IDENTIFICATION AND CHARACTERIZATION OF NOVEL GENETIC DETERMINANTS OF BIOFILM FORMATION IN *ENTEROCOCCUS FAECALIS*

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"I get by with a little help from my friends…" -John Lennon and Sir Paul McCartney

John Lennon and Paul McCartney were not writing about graduate school when they wrote "With a little help from my friends" for the Sergeant Pepper album. But it sums up exactly how I feel about my time in graduate school. I was tremendously fortunate to receive help, support and, encouragement from all sorts of friends and family throughout the odyssey of my graduate career. I shared both despair and triumph with a great group of people, and consider myself lucky. Throughout my PhD training, these experiences have defined and solidified me, both as a scientist and as a person. For my friends and for their support, I will always be grateful.

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DEDICATION

This dissertation is dedicated to

Blanche Geraldine Ballering

I carry your memory in my heart and hope that I made you proud

ABSTRACT

The nosocomial pathogen *Enterococcus faecalis* is a normal resident of the intestinal tracts of many vertebrates and invertebrates, including humans, and is readily isolated from many other environments. It has been well documented that *E. faecalis* can form biofilms on biotic and abiotic surfaces, and enterococcal biofilms likely play a role in virulence, persistence, and horizontal gene transfer of this organism. In my thesis research I used a large genetic screen to identify over 68 potential determinants of enterococcal biofilm formation. This, in conjunction with another genetic screen from our lab, constitute the first comprehensive examination of the core genome of *E. faecalis* for genetic determinants of biofilm formation.

I characterized the role in biofilm formation of a novel transcription factor, Enterococcal Biofilm Regulator (EbrA), identified in a Recombinase Based In Vivo Expression Techonology (RIVET) screen. This transcription factor was differentially expressed between biofilm and planktonic cells at 24 hours, and a mutant strain in which the open reading frame was deleted was defective in its ability to form biofilms as compared to wild-type. To determine the regulon of EbrA, I utilized methods that examined the proteome and the transcriptome and determined that EbrA is responsible for modulating the metabolic rate to promote survival of the biofilm population when it undergoes nutrient stress. As a function of this survival role, my experiments suggest that EbrA is also a negative regulator of the cell lysis mechanism, that is thought to mediate eDNA release for biofilm matrix production. The current model of eDNA release did not include a negative regulator.

Biofilm development is a dynamic process in which the population undergoes changes in both gene expression and metabolic activity. Previous characterizations have focused mostly on biofilms grown for at least 24 h. There is little information about the cellular processes associated with this transition from planktonic to biofilm growth in non-motile species, such as *E. faecalis*. The low amounts of biomass present in these initial stages of biofilm development preclude the use of standard expression profiling analyses of mRNA and protein. My thesis research also describes comparative analysis of the increase in biomass and adherent bacterial populations in the early stages of biofilm formation by the laboratory strain in relation to several clinical isolates. I then combined this analysis with high-resolution Field Emission Scanning Electron Microscopy (FESEM) analysis of the cell surface and the extracellular matrix of the developing biofilms. These studies revealed a dramatic temporal change in the appearance and biochemical composition of the extracellular matrix that has not been previously reported. The data also suggest that the biochemical composition of the biofilm matrix changes over time. One of those phenotypes has not been previously described. This work highlights the importance of careful kinetics studies, including very early time points, to identify novel phenotypes in complex biofilm growth systems.

FORWARD

This thesis is broken into three data chapters and an appendix: The kinetics of biofilm matrix formation in *Enterococcus faecalis*, Functional genomics of *Enterococcus faecalis*: multiple novel genetic determinants for biofilm formation in the core genome, Characterization of EbrA: a negative regulator of carbon metabolism and eDNA release in Biofilms and Proteome analysis of Enterococcal biofilms.

The first data chapter is based on a manuscript that will be submited for publication: Ballering, K.S., Dong, S., Wells, C., Henry-Stanley, M. Hallgren, A. and G.M. Dunny. Kinetic changes in biofilm matrix material in *Enterococcus faecalis*. All of the FESEM images were taken by Shen Dong. The lectin staining experiments were done with the help of Dr. Carol Wells and Dr. Michelle Henry-Stanley on biofilm cells that I grew.

The second data chapter is based on a paper that I published in the Journal of Bacteriology and reprinted here, with their permission, and with some additions: Ballering, K.S., C.J. Kristich, S.M. Grindle, A. Oromendia, D.T. Beattie, and G.M. Dunny. (2009) Functional genomics of *Enterococcus faecalis*: multiple novel genetic determinants for biofilm formation in the core genome. J. Bacteriol. 191:2806-2814. Copyright 2009. The American Society of Microbiology Press. The initial cloning of the RIVET strains was completed by Dr. David Beattie and Dr. Christopher Kristich. Anna Oromendia was responsible for the 2 hour RIVET screen results under my supervision. Suzanne Grindle helped with the bioinformatic analysis of the RIVET screen sequencing results.

The third data chapter is based on a manuscript that I will submit for publication soon: Ballering, K.S. and G.M. Dunny. Characterization of EbrA: a nutrient sensor in enterococcal biofilms and a negative regulator of eDNA release in *Enterococcus faecalis*. The iTRAQ experiments were completed at the Center for Mass Spectrometry at the University of Minnesota with helpful suggestions from Dr. LeeAnn Higgins and Dr. Lori Anderson. I would also like to thank Archana Deshpande of the University of Minnesota Microarray facility for her help with the microarray experiments. I also received helpful suggestions from Dr. Heather Haemig regarding the protein experiments and am indebted to Dr. Ron Jemmerson and Dr. Pat Schlievert for their help with the antibody generation and purification.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS……………………………………………………………………..i

DEDICATION……………………………………………………………………………v

ABSTRACT…………………………………………………………………………...vi

FORWARD…………………………………………………………………………..viii

TABLE OF CONTENTS……………………………………………………………….x

LIST OF TABLES……………………………………………………………………xii

LIST OF FIGURES…………………………………………………………………..xiii

CHAPTER 1:

Introduction………………………………………………………………………………..1

CHAPTER 2:

The kinetics of biofilm matrix formation in *Enterococcus faecalis*……………………..12

Introduction………………………………………………………………………………13

Materials and Methods……………………………………………………………………...14

Results……………………………………………………………………………………18

Discussion………………………………………………………………………………..35

CHAPTER 3:

Functional genomics of *Enterococcus faecalis*: multiple novel genetic determinants for biofilm formation in the core genome……………………………………………………...41

