulated > twofold ($\log_2 = 1$) in DBS01. Of these 151 genes, 98 were up-regulated and 53 were down-regulated. Nearly one-third (46 of 151) of the differentially regulated genes have unknown function, 20 are involved in metabolic functions, and 19 encode transport-related genes. Of the 98 up-regulated genes, 19 are up-regulated in mid-exponential phase only, 6 are up-regulated in late-exponential phase only, and 57 are up-regulated only in stationary phase (Figs. 2A and 2B). Of the 53 down-regulated genes 10 are down-regulated only in mid-exponential phase, 11 are down-regulated only in late-exponential phase, and 27 are down-regulated only in stationary phase (Fig. 2A). These data suggest that while PerA is primarily a negative regulator, it can also act as a dual regulator, as a positive influence on gene expression is also noted (Figs. 2A and 2B). Additionally, the PerA target genes show a high degree of growth-phase dependent

regulation, with the highest degree of influence occurring in stationary phase (Figs. 2A and 2B).

DBS01 shows altered expression of PAI-related genes. The 153 kb PAI carries virulence determinants (including cytolysin, Esp, and aggregation substance) adjacent to *perA* (17, 63). The proximity of the *perA* gene to genes with ascribed roles in virulence is suggestive of PerA regulation of PAI genes. In DBS01, 5 PAI genes were differentially regulated in any of the time points studied (Figs. 2B and 3). During mid-exponential growth the EF0579 gene was induced (Fig. 2B). This locus encodes a putative TetR-family protein with unknown function in *E. faecalis*. Four genes encoding hypothetical proteins (EF0488, EF0531, EF0532, EF0533) were down-regulated between 2 and 4 fold in DBS01 at late-exponential phase (Fig. 2B). In stationary phase the EF0579 gene was again induced, while the EF0488 gene was no longer differentially regulated (Fig. 2B). The microarrays used in this study were developed using the strain V583 sequenced genome. V583 is missing portions of the cytolysin operon, *nsr* and *gls24*-like genes, and the entire *esp* gene due to a spontaneous 17 kb deletion within the PAI (63). Therefore, quantitative reverse transcription PCR (qRT-PCR) was used to determine the expression of these PAI genes found in strain E99 but absent in V583. qRT-PCR revealed no differential regulation of these genes in DBS01 at any time point tested (data not shown). The differential regulation of PAI hypothetical genes, but not genes with previously ascribed roles in virulence, may indicate PerA-dependent control of genes with an unknown function in enterococcal pathogenicity; however this possibility remains to be studied.

The transcription of many housekeeping genes is altered in DBS01. AraC-type regulators are known to control a variety of cellular processes, including metabolism and other housekeeping functions. We mined the transcriptome to determine if any housekeeping genes were regulated by PerA, and found a number of genes differentially expressed in DBS01. A number of genes involved in basic cellular metabolism were down-regulated in DBS01, including *galK*, *rbsK* (EF2961) and *rbsD* (EF2960) (Fig. 2B). *galK* encodes for galactokinase, while *rbsK* and *rbsD* encode for ribokinase and a ribose transporter, respectively, and are required for transport and metabolism of galactose and ribose. Many housekeeping genes are induced in DBS01, including genes encoding ribosomal proteins (*rplQ*, *rpsP*, *rpsD* [EF3070], *rpmB* [EF3116] and *rpmH* [EF3333]) and pyrimidine nucleotide biosynthetic genes (*purA*, EF0014) (Fig. 2B). Lastly, putative peptide ATP-binding cassette (ABC) transporters were significantly induced in DBS01. While poorly studied in *E. faecalis*, these peptide transporters generally provide nutrients to bacteria in the form of amino acids or short peptides (23, 47)

PerA regulates biofilm-related genes in E99. *E. faecalis* E99 is a urinary-tract isolate possessing a high biofilm phenotype (71). Recently a ubiquitous enterococcal locus was characterized and named *ebp* (51). The *ebpABC* operon encodes the enterococcal biofilm-associated pilus and contributes to endocarditis, urinary tract infections (UTI), and biofilm formation (51, 66). The EbpABC proteins are polymerized through the activity of Bps (formerly, SrtC), and together are required for maximal biofilm production in *E. faecalis* (51). EbpR acts as a transcriptional activator of *ebpABC* and positively influences biofilm formation (10). As previously shown, the PerA regulator influences E99 biofilm formation in a medium-dependent manner (17). To determine if

PerA regulates *ebpABC* and *bps* gene expression, we compared the transcriptome of DBS01 to E99 during mid-exponential, late-exponential, and stationary phase. In DBS01, the *ebpABC* operon and associated *bps* gene was induced between 4 and 8-fold during mid-exponential and stationary phases (average operon induction = 6.2-fold) (Fig. 4). The transition from mid-exponential to late-exponential growth was concomitant with an increase in expression of the *ebpABC* operon (average operon induction = 8.8-fold) (Fig. 4). Induction of the *ebpABC* and *bps* genes was confirmed by using qRT-PCR (Table 2). The high degree of *ebpABC* up-regulation shown here, as well as the increase in biofilm formation previously shown in DBS01 (17), suggests that PerA may act as a repressor of the *ebpABC* operon and associated *bps* gene in E99.

Next we sought to examine if PerA regulates other biofilm-related genes found in E99, including *esp*, the *bee* locus, and *fsrABCD* operon. *esp* encodes for enterococcal surface protein, a high-molecular weight protein that is enriched among infection-derived enterococcal isolates (72). The *esp* gene has been shown to enhance biofilm formation (70). The *bee* locus is a unique five-gene system that contributes to the high biofilm phenotype found in E99 (71). The *bee* locus is located on a large (~80 kb) plasmid, and can transfer at high frequencies by conjugation (71). The microarrays used for this experiment were derived from the *E. faecalis* V583 sequenced genome. V583 is missing the *esp* gene due to a 17 kb PAI deletion (63), and does not contain the conjugative plasmid harboring the *bee* locus (unpublished results). Therefore it was impossible to examine gene expression of these by using microarrays. qRT-PCR was used to determine possible changes in gene expression for the *esp* and *bee* loci. When comparing

DBS01 and E99 using qRT-PCR, no significant differential regulation of the *esp* or *bee* loci in any of the three growth phases tested was observed (data not shown).

The *fsr* system, encoded by the *fsrABCD* operon, is similar to the *argABCD* operon found in *Staphylococcus aureus* (48). *fsr* is a quorum-sensing system that mediates the production of a cyclic peptide termed gelatinase-biosynthesis activating pheromone (GBAP) (48, 49). Through the production of GBAP, *fsr* activates two genes encoding a gelatinase (*gelE*) and a serine protease (*sprE*) resulting in biofilm formation (32, 57, 58). Though little is known about the *fsr* or *gelE-sprE* loci in E99, approximately 60% of *E. faecalis* clinical isolates produce gelatinase (20). We searched the microarray data and found no differentially regulated genes in either the *fsr* or *gelE-sprE* loci in DBS01. Taken together these data suggest that PerA may act to repress the *ebpABC* operon and associated sortase while having little to no influence on the expression of the *esp, bee* or *fsr* loci under the conditions tested.