Introduction………………………………………………………………………………42

Materials and Methods……………………………………………………………………...45

Results……………………………………………………………………………………51

Discussion………………………………………………………………………………..68

CHAPTER 4:

Characterization of EbrA: a negative regulator of carbon metabolism and eDNA release in Biofilms……………………………………………………………………………73

Introduction……………………………………………………………………………74

Materials and Methods…………………………………………………………………...75

Results……………………………………………………………………………………81

Discussion………………………………………………………………………………..96

CHAPTER 5:

Conclusion ……………………………………………………………………………..102

Conclusion……………………………………………………………………………...103

Future Directions……………………………………………………………………….109

REFERENCES………………………………………………………………………….114

APPENDIX I:

Proteome analysis of Enterococcal biofilms……………………………………………128

APPENDIX II:

Development of biochemical tools to examine the direct effect of EbrA binding…………………………………………………………………………………133

LIST OF TABLES

Table 1. Genes known to be involved in *E. faecalis* biofilm formation………………….7

Table 2. Primers used to create mutants used in this study…………………………...46

Table 3. Genetic determinants of biofilm formation identified in RIVET screen………55

Table 4. Non-sibling clones isolated more than twice…………………………………...59

Table 5. Predicted DNA binding proteins identified in RIVET screen…………………67

Table 6. RIVET clones that do not follow convention…………………………………70

Table 7. iTRAQ-identified proteins differentially expressed between Δ*ebrA* biofilms and OG1RF biofilms……………………………………………………………………82

Table 8. Transcriptional differences in gene expression………………………………...91

Table 9. Proteomic analysis of biofilm and planktonic cells…………………………...129

Table 10. Conditions used for optimal protein over-expression………………………134

Table 11. Oligonucleotides used in EMSA……………………………………………..137

LIST OF FIGURES

Figure 1. The stages of biofilm formation……………………………………………..4
Figure 2. A description of the advantages of RIVET to investigate biofilm formation…………………………………………………………………………………10
Figure 3. Comparison of biofilm formation abilities of clinical isolates of *E. faecalis*………………………………………………………………………………...20
Figure 4. Early biofilm growth kinetics in *E. faecalis*…………………………………...21
Figure 5. Late biofilm growth kinetics in *E. faecalis*……………………………………23
Figure 6. Analysis by FESEM of early biofilm growth kinetics of *E. faecalis* OG1RF and VA1128 on cellulose membranes………………………………………………………..24
Figure 7. Close up images of the two matrix phenotypes………………………………..27
Figure 8. Analysis by FESEM of 24 hour biofilm growth of *E. faecalis* OG1RF on cellulose membranes……………………………………………………………………..30
Figure 9. Lectin and eDNA staining of *E. faecalis* biofilms shows differing phenotypes for different matrix materials……………………………………………………………31
Figure 10. Four and Twenty-four hour concanavalin A stained OG1RF biofilms………32
Figure 11. qPCR analysis of *epa* locus gene expression in OG1RF Biofilms……………………………………………………………………………….34
Figure 12. RIVET Plasmid and Strains…………………………………………………48
Figure 13. Genome Distribution of Determinants Identified in RIVET Screen………..54
Figure 14. Confirmation of Differential Expression of RIVET-Identified Genes During Biofilm Growth…………………………………………………………………………61
Figure 15. Biofilm Defects of *E. faecalis* Strains carrying Null Mutations in RIVET-identified Genes…………………………………………………………………………64
Figure 16. RIVET and Transposon Screens for Biofilm Determinants Identify Unique, but Linked Genes……………………………………………………………………..65
Figure 17. Exogenously added glucose rescues Δ*ebrA* growth defect…………………84
Figure 18. High levels of exogenous carbon rescue Δ*ebrA* biofilm growth defect……………………………………………………………………………………86

Figure 19. Planktonic growth of Δ*ebrA* is equivalent to OG1RF in M9-YE medium supplemented with three different carbon sources……………………………………….88
Figure 20. qPCR analysis of *gelE* and *sprE* expression levels in biofilms of Δ*ebrA* vs. OG1RF……………………………………………………………………………...94
Figure 21. *ebrA* and *gelE* kinetic expression patterns are inverse to each other………..95
Figure 22. New model for EbrA function……………………………………………100
Figure 23. Purification and testing the polyclonal EbrA Antibody……………………140

CHAPTER 1:

INTRODUCTION

Enterococcus faecalis is a Gram-positive commensal organism of the gastrointestinal (GI) tract of many organisms, including invertebrates and humans. The enterococci comprise a significant proportion of the GI flora of healthy humans reaching easily cultural levels in feces (46). In addition to the GI, tract enterococci are present in the oral cavity and female genital tract. The enterococci are extremely hardy and can withstand levels of cell envelope stress induced by bile and salt, as well as temperature extremes that would kill most other non-spore forming bacteria. In addition the enterococci are intrinsically resistant to several antibiotics and can acquire new mechanisms of antibiotic resistance, including resistance to "antimicrobials of last resort" such as vancomycin and teicoplanin. This occurs through the exchange of mobile genetic elements such as plasmids, transposons and pathogenicity islands (PAI). In turn, the enterococci are adept at transferring these mobile genetic elements to other previously susceptible enterococci and, more worrisome, to other pathogens such as *Staphylococcus aureus* (80) potentially when growing in multispecies biofilms.

E. faecalis because of its hardy nature and high levels of antibiotic resistance has emerged recently as an important nosocomial pathogen, the second most common in the United States (81). *E. faecalis* is known to infect the urinary tract, bloodstream, heart valves and surgical sites (67). Implanted medical devices provide another niche for enterococcal growth and *E. faecalis* has increasingly been associated with infections from catheters, stents and prostheses. The results from 10 studies from different laboratories examining the biofilm formation abilities of clinical isolates of *E. faecalis* have shown that, on average, 79% of clinical *E. faecalis* isolates are able to form biofilms when tested

in vitro (63). These results strongly implicate biofilm formation as a virulence factor for *E. faecalis* pathogenesis.

Biofilms are comprised of a population of microbial cells that are attached to a surface or each other at an interface (i.e. air/liquid) and are encased in a hydrated extracellular matrix composed of some combination of proteins, polysaccharides or nucleic acids. The biofilm phenotype is different than that of planktonic (free floating, single cell) cells of the same species (19). Growth as a biofilm offers many advantages to the bacteria that reside inside the biofilm. These advantages include, but are not limited to; antibiotic resistance of up to 10-1000 fold that of planktonic cells and resistance to phagocytosis, both of which allow cells growing in a biofilm to thrive inside what is often a hostile environment inside of a host (56). Biofilms are thought now to be involved in over 80% of microbial infections and this is likely due to the previously described traits and advantages of biofilm growth (56).

Microbial biofilms develop through a set of highly regulated, complex steps. First the cells must interact with the surface and attach, form microcolonies, and then form confluent biofilms that are highly ordered and often comprise a recognizable 3-dimensional architecture (71) this process is demonstrated for a generic organism in figure 1. Examples of the different stages of growth have been well elucidated throughout the bacterial biofilm literature. For example the steps needed to transition from reversible to irreversible attachment (steps 1 and 2 from figure 1) bacteria must express adhesins on their surface. This increases the individual cell's ability to attach to the surface as well as to other cells. In the case of staphylococcal species, Bap (39) is one of the adhesins important for biofilm attachment to surfaces and other cells. In the

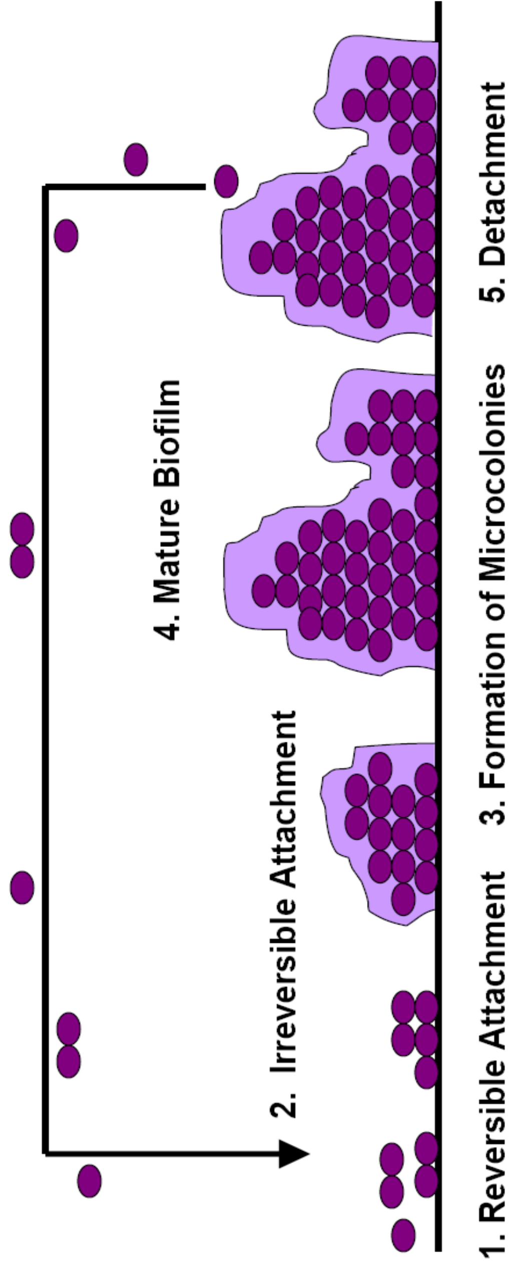


Figure 1. The stages of biofilm formation. This diagram shows the stages of biofilm growth on a generic surface.

case of the enterococci, it has been shown that a biofilm specific pilus is required for initial and irreversible attachment and subsequent biofilm formation (12, 53). In motile species like *Bacillus subtilis,* motility often needs to be "lost" or turned off in order to allow for attachment (13). One of the hallmarks that separate biofilm cells from non-biofilm cells is their ability to produce and extracellular matrix material, which can be made of polysaccharide, protein or nucleic acids. This is thought to be required, perhaps to help solidify cell-cell adhesion, for formation of microcolonies (step 3 of Figure 1). One of the best studied components of bacterial extracellular matrix is the polysaccharide alginate of *Pseudomonas aeruginosa* (21). In step 4 of figure 1 the complex structure of the biofilm is formed. Most global studies of the phenotypic differences between biofilm and planktonic cells have been done on mature biofilms (7, 8, 22, 33, 62, 79, 85, 86, 102) demonstrating that mature biofilms are phenotypically different from planktonic cells of the same age. The fifth and last stage of biofilm formation is the dispersal stage (step 5 of Figure 1). It is as this stage that some cells are sloughed off of the biofilm and are released out into the environment (90). This process is highly regulated in *S. aureus* through heterogeneous expression of the Agr quorum sensing system (104).

It is important to understand the biology and genetics of this biofilm forming nosocomial pathogen, *E. faecalis*, in the hopes that through understanding of the biological processes at work we will be able to develop targeted therapeutics that would target the biofilm form of the enterococci to ameliorate the morbidity and mortality associated with biofilm infections with this organism.

Most of the initial experiments examining genetic determinants of biofilm formation in *E. faecalis* relied heavily on comparisons of biofilm forming and non-

biofilm forming clinical isolates. These studies looked for genetic differences between the strains that might account for their differences in biofilm formation abilities. In addition other researchers looked for enterococcal genes with homology to known biofilm required genes in other organisms like *S. aureus*. These approaches identified a number of genes that were shown to be involved in biofilm formation (table 1). However the complicating affects of differences in strain composition, growth media and experimental methods soon became apparent when trying to apply these results broadly to all/most enterococci.

Due to strain and experimental differences some of the early literature was hard to compare and often in what appeared to report conflicting results. For example, initial reports indicated that *esp*, a gene located in a PAI, was present in 93.5% of clinical isolates that were able to form biofilms and all of the biofilm-negative isolates in that study did not contain the *esp* gene (98). However later experiments done in our lab with OG1RF, which does not contain *esp* or the PAI that it resides in, show that this strain forms robust biofilms without the need for *esp* (51). Another study with clinical isolates showed that 77/89 *esp*-negative isolates were able to form biofilms (64). In addition to the confusion with *esp*, there has been confusion over the role of nutrients in growth of this organism. One study showed that the addition of 1% glucose to Trypticase Soy Broth without dextrose growth medium (TSB) enhanced biofilm formation. In contrast work in our lab showed that increasing the glucose concentration to 0.2% from baseline amounts (again in TSB) decreased the amount of biofilm produced