***perA* and *ebpABC* respond to the presence of bicarbonate in E99.** Using β-gal assays and qRT-PCR, Bourgogne et al. have recently shown that *E. faecalis* OG1RF *ebpABC* expression increases when grown in sodium bicarbonate in an *ebpR*-dependent manner (11). Our data suggest that PerA acts as a repressor of the *ebpABC* locus (Figs. 2B and 4). Furthermore, AraC-type regulators are known to respond to bicarbonate, including RegA in *Citrobacter rodentium* and ToxT in *Vibrio cholerae* (3, 77). Given that OG1RF lacks the *E. faecalis* PAI, including *perA*, we were curious to determine the effects of bicarbonate on *ebpABC* expression in E99. To do this we analyzed the transcriptome of E99 grown in THB supplemented with 100mM sodium bicarbonate. When compared to E99 grown in THB, *perA* was down-regulated in the presence of bicarbonate while *ebpR*

(the activator of *ebpABC*) was moderately induced (Fig. 5). Furthermore, the average *ebpABC* expression increased approximately 7-fold (*ebpA* = 8.0, *ebpB* = 7.7, *ebpC* = 4.9), with the biofilm and pilus-associated sortase (*bps*) being induced 4-fold (Fig. 5).

We reasoned that if PerA represses the *ebpABC* locus, a down-regulation of *perA* in the presence of bicarbonate would cause a response similar to that seen in DBS01 (Δ*perA*). When comparing the transcriptome of E99 grown in THB supplemented with 100mM sodium bicarbonate to DBS01 grown in THB, similar trends in *perA*, *ebpR-ebpABC* and *bps* gene expression are observed (Fig. 5). These results suggest that *perA* is down-regulated in the presence of bicarbonate, concomitant with an induction of the *ebpR-ebpABC* and *bps* loci.

Effect of the *perA* mutation on expression of ADI pathway. The arginine deiminase (ADI) system is used by many microorganisms to generate ATP via arginine fermentation (22). Genes comprising the ADI pathway in *E. faecalis* are arranged as the *arcABCRD* operon (ArcA, arginine deiminase, ArcB, ornithine carbamolytransferase; ArcC, carbamate kinase; ArcR, Crp/Fnr regulator, ArcD arginine/ornithine antiporter), and are known to be transcribed in the presence of arginine (6). The ADI pathway has a complex regulatory scheme with binding sites for two arginine-sensitive regulators (ArgR1 and ArgR2), a catabolite control protein (CcpA), as well as a protein involved in *E. faecalis* pathogenicity (Ers) (6, 60). In DBS01 the *arcABCRD* operon is highly up-regulated in all time points tested (Fig. 4). On average, the *arcABCRD* operon is induced 7.6-fold during mid-exponential growth and plateaus upon entrance into late exponential phase induced 11-fold. The average expression of the *arcABCRD* genes is up-regulated 3-fold during stationary phase. This pattern of ADI pathway regulation is similar to that

previously observed in *E. faecalis*. Bourgogne et al. found that the enterococcal FsrB transcriptional regulator negatively influences *arcABC* expression during transition from exponential to stationary phase; though it is unclear if this regulation is direct or indirect (9). Riboulet-Bisson et al. have shown that the Ers regulator activates *arcABC* expression by binding upstream of the *arcA* gene (60). For unknown reasons and in contrast to this study, *arcRD* gene expression was not differentially regulated by FsrB or Ers (9, 60). In DBS01 *argR1* gene expression was induced at all time points tested (Fig. 4) while the *argR2* gene was not differentially regulated (data not shown). The *argR1* and *arcABCRD* genes account for 60% (6 out of 10) of the genes up-regulated in all time points tested (Figs. 2A and 4), suggesting the PerA regulator may act as a repressor of arginine catabolism in *E. faecalis*.

PerA regulates a putative temperate bacteriophage in E99. Temperate bacteriophages are disseminated throughout many gram-positive bacteria, including *E. faecalis*. The *E. faecalis* V583 sequenced genome contains seven regions arising from integrated phages (56). Though the role of these phages in *E. faecalis* virulence has yet to be discovered, each of these mobile elements contains homologs of virulence determinants from *Streptococcus mitis* phage SM1 (56, 78). We mined the microarray data for each of these putative phage-related genes, and found a cluster of genes similar to phage 04 in V583 that was differentially regulated in DBS01 (Figs. 3 and 6). This element spans *ef1985-ef2043* and contains putative replication, integration and virulence functions. The majority of genes on the phage display either no change or non-significant induction or repression in DBS01 throughout all growth phases. However a group of genes show significant growth phase-independent repression in DBS01,

including homologs of *pblA*, *pblB* and a gene encoding a putative lysin (Fig. 6). PblA and PblB mediate bacterial attachment to platelets in *S. mitis* (7). The lysin protein serves a dual purpose: permeablizing the bacterial cell wall, thus permitting release of PblA and PblB, and binding to platelets through interaction with fibrinogen and fibrinogen receptors (45, 62). *E. faecalis* is known to aggregate human platelets, yet the molecular mechanisms coordinating this process have not been discovered (59). The repression of *pblA*, *pblB* and lysin in DBS01 suggests that PerA influences the expression of genes putatively involved in platelet binding and cell wall permeability residing on a temperature bacteriophage in E99.

PerA influences the binding to human platelets. PerA differentially regulates two distinct loci potentially important in bacterial attachment to human platelets. First are the putative *pblA*, *pblB* and lysin genes residing on a temperate bacteriophage. Next is the Ebp pilus, which has recently been shown to mediate bacterial attachment to human platelets (50). Given that genes potentially involved in platelet binding were both induced and repressed in DBS01 (the *ebp* and phage-related loci, respectively), we sought to determine if DBS01 showed an altered ability to bind human platelets. To assess this we compared the ability of E99 and DBS01 to adhere to human platelets immobilized in microtiter plates. As shown in Fig. 7, DBS01 binds human platelets significantly ($P < 0.0005$, unpaired *t*-test) better than the E99 wild-type strain. DBS01 bound platelets approximately 5-fold better than E99. When DBS01 contained a plasmid-encoded copy of *perA* (pGT101), platelet-binding abilities were restored to the wild-type levels (Fig. 7). These results suggest that the inactivation of *perA* increases platelet binding in DBS01, possibly through the derepression of the *ebpABC* locus.

Discussion

The *perA* gene is located on the *E. faecalis* PAI, adjacent to loci with ascribed roles in virulence and genes with putative metabolic functions (63). Given its location, it was our hypothesis that the primary function of PerA was to regulate the expression of PAI genes in *E. faecalis*. However, transcriptional analysis revealed that in DBS01 only 5 PAI genes of unknown function were altered in gene expression during the time course study. To our surprise the overwhelming majority of genes regulated by PerA were chromosomally located yet not residing within the PAI. McBride et al. (43) have recently suggested that the enterococcal PAI is comprised of clusters of genes that likely undergo horizontal transfer as modules. Additionally, portions of the enterococcal PAI have been shown to conjugatively transfer both *in vitro* and *in vivo* (15). These findings raise the possibility that PerA is able to transfer to strains lacking the PAI and subsequently exert alien control of native genes. In this scenario, the acquisition of the transcriptional regulator PerA could effect a rapid physiological change in the recipient. In *Salmonella*, HilD, a transcriptional regulator encoded on the *Salmonella* pathogenicity island SPI-1, has been shown to regulate genes on the evolutionary distinct SPI-2 pathogenicity island (12). Furthermore, *E. coli* strain K12 genes can be regulated by Ler, a regulator located on the locus for enterocyte effacement (LEE) pathogenicity island of strain O157:H7 (1). Our data suggest that PerA may have the ability to control native chromosomal genes upon entry into a recipient; however, the ability of PerA to transfer into an enterococcal strain lacking the PAI and regulate native genes remains to be tested.

Biofilm formation is often a key component of bacterial pathogenesis (42, 46, 79). Though not necessarily a virulence trait, as biofilms are also produced by many avirulent

bacteria, biofilms contribute to pathogenicity by increasing resistance to antibiotics and environmental stresses (21). In *E. faecalis*, biofilms are correlated with infective endocarditis (10) and urinary tract infections (66), and promote bacterial survival inside phagocytes (5). PerA has been shown to influence biofilm formation in a medium specific manner, as a *perA*-deficient strain designated DBS01 produced more biofilm than the WT strain E99 (17). Transcriptional profiling revealed that PerA negatively regulates the enterococcal biofilm associated pilus (*ebp*) locus, a ubiquitous determinant important for maximal biofilm production (51). This makes possible the interesting scenario where the PAI-residing *perA* could transfer to recipient strains and influence biofilm formation through regulation of the *ebp* locus.