Table 1. Genes known to be involved in *E. faecalis* biofilm formation

Gene/locus	Protein/function
atn/AtlA	Autolysin
bee	Biofilm enhancer in *Enterococcus*
bop	Biofilm on a plastic surface (putative sugar binding transcriptional regulator)
dltA	D-alanine lipoteichoic acid
ebp locus	Endocarditis and Biofilm associated pilus
ebpR	Regulator of Ebp locus
epa	Enterococcal polysaccharide antigen
esp	Enterococcus surface protein
fsr locus	*E. faecalis* regulator (two-component system)
ace	Collagen binding protein
gelE	gelatinase
sprE	protease
salA	Secretory antigen like-A
salB	Secretory antigen like-B
srtC	Sortase C

relative to planktonic growth (51). As in all areas of microbiology, small differences in the genetic composition of different strains can dramatically impact experimental results as has been demonstrated for the role of *esp* in biofilm formation in *E. faecalis*. The *esp* gene is harbored on a pathogenicity island not shared by all enterococci that are able to form biofilms. It has been determined that *esp* does enhance biofilm formation, but it is neither necessary or sufficient for biofilm formation. This highlights the need for work done in a strain that does not contain mobile genetic elements, like plasmids, and pathogenicity islands like *E. faecalis* OG1RF in order to elucidate the genetic determinants of biofilm formation in the core genome of this organism.

Work done concurrent to my thesis work has elucidated the regulation of eDNA release into the biofilm matrix of *E. faecalis* (95-97). This work showed that two genes regulated by the Fsr quorum sensing system encode proteins that enhance or reduce cell lysis. Both proteins, GelE and SprE act on the autolysin, AtlA. However the GelE/AtlA interaction induces cell lysis where as the SprE/AtlA interaction does not. The lysed cells are thought to provide the eDNA that is known to be found in *E. faecalis* biofilms. While counterintuitive, it is thought that selective lysis of biofilm subpopulations can have advantageous effects on the biofilm population as a whole as demonstrated by this work on the release of eDNA (95-97). Interestingly, in chapter 4 I describe experiments that suggest that there is a novel negative regulator of this system.

At the time that I started my thesis research there was a need for a comprehensive examination of the genetics and biology of biofilm formation in this organism. To accomplish this objective I decided to use *E. faecalis* OG1RF because it does not contain any plasmids or the PAI that other enterococcal species contain, however it forms robust

biofilms in TSB indicating that the core genome of this strain contains all of the necessary and sufficient genetic components required for biofilm formation. We hypothesized that the biofilm required genes identified in this strain might be conserved biofilm determinants in all *E. faecalis* strains. In addition I planned to use the same/similar growth conditions for all of my experiments to establish an internal control and allow for comparison of results across experiments. Some of the initial research presented in this thesis was done to determine appropriate conditions to examine biofilm formation in this organism.

The questions I wanted to answer regarding biofilm formation in *E. faecalis* were very broad. I wanted to characterize the growth of biofilm formation in *E. faecalis* and also I wanted to know what genes were required for biofilm formation. I hypothesized that there were a set of genes that were expressed only in biofilm cells, not their planktonic counterparts and those genes comprised of the set of enterococcal biofilm required genes. In addition I hypothesized that the process of forming a biofilm in *E. faecalis* underwent characteristic changes if followed from initial attachment to maturity. I asserted that by understanding and characterizing the spatial and temporal nature of those changes more educated and focused experiments could then be undertaken.

To test these hypotheses I utilized a variety of genetic and molecular biological techniques. To look at the kinetics of biofilm formation I utilized high-resolution Field Emission Scanning Electron Microscopy coupled with other biological and biochemical measures of biofilm formation and the production of biofilm related compounds. At the

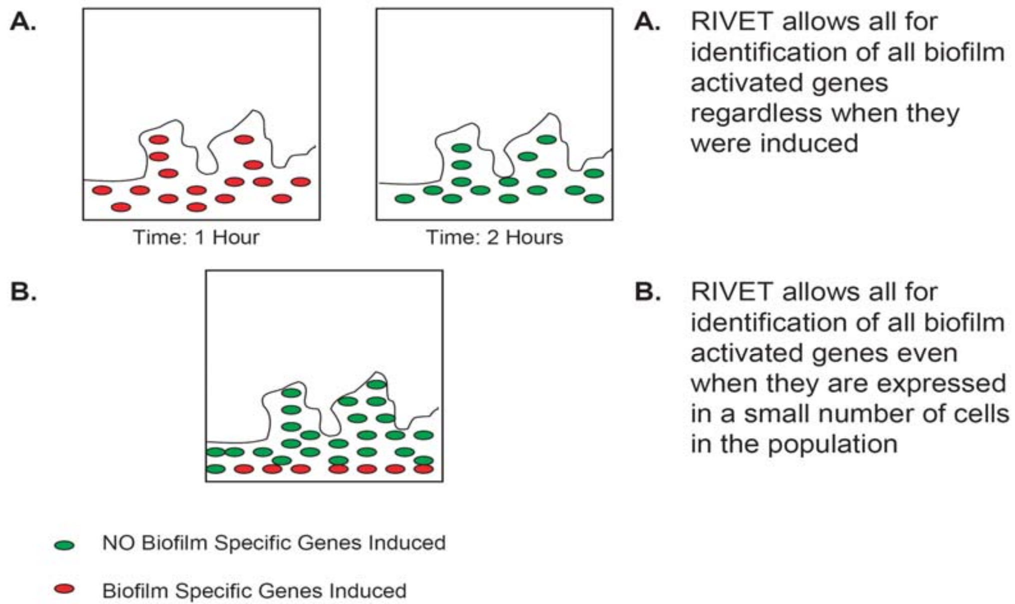


Figure 2. A description of the advantages of RIVET to investigate biofilm formation. This diagram depicts two situations shown in part A and part B that describe how a RIVET screen circumvents artifacts introduced by other types of technologies such as Microarrays.

outset of these experiments randomly inserting transposons, which are a staple experimental tool to look for genetic components of a given system, were shown to be non-random for the subset used commonly in *E. faecalis* (37). So I was not able to use them for my genetic studies. However, our lab later generated and used a new transposon system to investigate biofilm formation (53). Instead, I utilized a Recombinase-based In Vivo Expression Technology (RIVET) screen which is based on the theory of promoter trap cloning in that this screen will identify promoters that are turned-on during a condition of interest. There are many advantages to using a RIVET screen when examining a complex growth process such as the fact that it allows for identification of genes that are turned on from the beginning of the screen to the end instead of at one time point as in microarray-type experiments. In addition, the heritable genotypic change native to RIVET screens allows for identification of genes that are turned on in a small subset of cells that might not been seen when averaging together the gene expression of a whole population (figure 2). The results of this work have provided critical insight into the kinetics of biofilm formation as well as the composition of biofilms and also the genetic composition and regulation of biofilm formation in *E. faecalis*.

CHAPTER 2:

Kinetics, population increases and changes in the biofilm matrix material in *Enterococcus faecalis*

The physiological changes that initiate the transition from planktonic growth to production of biofilms by microorganisms are not well understood. To begin to address this question for *Enterococcus faecalis*, I used high-resolution field emission scanning electron microscopy to examine the temporal changes in bacterial cell surface and the extracellular matrix during biofilm development, with particular emphasis on the first few hours of the process. I observed an abundant, densely interwoven “sweater-like” material coating the bacterial cells and the adjacent substratum during the first few hours of biofilm development when the adherent cell population was increasing rapidly. Within eight hours, the appearance of the matrix changed to a fibrillar structure that did not obscure the bacterial cell surface; similar changes in the matrix were observed in two unrelated *E. faecalis* strains. The temporal changes in the appearance of the biofilm matrix correlated with changes in polysaccharide content of the matrix as detected by lectin staining, and with expression of an operon (*epa*) implicated in exopolysaccharide production. In contrast to the temporal changes in the carbohydrate component of the matrix, extracellular DNA was detected at similar levels in early and mature biofilms.

INTRODUCTION

The nosocomial pathogen *E. faecalis* is a normal resident of the intestinal tracts of many vertebrates and invertebrates including humans, and is readily isolated from many other environments. It has been well documented that *E. faecalis* can form biofilms on biotic and abiotic surfaces, and enterococcal biofilms likely play a role in virulence, persistence, and horizontal gene transfer. Our group has previously used genetic screens (5, 53) to identify over 80 potential determinants of enterococcal biofilm formation (also described in Chapter 3). Previous characterization has focused mostly on biofilms grown for at

least 24 h. In the case of motile organisms, there is mounting evidence for a concomitant loss of motility in the initial transition from planktonic to biofilm growth (101). However, there is little information about the cellular processes associated with this transition in non-motile species such as *E. faecalis*, and the small numbers of cells present at the early stages of biofilm formation make it difficult to use standard expression profiling analyses of mRNA and protein. This chapter describes comparative analysis of the increase in biomass and adherent bacterial populations in the early stages of biofilm formation by *E. faecalis* OG1RF in relation to several clinical isolates. I combined this analysis with high-resolution FESEM analysis of the cell surface and the extracellular matrix of the developing biofilms. These studies revealed a dramatic temporal change in the appearance and biochemical composition of the extracellular matrix that has not been previously reported. The data provide potential phenotypes and gene expression patterns that suggest more focused approaches to examining biofilm formation are important at early time points. The data also suggest that the biochemical composition of the biofilm matrix that change over time. Some of these changes happen early in biofilm development and have not been previously described. This work highlights the importance of careful kinetics studies, including very early time points, to identify novel phenotypes in complex growth systems like biofilms.

MATERIALS AND METHODS

Bacterial Strains: *E. faecalis* strain OG1RF (26), has been described. VA1128 and VA1318 were isolated from human urinary catheters and were provided for this study by Dr. James Johnson of the Minneapolis Veterans Administration. Hospital strains Gbg9 and Lin59 were isolated from hospitals in Norway and were provided for this study by

Dr. Anita Hallgren. Bacteria were stored at -80°C in Brain Heart Infusion broth (BHI; prepared according to the manufacturer's instructions) supplemented with 50% glycerol. Unless otherwise indicated, all culture media were from Difco. Culture media were as follows: TSB, prepared according to the manufacturer's instructions, without additional exogenous carbohydrate and brain heart infusion (BHI) medium, prepared according to the manufacturer's instructions. All bacterial strains were grown at 37°C without any form of aeration unless otherwise noted for a specific assay.

Biofilm assays: Cells were grown on 1 cm^2 cellulose membranes (SpectraPor RC, Spectrum Labs) for CFU assays or 10cm^2 cellulose membranes (SpectraPor RC, Spectrum Labs) for qPCR, lectin or eDNA assay, and submerged in TSB after being inoculated with a 1:100 dilution of an overnight culture. In the case of any comparison experiments, overnight cultures were measured using OD 600 and then adjusted using sterile medium so that the OD 600 values were normalized to each other before inoculating. The biofilms were harvested as previously described (5, 28). Briefly the small (1 cm^2) membranes were votexed for 1 min, 2 times in a total of 500 ul potassium phosphate buffered saline (KPBS) and the large (10 cm^2) membranes were scraped using sterile flat plastic or metal scrapers by hand into 35mls KPBS. After harvesting cells at the indicated time point, cells were serially diluted in KPBS and plated to enumerate the CFU/cm^2 or used for isolating RNA or for lectin staining.

Colorimetric biofilm assay: Biofilms were grown for desired period of time in TSB in a transwell plate that contained a membrane made of PET plastic (BD Falcon 353495). A medium only transwell was used as a control. Overnight cultures of the enterococcal strain used were inoculated into the transwell membrane at a dilution of 1:100 into the

800ul of medium that the transwell holds. Once the incubation period was reached the medium was aspirated from the wells. This was then rinsed five times with sterile water. 1mL 1% crystal violet (Sigma Aldrich) was added to the wells. This was left to stain for 20 min at room temperature and then washed 5 times with sterile water. The remaining strain was released using 1mL 95% ethanol and allowed to destain for 10 min in the wells. The ethanol/crystal violet mixture was mixed by pipetting up and down and transferred to cuvettes to read at OD595. This protocol was adapted from (23).

FESEM imaging: Samples were processed for SEM as described (53) with minor modifications, with all aldehyde fixatives and buffers obtained from Electron Microscopy Sciences (Hatfield, PA). Briefly, samples were rinsed, fixed overnight in a mixture of 2% glutaraldehyde, 2% paraformaldehyde, 4% sucrose, and 0.15% alcian blue in 0.15M sodium cacodylate buffer, washed in cacodylate buffer, postfixed in 1% osmium tetroxide and 1.5% potassium ferricyanide in cacodylate buffer, rinsed, dehydrated through a graded ethanol series, followed by critical point drying with CO_2. Samples were coated with 1 to 2 nm of platinum with an argon ion beam coater (Denton DV-502), and viewed with a Hitachi S-4700 field emission scanning electron microscope operated at 2 to 3 kV. Images were collected using Quartz PCI software and stored in TIFF format.

qPCR: The RNA isolation and synthesis of cDNA was performed as described (5). The qPCR was done as described in (5) as well as chapter 3. The primers for *gyrB* (reference gene) and *epaB* and *epaL* were previously published (12, 94).