E. faecalis is known to aggregate platelets (59) a phenotype mediated, at least in part, by the Ebp pilus (50). When comparing the ability of DBS01 and E99 to bind human platelets, DBS01 was found to adhere to platelets significantly (~ 5 fold) better than E99 (Fig. 7). This ability to bind platelets is frequently implicated in promoting infective endocarditis (26, 68). When the heart valves become damaged, platelet aggregation on the damaged tissue can serve as binding foci for circulating bacteria. In animal studies, these vegetations cause the further accumulation of platelets and bacteria onto the infected surface, a condition that may lead to heart failure or death (24).

PerA influenced the expression of a number of genes involved in amino acid metabolism. The majority of these genes comprise the ADI pathway (*arcABCRD*) in *E. faecalis*. The ADI pathway is used by *E. faecalis* to produce ATP via arginine fermentation (35, 67). Expression of *arcABCRD* is tightly controlled as the ADI promoter region contains multiple binding sites for transcriptional regulators and

catabolite repression elements (6). Riboulet-Bisson et al. (60) recently identified an *Ers* (enterococcal regulator of survival) binding site upstream of the *arcA* gene, and suggested an activator role for this protein. In the current work, microarray analysis revealed that the ADI pathway is highly induced in DBS01, which is suggestive of PerA repression of these genes. Of interest is the increase in *arcABCRD* gene expression concomitant with the induction of the *ebp* locus in DBS01 (17). During an infection, it is possible that these coordinately PerA-regulated genes perform a related function. In the presence of host proteins or amino acids, the de-repression of the *arcABCRD* operon would permit the transport and degradation of liberated arginine. In this scenario arginine fermentation may provide energy for biofilm formation during pathogenesis. The biofilms could then serve to increase bacterial persistence inside the host and further the invasion of nutrient-rich host tissue. Furthermore, the PerA regulon comprises genes encoding a putative peptide ABC transport system (Fig. 3). These peptide transport systems provide nutrients to the cell by internalizing amino acids and short peptides, and are often critical for the survival of auxotrophic lactic acid bacteria (23). Zhu et al. (79) found that clinical isolates of *Staphylococcus aureus* selectively extracted arginine from growth media during biofilm formation. Chaussee et al. (13) found that in *Streptococcus pyogenes* the expression of virulence factors is coordinately regulated with amino acid catabolism. In this work, we show that PerA regulates genes involved in amino acid catabolism and biofilm formation, which further suggests a regulatory, if not functional, correlation between amino acid degradation and biofilm formation. While intriguing, the correlation between arginine metabolism and biofilm formation in *E. faecalis* remains to be studied.

Bicarbonate production is important for maintaining pH homeostasis in the small intestine, as it neutralizes acid in the intestinal lumen and prevents damage to the adherent mucus layer (4, 38). Many pathogens use the presence of bicarbonate as an environmental signal to coordinate the expression of virulence traits and frequently AraC-type regulators are involved (2, 3, 77). Bourgogne et al. have shown that the transcription of the *E. faecalis* OG1RF *ebp* locus is enhanced in the presence of bicarbonate, yet the regulatory cascade linking bicarbonate to *ebp* expression is unclear. In E99, PerA appears to be a repressor of *ebpABC* expression (Figs 2B and 4). In the presence of bicarbonate *perA* was down-regulated concomitant with an induction of *ebpR-ebpABC* and *bps* expression (Fig. 5). This suggests that in E99, PerA may be part of the regulatory cascade controlling *ebp* expression in response to bicarbonate whereby the production of bicarbonate in the intestine causes a down-regulation of *perA*, which leads to the production of the Ebp pilus. In this scenario, the sensing of environmental bicarbonate ultimately stimulates the production of an adhesin that could aid in colonization of the intestine.

From our data we are unable to determine if PerA directly responds to bicarbonate or if it is influenced by other regulatory mechanisms that detect bicarbonate. AraC-type regulators are comprised of a conserved C-terminal DNA-binding domain and a N-terminal domain important for ligand binding. Comparisons of the PerA sequence to other AraC-type regulators that are known to detect bicarbonate (*C. rodentium* RegA and *V. cholerae* ToxT) reveal that PerA exhibits C-terminus similarity, yet virtually no N-terminus sequence similarity exists (data not shown). Furthermore, we have previously shown that the PerA N-terminus contains no similarities with other AraC-type regulators

(17). It is possible that PerA senses bicarbonate using a unique bicarbonate-binding motif, however it is also possible that other regulators that sense bicarbonate may control *perA* expression. In regards to the latter possibility, *E. coli* MarA and SoxS are AraC-type regulators known to regulate transcription without directly detecting a ligand (29, 34).

PerA also appears to influence the expression of a number of housekeeping genes. Perhaps most notably is the down-regulation of genes in DBS01 involved in the basic metabolism of the cell, concomitant with an induction of genes responsible for biofilm formation and attachment to host cells (Fig. 2B). It is possible that at the site of infection E99 uses PerA as a global dual-regulator to orchestrate the down-regulation of many housekeeping genes non-essential to pathogenicity while inducing genes responsible for colonization and infection of the host.

We have previously shown that PerA contributes to *E. faecalis* survival in the macrophage (17). However, finding the PerA-regulated genes that coordinate macrophage survival using our current strategy has, thus far, proven inconclusive. We are keen to realize the harsh phagosomal environment encountered by *E. faecalis* during phagocytosis is almost certainly drastically different than the conditions in this study. Though studies seeking to determine the *E. faecalis* intracellular survival strategy have increased our understanding of the challenges faced upon phagocytosis, the whole-genome transcriptional response used by *E. faecalis* during macrophage survival has yet to be revealed. This information would not only yield a better understanding of the phagosomal landscape during *E. faecalis* infection, but it would also illuminate the *E. faecalis* macrophage survival strategy. During intracellular survival, it is possible that

basal (or perhaps enhanced) expression of *perA* influences the transcription of hypothetical function genes, thus impacting persistence within the macrophage.

In the current study we used whole-genome *E. faecalis* V583 microarrays to determine the PerA regulon in E99. Though we used qRT-PCR to interrogate PAI genes in E99 that are missing from V583, we realize there could be other genes present in E99 yet absent from the V583 microarray. E99 contains a large, conjugative plasmid (pBEE99) comprised of genes that confer a high biofilm phenotype and increased ultraviolet radiation resistance (16). Additionally pBEE99 contains genes putatively encoding an aggregation substance and a two-component bacteriocin (16). Under the conditions tested PerA did not regulate either the PAI genes or the bee locus. However, the expression of the remaining pBEE99 genes in DBS01 remains to be determined. Furthermore, since the E99 genome has yet to be sequenced, this strain could possess unknown loci that are potentially regulated by PerA and contribute to virulence.

In conclusion, our data suggests that PerA is a global transcriptional regulator that coordinately controls genes important for pathogenicity. We can now propose a mechanism of how E99 achieves pathogenicity by using PerA as part of a regulatory network controlling expression of virulence genes. When appropriate environmental signals are sensed (quite possibly the presence of bicarbonate), the cell quickly and efficiently creates a rapid physiological change by down-regulating one gene: *perA*. In response to the environmental signal, the reduced levels of PerA would alleviate repression of genes important for biofilm formation and colonization of host tissues. Concurrently, metabolic and substrate transport pathways critical for cell nutrition are

induced while unnecessary housekeeping genes are repressed, thus ensuring the cell has the proper nutrients for pathogenicity.