Lectin Binding: Concanavalin A and Wheat Germ Agglutinin (Vector Laboratories ABC Elite kit) were used to stain polysaccharide present in biofilm cultures of both OG1RF

and TX5179. Biofilms of each strain were grown for either 4 or 24 hours as described above. Biofilm cells were harvested via aseptic scraping (not vortexing and sonication) into potassium phosphate buffered saline pH 7.4 (KPBS) and pelleted by centrifugation. The biofilm cell pellets were resuspended in 50-200ul of KPBS and normalized via OD 600 value to each other. A 50ul aliquot of normalized cell suspension was spotted onto charged slides (Fisher SuperFrost/Plus slides) and allowed to air dry. Cells were fixed to the slides by exposing the slides for 10 min at room temperature in methanol containing 1% hydrogen peroxide. Slides were allowed to dry before proceeding with the assay. Lectins were reconstituted to 2mg/ml with sterile highly pure water. Lectins were diluted in Lectin Buffer B + 1% BSA (provided in kit) to a working concentration of 10ug/ml. Slides were prepared according to manufacturer directions. Streptavidin was added to a concentration of 15ug/ml for fluorescent detection of lectin binding. Labeled slides were evaluated using an EXFO X-Cite 120 arc lamp (Mississauga, ON) at a magnification of 100x using the Photometrics (Tucson, AZ) CoolSNAP ES camera using MetaVue software (Molecular Devices, Downingtown, PA), images were stored as TIFF files (selection shown in figure 10). Arbitrary fluorescence units were collected using MetaVue software by taking an average of 10 areas 3500 pixels each on each slide.

eDNA Staining: Biofilms were grown as described above for either 4 or 24 hours. Cells were harvested via scraping from the biofilms and pelleted by centrifugation. Planktonic cells of the same ages as the biofilms were also pelleted and both the cell pellets and supernatants of the planktonic cells were treated in the same way as the biofilm cells. The cell pellets were saved and plated to enumerate cells present in the biofilms and planktonic cultures. The cell supernatants were stained, in triplicate, with SYTOX green

dye at a final concentration of 1uM (Invitrogen) according to the manufacturers instructions. This protocol was adapted from (95). SYTOX dye is specific for double stranded DNA and is membrane impermeable. The stained supernatants were read on a Turner Biosystems Modulus Microplate Multimode Reader 9300-010 using the blue filter set (excitation 490nm and emission 510-570nm). The ng of eDNA per sample were calculated by using a standard curve made from *E. faecalis* OG1RF genomic (ds)DNA and the values given are normalized to the number of cells present in the sample obtained from plating the cell pellets (eDNA/cell).

RESULTS

Comparison of biofilm-forming abilities of clinical isolates of *E. faecalis* demonstrates potential for differential results based on assay. A collection of 5 *Enterococcus faecalis* strains were tested for their ability to form biofilms in two different assays of biofilm biomass at 96 hours. Four of the isolates were clinical strains either circulating in hospitals in Sweden (Gbg9 and Lin59) or isolated from human urinary catheters (VA1128 and VA1318). *E. faecalis* OG1RF is a common lab strain shown previously to form biofilms (51). In one assay the strains were grown on flat membranes made of the same cellulose material from which kidney dialysis membranes were once made. Biofilm cells that adhered to the membranes were harvested as described in the materials and methods. The cells were then serially diluted and plated for cell counts. Since this is only a measure of viable cells, a colorimetric assay was also used that could give a measure of the total adherent biomass. This assay involved crystal violet staining of the washed membranes. The procedure is described in the materials and methods. The results of these experiments are shown in figure 3. Strains OG1RF,

Gbg9 and VA1128 show similar results in both assays. Strains VA1318 and Lin59 showed more CFU/cm^2 than produced in the same time by OG1RF. However in the colorimetric assay VA1318 and Lin59 had a far lower OD595 than does OG1RF. Strain VA1128 appeared to be able to form the most biofilm biomass based on the conditions used in both assays.