Table 1. qRT-PCR primers used in this study

Primer	Sequence (5` - 3`)
arcA-F	AAGCCAATATTCGCAGCGAA
arcA-R	AATGCCTGCAATCGCTTTTT
arcB-F	TTTGACGGGATTGAGTTCCG
arcB-R	TGCCATTGATCCGTTAAACCA
arcC-F	ATGATGCTAGCGCACATGCA
arcC-R	GCCATGTGAAACAATCAACCG
arcR-F	TCCGAGAATCGGACTGTTTCA
arcR-R	AACGCTCAAACAGTTTAACTGGC
ebpA-F	ACCGCGGATGAAAGCTATCA
ebpA-R	CCAGGAACTGCTAATTCACGG
ebpB-F	CGTACAGGCGGCAAGTCTTT
ebpB-R	AGGTATTCCCCCGCTTGATT
ebpC-F	GAATTTTACGAGCAACGAGCG
ebpC-R	TCGGTGGTTCCTTGAGCAAC
bps-F	CATTTCAGGCCATCGTGGTC
bps-R	GCGTCTTCCCATTGACTTCG
16S-F	AGCCGGAATCGCTAGTAATCG
16S-R	TCGGGTGTTACAAACTCTCGTG

Table 2. Members of the PerA regulon confirmed by qRT-PCR

		Fold-Change*		
Gene	Product	0.05	0.5	1.0
ebpA	von Willebrand factor	7.8	30.0	21.1
ebpB	Cell wall surface protein	14.0	30.0	19.7
ebpC	Cell wall surface protein	14.0	27.9	19.7
bps	Sortase	2.6	2.8	2.0
arcA	Arginine deiminase	30.0	274.4	9.8
arcB	Ornithine carbamoyltransferase	24.3	181.0	13.9
arcC	Carbamate kinase	8.0	73.5	19.7
arcR	Transcriptional regulator Crp/Fnr	4.3	64.0	18.4

* Change in DBS01 gene expression (DBS01 : E99) at OD600 = 0.05, 0.5 and 1.0

Figure legends

Figure 1. Growth curves for E99 (A) and the Δ*perA* derivative DBS01 (B). Arrows indicate sampling times for microarray analysis and correspond to mid-exponential, late-exponential, and stationary phases (OD_{600} of 0.05, 0.5 and 1.0, respectively). OD_{600}, optical density at 600 nm.

Figure 2. Comparisons of microarray results for E99 and DBS01. Control RNA was extracted from E99 and used to normalize the test RNA extracted from DBS01 (DBS01 : E99). All data presented here shown as fold change in gene expression (test : control). (A) Upper diagram: Venn diagram comparing significantly up-regulated genes (> 2 fold) in DBS01 during mid-exponential, late-exponential and stationary phase. Lower diagram: Venn diagram comparing significantly down-regulated genes (> 2 fold) in DBS01 during mid-exponential, late-exponential, and stationary phase. (B) Hierarchically-clustered heat map of all genes differentially regulated > twofold between DBS01 and E99.

Figure 3. All genes differently regulated in DBS01 mapped onto the *E. faecalis* chromosome. The outer ring displays those genes differentially regulated during mid-exponential phase. The middle ring displays those genes differentially regulated during late-exponential phase. The inner ring displays those genes differentially regulated during stationary phase. The innermost circle displays the location relative to position zero in millions of base pairs of the *E. faecalis* V583 genome. The locations of the

arginine deiminase (ADI) and enterococcal biofilm-associated pilus (Ebp) operons, the *E. faecalis* pathogenicity island (PAI), and a phage related element are indicated.

Figure 4. Plots comparing the log_2 expression ratios of the arginine deiminase (ADI) and enterococcal biofilm associated pilus (Ebp) operons in DBS01.

Figure 5. *perA, ebpR-ebpABC* and *bps* gene expression in E99 grown in THB supplemented with 100 mM sodium bicarbonate (dark bars) or DBS01 grown in THB (light bars). The values shown are mean expression intensities (mean ± SD).

Figure 6. Map of *E. faecalis* V583 phage 04. The putative proteins were compiled using the annotated V583 sequence. The direction of transcription is shown in blue (reverse) and red (forward). Heat maps of expression ratios (fold change) for DBS01 are shown for mid-exponential (O.D. 600 = 0.05), late-exponential (O.D. 600 = 0.5) and stationary phase (O.D. 600 = 1.0).

Figure 7. Platelet binding activity of E99 and DBS01. The values shown are percent of wild-type (E99) binding (mean ± SD). Asterisks indicate binding activities that were significantly different than E99 (P = 0.05). Platelet binding assays were performed in triplicate and each experiment was repeated twice (n = 6).

Figure 1.

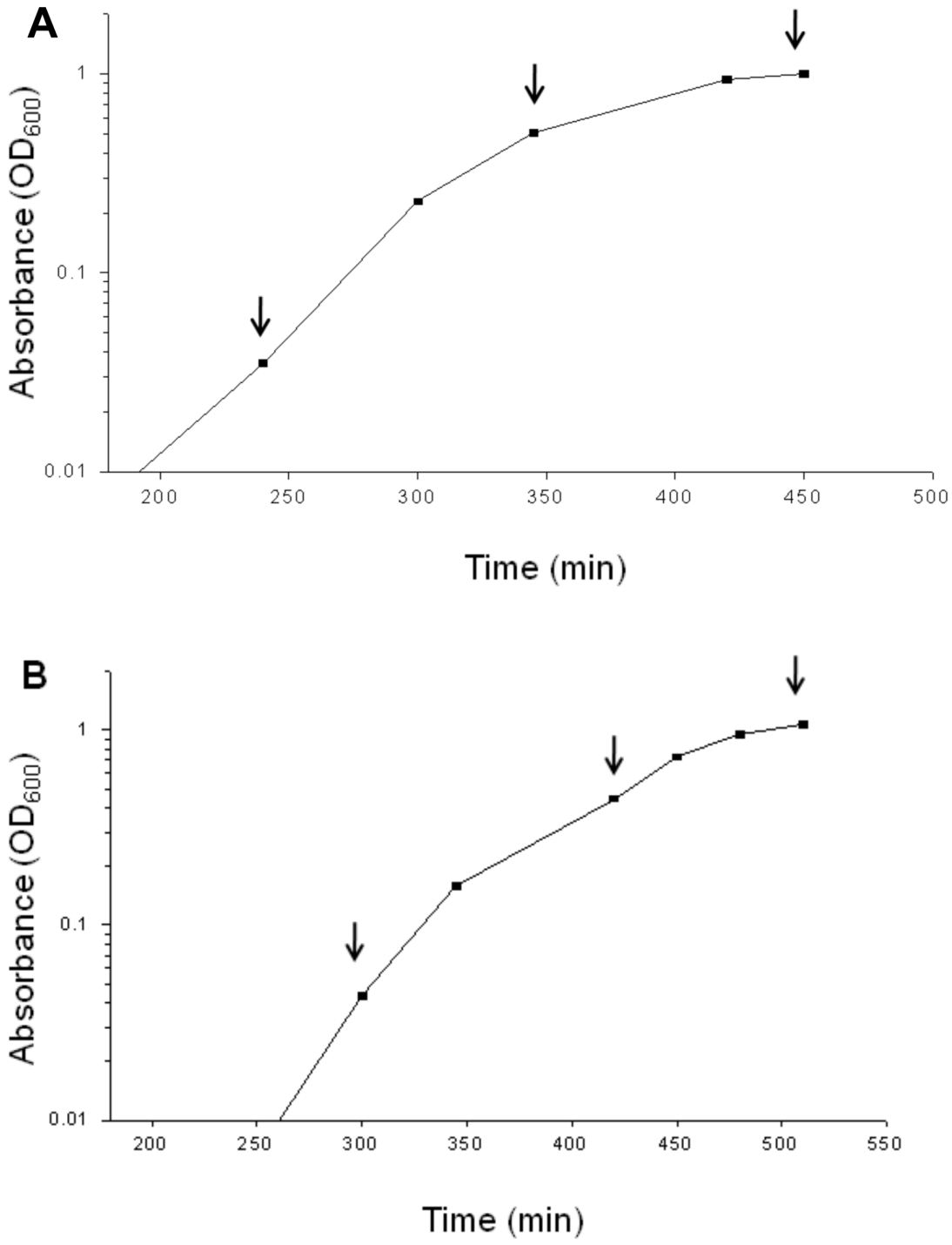

A
Absorbance (OD600)
1
0.1
0.01
200
250
300
350
400
450
500
Time (min)
B
Absorbance (OD600)
1
0.1
0.01
200
250
300
350
400
450
500
550
Time (min)

Figure 2.