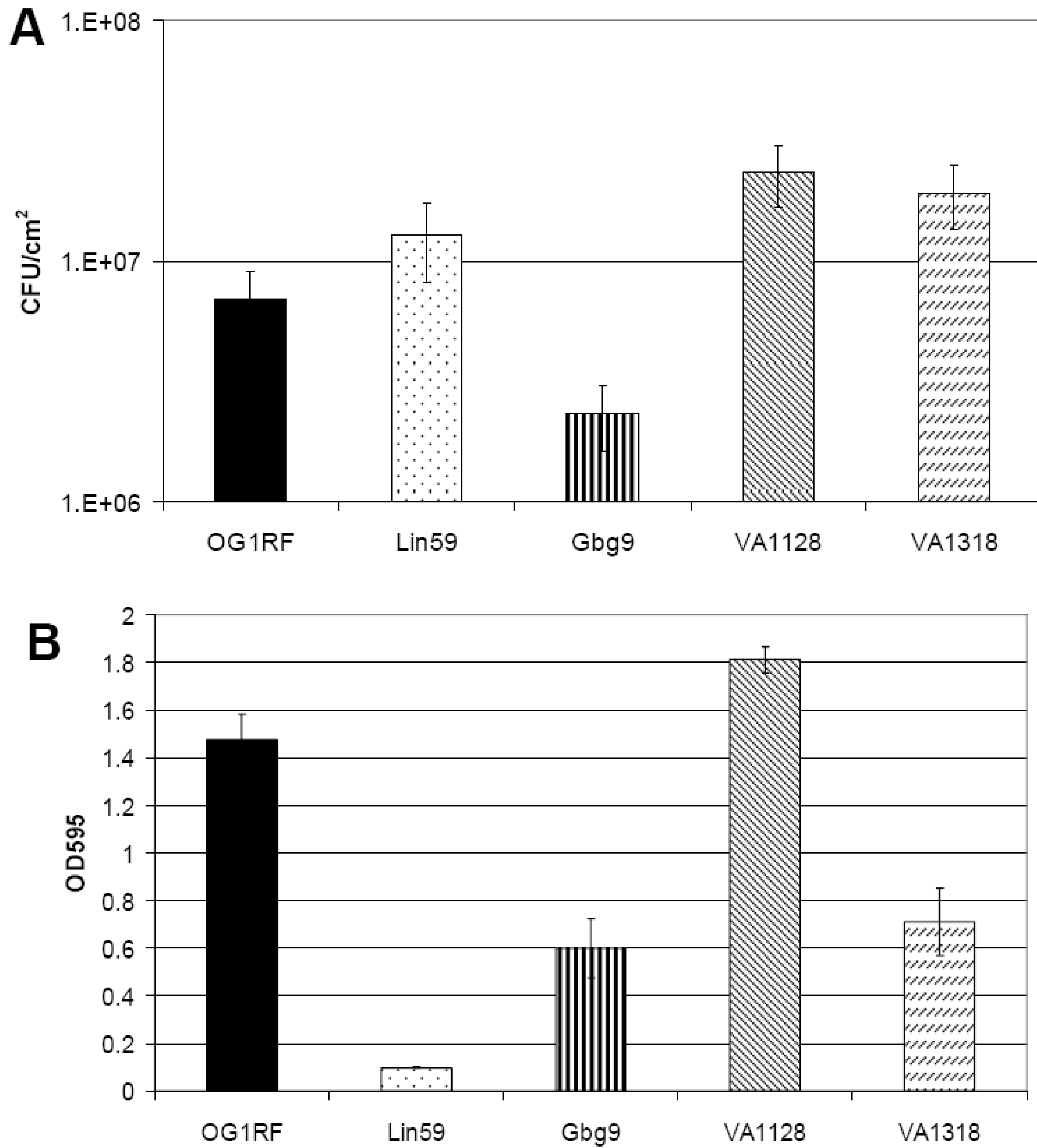


Figure 3 Comparison of biofilm formation abilities of clinical isolates of *E. faecalis*. Strains in panel A were grown for 96 hours in TSB on cellulose membranes as described in the materials and methods section. The graph shows the resulting CFU/cm^2 for each strain after surface adherent bacteria were enumerated Strains in panel B were also grown for 4 days in TSB however a PET membrane was used as the biofilm growth substrate as described here in the materials and methods. The resulting biofilms were stained with crystal violet as described in the materials and methods. The colorimetric reading in Panel B is expressed as the reading at OD595. Error bars in A and B represent 1 standard deviation.

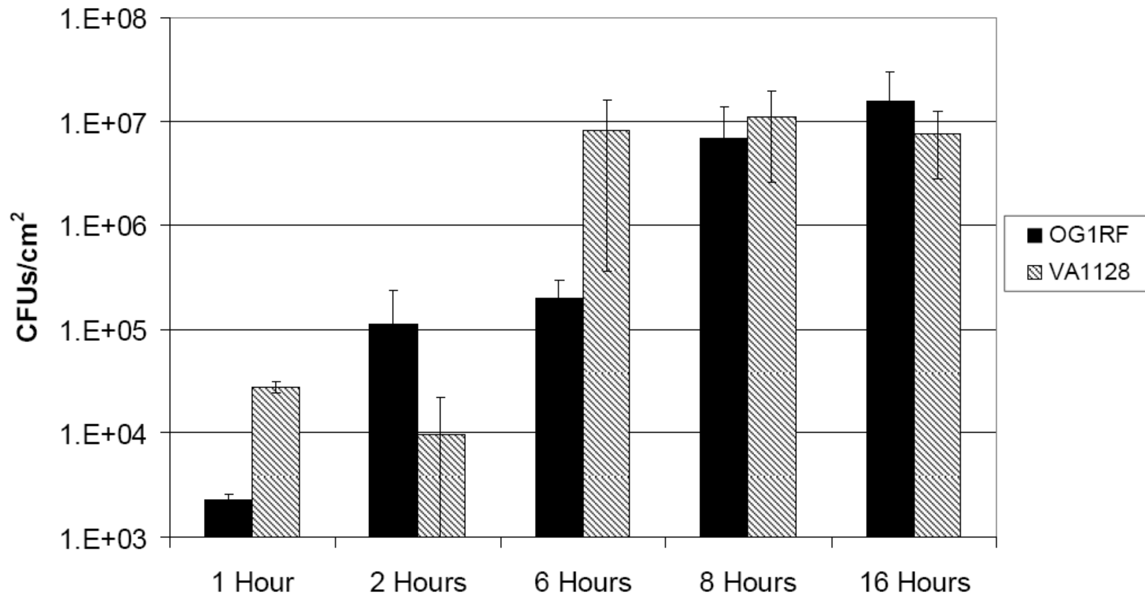


Figure. 4 Early biofilm growth kinetics in *E. faecalis*. Biofilms of *E. faecalis* OG1RF or VA1128 were grown for the stated period of time on cellulose membranes as described in the materials and methods. Membrane adherent CFU were enumerated and expressed as CFU/cm^2. Error bars represent 1 standard deviation.

Early biofilm growth kinetics and appearance of *E. faecalis* strains OG1RF and VA1128. I used a CFU assay and high-resolution field emission scanning electron microscopy (FESEM) in a comparative temporal analysis of biofilm formation by OG1RF and VA 1128. Figure 4 depicts the increase in adherent bacterial populations of each strain during 16 h of incubation of the submerged membranes in a batch culture of TSB medium. While the number of adherent VA1128 cells after 6h was significantly greater than that of OG1RF, both strains produced adherent populations of ~ 10^7 CFU/cm^2 after 8h of growth. This value did not substantially increase between the 8h and 16h time points, probably due to nutrient depletion. To examine longer time points, I carried out additional experiments where the medium was replaced, and the adherent bacteria were enumerated every 24 hr for 4 days. Under these conditions the adherent populations of both strains reached values exceeding 10^8/cm^2 (figure 5).