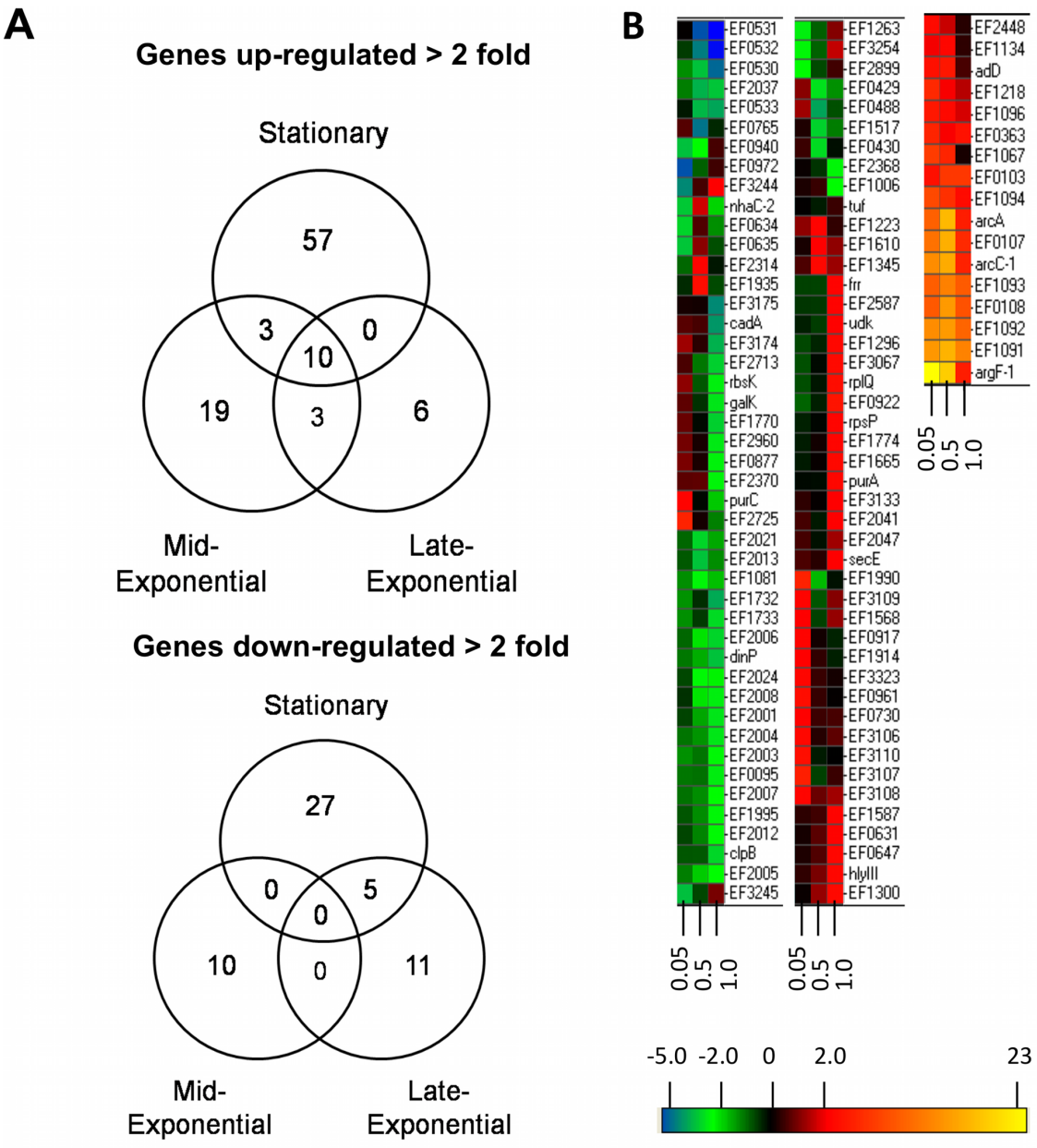

A
Genes up-regulated > 2 fold
Stationary
57
3
0
10
19
3
6
Mid-
Exponential
Late-
Exponential
Genes down-regulated > 2 fold
Stationary
27
0
5
0
10
0
11
Mid-
Exponential
Late-
Exponential
B
EF0531
EF0532
EF0530
EF2037
EF0533
EF0765
EF0940
EF0972
EF3244
nhaC-2
EF0634
EF0635
EF2314
EF1935
EF3175
cadA
EF3174
EF2713
rbsK
galK
EF1770
EF2960
EF0877
EF2370
purC
EF2725
EF2021
EF2013
EF1081
EF1732
EF1733
EF2006
dinP
EF2024
EF2008
EF2001
EF2004
EF2003
EF0095
EF2007
EF1995
EF2012
clpB
EF2005
EF3245
EF1263
EF3254
EF2899
EF0429
EF0488
EF1517
EF0430
EF2368
EF1006
tuf
EF1223
EF1610
EF1345
frr
EF2587
udk
EF1296
EF3067
rplQ
EF0922
rpsP
EF1774
EF1665
purA
EF3133
EF2041
EF2047
secE
EF1990
EF3109
EF1568
EF0917
EF1914
EF3323
EF0961
EF0730
EF3106
EF3110
EF3107
EF3108
EF1587
EF0631
EF0647
hlyIII
EF1300
EF2448
EF1134
adD
EF1218
EF1096
EF0363
EF1067
EF0103
EF1094
arcA
EF0107
arcC-1
EF1093
EF0108
EF1092
EF1091
argF-1
0.05
0.5
1.0
-5.0 -2.0 0 2.0 23

Figure 3.

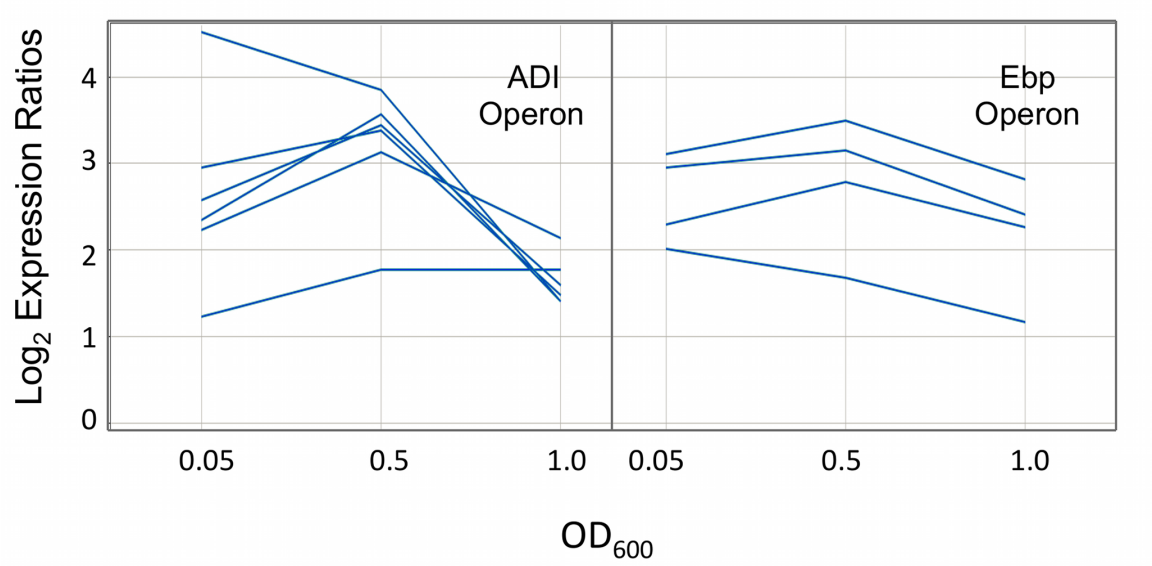

Log2 Expression Ratios
4
3
2
1
0
ADI
Operon
Ebp
Operon
0.05
0.5
1.0
0.05
0.5
1.0
OD600

Figure 4.

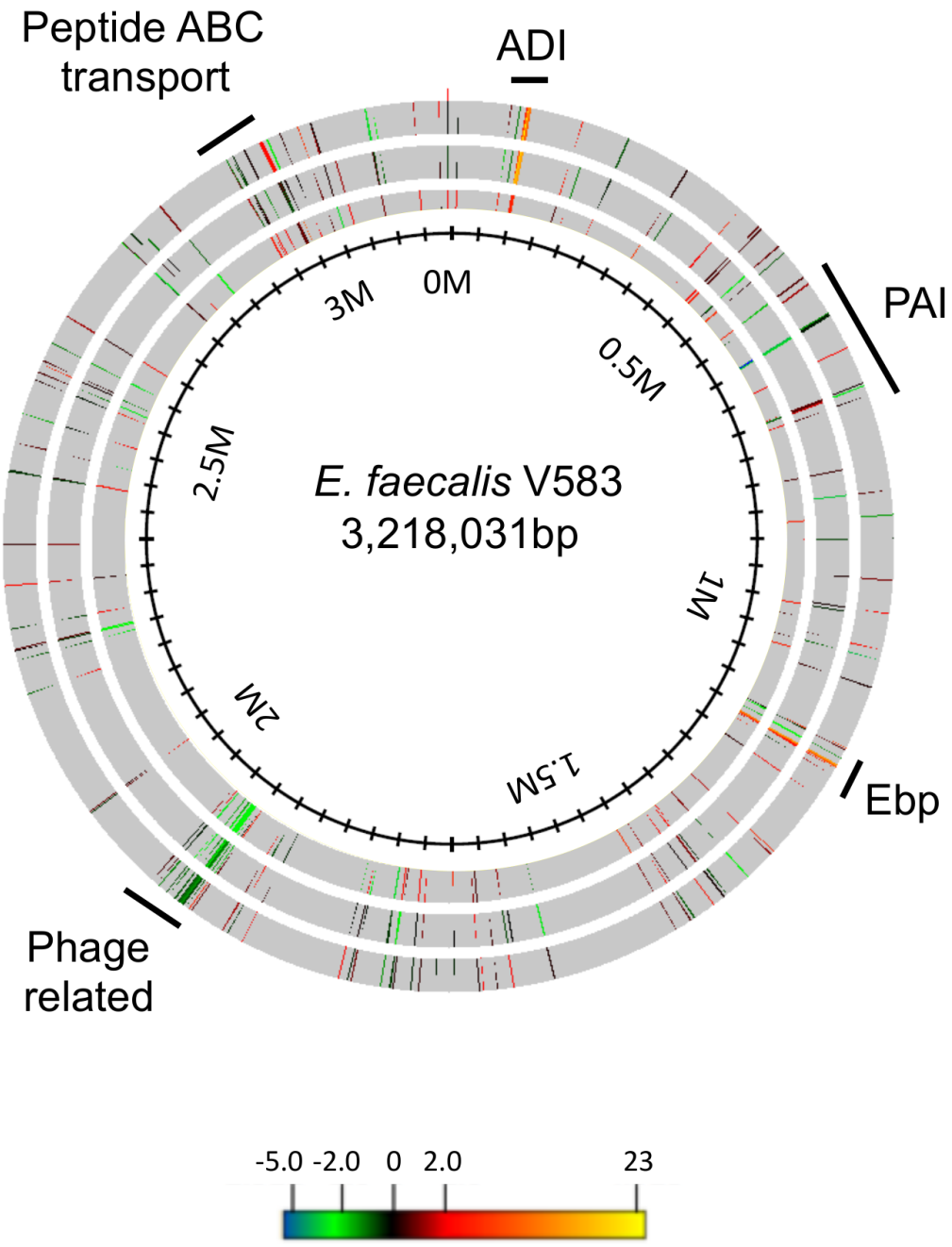

Peptide ABC transport
ADI
PAI
Ebp
Phage related
0M
0.5M
1M
1.5M
2M
2.5M
3M
E. faecalis V583
3,218,031bp
-5.0 -2.0 0 2.0 23

Figure 5.

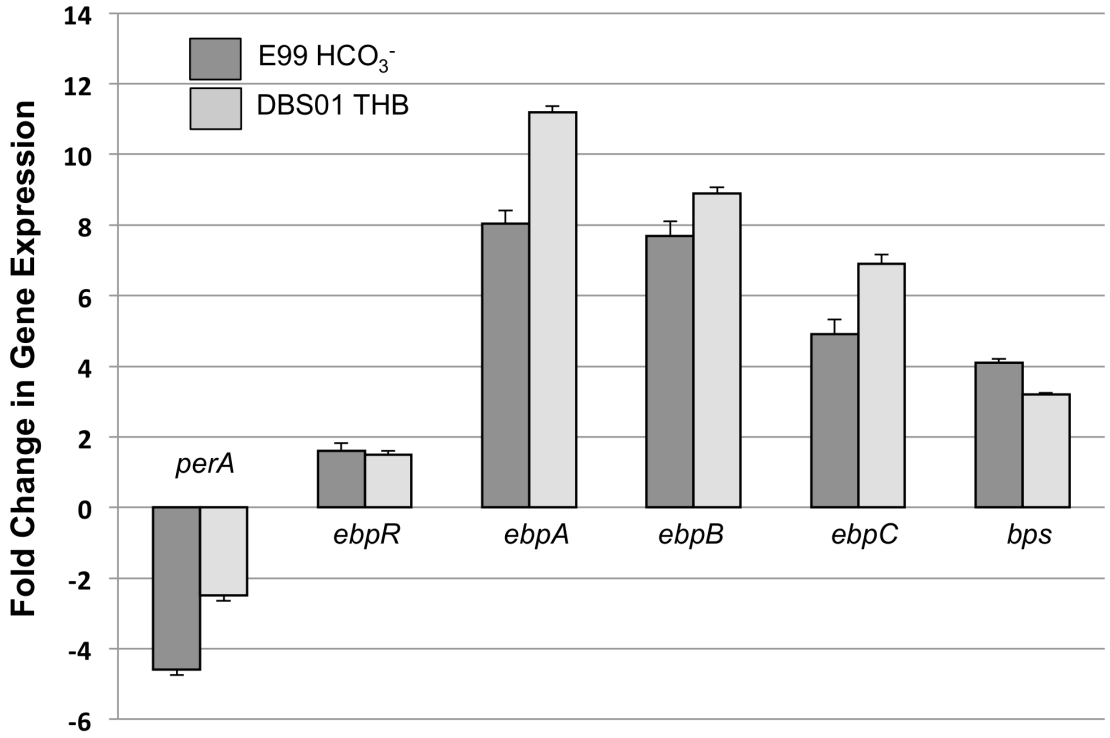

Fold Change in Gene Expression
14
12
10
8
6
4
2
0
-2
-4
-6
E99 HCO3-
DBS01 THB
perA
ebpR
ebpA
ebpB
ebpC
bps

Figure 6.

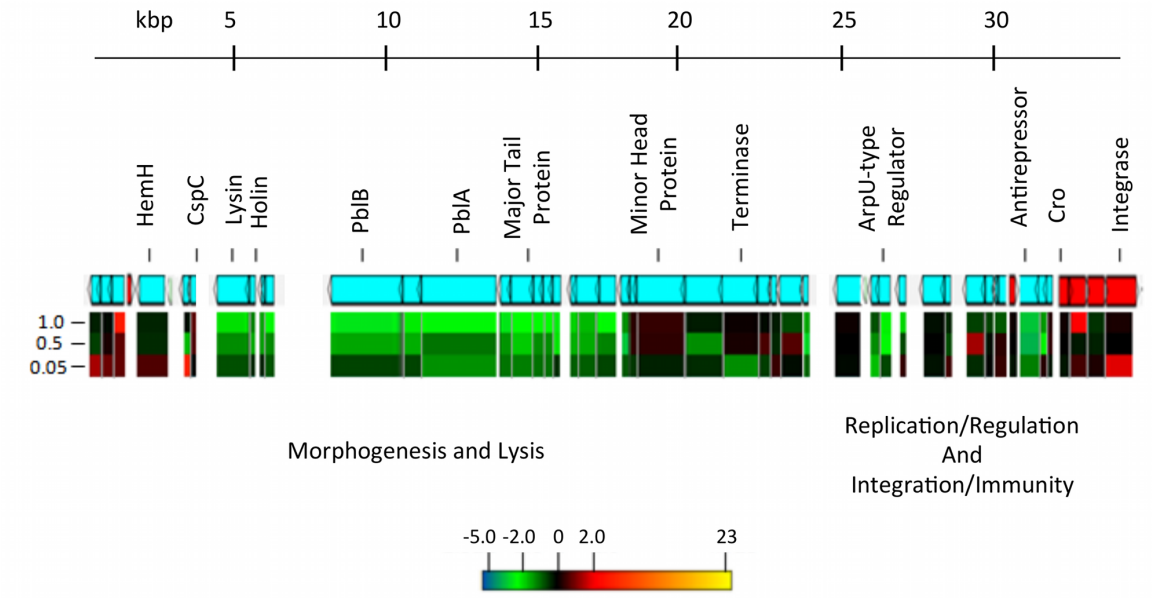
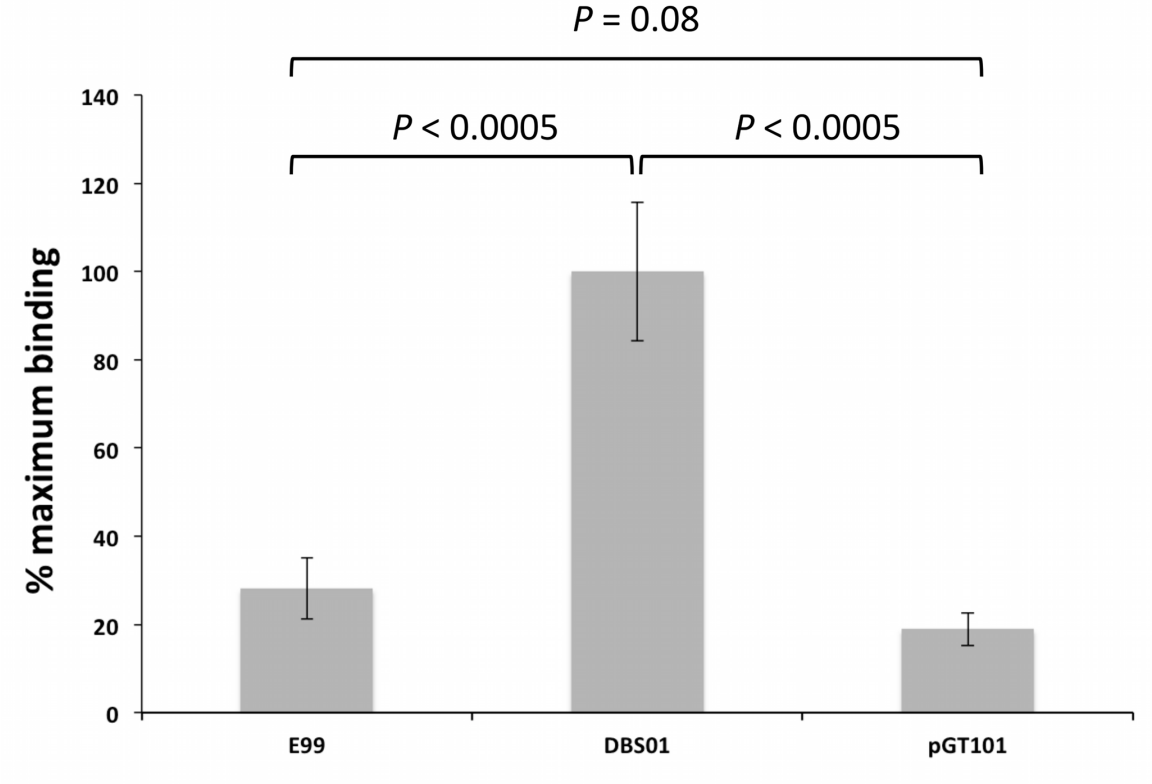

kbp
5
10
15
20
25
30
HemH
CspC
Lysin
Holin
PblB
PblA
Major Tail Protein
Minor Head Protein
Terminase
ArpU-type Regulator
Antirepressor
Cro
Integrase
1.0
0.5
0.05
Morphogenesis and Lysis
Replication/Regulation And Integration/Immunity
-5.0 -2.0 0 2.0 23

Figure 7.


P = 0.08
P < 0.0005
P < 0.0005
% maximum binding
140
120
100
80
60
40
20
0
E99
DBS01
pGT101

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Chapter 4: Conclusions and Outstanding Questions

The experiments described in this thesis have detailed the *E. faecalis* V583 intracellular transcriptome and identified genes regulated by the AraC-type transcriptional regulator, PerA. In this final chapter, the main points from of the previous chapters are reviewed and general trends discussed. Additionally, outstanding questions that warrant future study are addressed.

Chapter two summary. The experiments presented in Chapter two examined the response of *E. faecalis* V583 to the intracellular environment within a macrophage. By interrogating the bacterial transcriptome during macrophage survival we are able to determine not only the *E. faecalis*-containing phagosomal environment but also the genetic response to such harsh conditions. We found that a macrophage infected with *E. faecalis* is limited for substrates required for growth, including sources of carbon, amino acids and nucleotides. These conditions were sufficient to elicit the stringent response in V583, which resulted in the down-regulation of genes comprising the transcriptional and translational apparatus. Furthermore, we found the phagosome to be devoid of copper, phosphate, iron and potassium. The macrophage produced a robust oxidative burst following phagocytosis, as was evidenced by the induction of *E. faecalis* oxidative stress response genes. The induction of the bacterial SOS and heat-shock stress responses suggest that cellular components were damaged. These findings provide a detailed analysis of the intracellular environment and the bacterial systems used during survival within macrophages.

Chapter three summary. The experiments presented in Chapter three determined the PerA regulon. PerA resides on a mobile PAI yet the overwhelming majority of PerA-regulated genes are chromosomally located (not on the PAI). PerA coordinately regulates genes involved in metabolism, amino acid degradation, biofilm formation and platelet binding. Using an *in vitro* platelet-binding assay, we showed that PerA mediates the binding of *E. faecalis* to human platelets. Finally, we show that PerA responds to bicarbonate, an intestinal ion frequently used by pathogens to determine the site of infection. These results indicate that PerA is a global transcriptional regulator that coordinately regulates genes responsible for enterococcal pathogenicity. Given the ability of the *E. faecalis* PAI to undergo horizontal transfer into recipients that lack the PAI, this likely represents a novel strategy for coordinate regulation of genes in the core genome by a PAI-encoded regulator.

Synthesis and Outstanding questions. When analyzing the *E. faecalis* intracellular transcriptome, it was my goal to describe the V583 response to phagocytosis in light of what is known about other intracellular pathogens (specifically *L. monocytogenes*, *S. typhimurium* and *M. tuberculosis*). In each comparison similarities and differences were observed. For example, *E. faecalis* and *S. typhimurium* both experience oxidative stress within the macrophage and repair oxidized protein using homologous systems (Chapter 3, Fig. 3) (6). Furthermore, each induce members of the bacterial SOS response, suggesting that cellular components were damaged during intracellular survival (Chapter 3, Fig. 3) (6). However, *S. typhimurium* replicates within the SCV using energy obtained through the metabolism of carbohydrates via the Entner-Doudoroff pathway (6), while V583 does not appear to replicate or metabolize within the macrophage (Chapter 2, Fig. 3) (9). In

contrast to *S. typhimurium* and *M. tuberculosis* (intracellular pathogens that persist within modified phagosomes), *L. monocytogenes* produces a lysin to escape the phagsome and enter into the macrophage cytoplasm (8, 13). Here, the bacteria find conditions suitable for replication and eventually commandeer host proteins to facilitate cell-to-cell spread (4, 16). Unfortunately, the amount of information known about the *E. faecalis* intracellular survival strategy trails that of other intracellular pathogens. While it is clear that the *E. faecalis*-containing phagosome fuses with lysosomes (1), we do not know if this fusion is delayed, as occurs in macrophages infected with *M. tuberculosis* (14). Also, *E. faecalis* induces a gene encoding a hemolysin (Chapter 3, Fig. 3) and is able to escape the phagosome and enter the cytoplasm (much like *L. monocytogenes*) (9). Yet unlike *L. monocytogenes*, *E. faecalis* is apparently unable to replicate within the macrophage (9). Why not?

The enterococci are fastidious bacteria that require relatively rich growth conditions; in fact, there is currently no chemically defined minimal medium that supports growth of *E. faecalis*. Furthermore, like most lactic acid bacteria, *E. faecalis* is auxotrophic for many amino acids and, therefore, must obtain them from the surrounding environment. Previous studies have determined that macrophage infected with other intracellular pathogens vary in regards to carbohydrate and amino acid availability (2, 5, 15). The data presented in this thesis (specifically in Chapter 2) suggest that the macrophage infected with *E. faecalis* is devoid of carbohydrates and amino acids. Whether the lack of enterococcal replication during intracellular survival can be directly attributed to the lack of carbon and amino acids remains to be studied. However, the data in Chapter 2 suggest 1) that the macrophage is limited for key compounds required for

growth of *E. faecalis,* and 2) the intracellular environment varies depending upon the pathogen phagocytosed. I'd like to think that Dirk Schnappinger was right when he stated, "There is no one phagosome. Phagosomes differ depending on the cell in which they arise, their point along the developmental cycle of the organelle, and the nature of the microbe resident within them" (15).

On the other hand, perhaps *E. faecalis* does not need to replicate within the macrophage to cause disease, unlike other intracellular pathogens. The genes expressed during intracellular survival, combined with intrinsic survivability in harsh conditions, may permit *E. faecalis* to remain quiescent in the macrophage. Thus, this opportunistic pathogen may simply persist in the macrophage, thereby using an immune cell as a vehicle for translocation to extra-intestinal sites.

I have often been told that in science the quest to answer one question often leads to many others. That was certainly the case in the experiments highlighted in Chapter 3. It was our goal to define the PerA regulon, which led to the interesting discovery that this PAI-encoded transcriptional regulator primarily influences the expression of genes not located on the PAI. Also surprising were the findings that PerA contributes to platelet binding and responds to bicarbonate. Despite this advance in the understanding of coordinate regulation in *E. faecalis*, many unanswered questions remain. We currently do not know the set of genes directly regulated by PerA. Undoubtedly the PerA regulon defined in Chapter 3 is comprised of genes that are both directly and indirectly PerA-regulated. Knowing the set of genes directly regulated by PerA would allow us to determine a consensus PerA-binding motif. With this information we could then mine

the *E. faecalis* genome for potential PerA-regulated genes that did not respond in our growth conditions.

Coburn et al. showed that PerA contributes to the survival of *E. faecalis* within a macrophage (3). The experiments outlined in Chapter 3 defined the PerA regulon. However, from our data we are unable to determine which PerA-regulated genes are important for intracellular survival as the growth conditions used in this study in no way mimic the intracellular environment. Obtaining the intracellular transcriptome of E99 or DBS01 (Δ*perA*) would provide clues as to which PerA-regulated genes are important for survival in the macrophage. We are keen to realize that the numerous PerA-regulated genes of unknown function might contribute to intracellular survival, yet this possibility remains to be studied.

Although PerA is clearly important for the survival of *E. faecalis* E99 in the macrophage (3), many strains lacking the *perA* gene persist during intracellular survival (9). There are many potential explanations for this observation; a few are discussed below. PerA is an AraC-type protein and as such it is one of the most common transcriptional regulators found in bacteria (7). In fact, the V583 genome contains 9 AraC-type regulators (12). It is possible that other AraC-type regulators function in place of PerA in *E. faecalis* strains lacking this protein. For this to occur it would be necessary for the DNA-binding domains to be very similar. Fortunately, the N-terminal DNA-binding domains are highly conserved amongst AraC-type regulators (7). Another possibility is that other *E. faecalis* strains capable of intracellular survival possess loci not found in E99. These loci might contribute to persistence within the macrophage regardless of the presence of PerA.

E. faecalis strains lacking PerA have a decreased bacterial load in the liver and spleen (3). Though it is possible the decreased bacterial load of DBS01 in the liver and spleen is due to the attenuated survival of this strain in the macrophage, it is also possible PerA-regulated genes are critical for infection of these tissues. It is not known if *perA* is expressed in the liver or spleen, however obtaining this information would provide clues as to the role of PerA and its regulon in these sites.

The data presented in Chapter 3 (specifically those regarding the response of PerA in the presence of bicarbonate) suggest PerA does not directly detect bicarbonate, but rather is influenced by other regulators. Additionally, AraC-type regulators are capable of directly sensing environmental stimuli, however these proteins are also known to regulate transcription without directly detecting a ligand (10, 11). *per*A is down-regulated in bicarbonate concomitant with the induction of members of the PerA regulon (Chapter 3, Fig. 5). We reason that if PerA were auto-regulating its own expression, the *perA* transcript would increase in abundance under PerA de-repression conditions. We observed the opposite. Furthermore, the PerA C-terminus contains no known bicarbonate-binding domains (data not shown), further suggesting another cellular component senses ion concentration then influences *perA* transcription. Defining the position of PerA within the *E. faecalis* E99 regulatory network would not only increase our understanding of transcriptional regulation in this pathogen, but also permit the search of regulators with similar functions in other strains.

Clearly many questions remain. However, I hope the data presented here advances the understanding of enterococcal pathogenicity. More broadly, it is my hope

that the information provided here can be used to further delineate commensal vs. pathogen relationships.

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