To examine the cellular and extracellular architecture of these developing biofilms, I used FESEM analysis with alcian blue incorporated into the fixative to stabilize and facilitate subsequent visualization of the enterococcal biofilm extracellular matrix (28). This analysis provided some interesting and unexpected results, especially for the very early time points. After 2h of incubation (figure 6. A, E, I), the total number of attached bacteria was low, but all the cells examined were covered with a dense interwoven material that also extended from the cells onto the surrounding substratum. Because of its appearance, we refer to this material as the “sweater” matrix; for cells covered by this material, many of the surface features of *E. faecalis* typically seen in FESEM images are obscured. In the case of strain OG1RF, the 6h time point (figure 6. B, F, J) showed an increased density of attached bacteria and some multi-layered groups

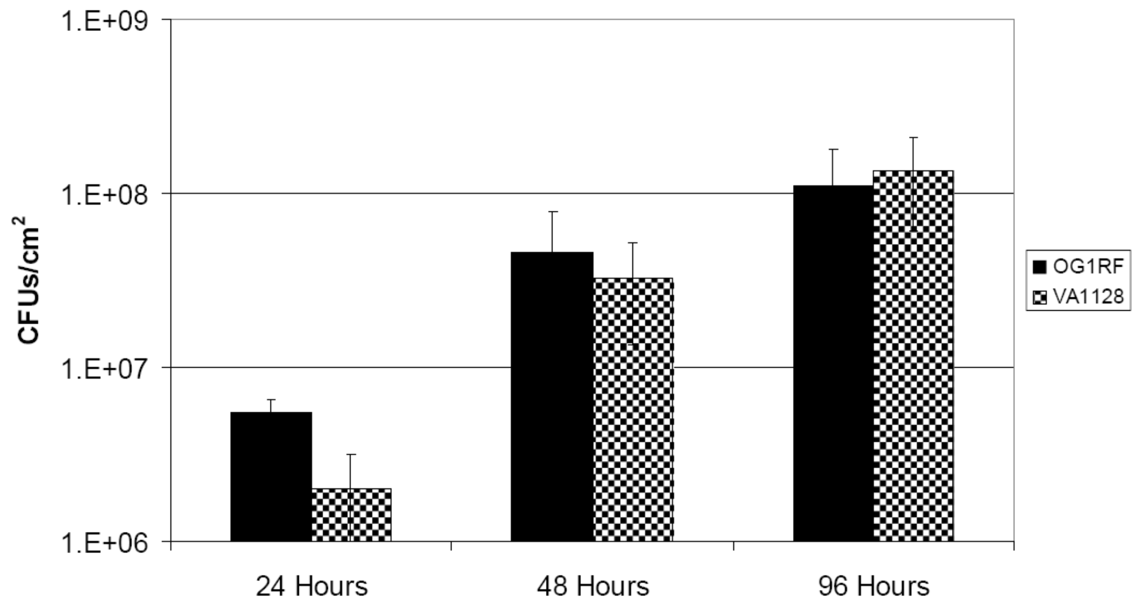


Figure 5. Late biofilm growth kinetics in *E. faecalis*. Biofilms of *E. faecalis* OG1RF or VA1128 were grown for the stated period of time on cellulose membranes as described in the materials and methods. The medium was replaced every 24 hours. Membrane adherent CFU were enumerated and expressed as CFU/cm^2. Error bars represent 1 standard deviation.

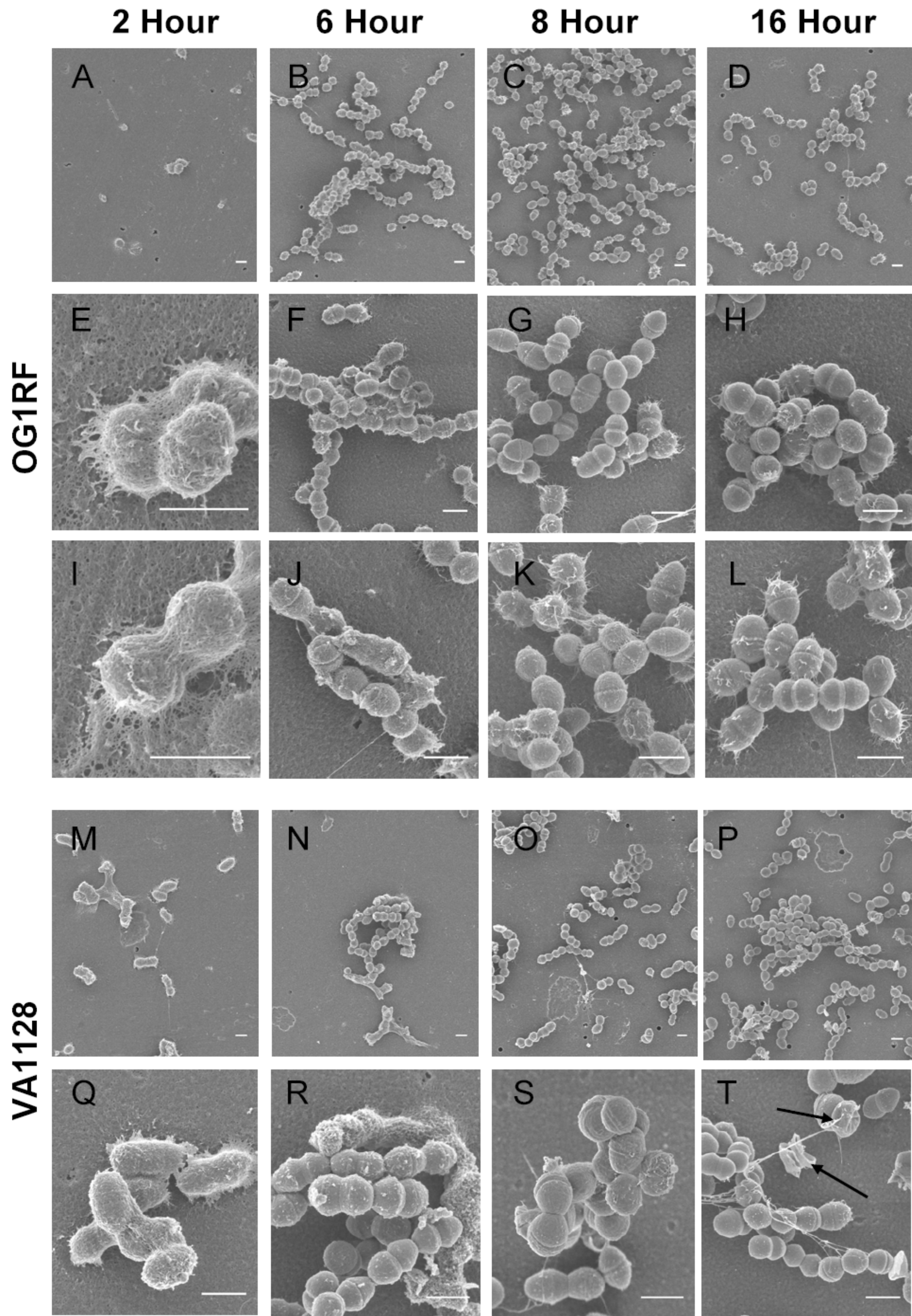

2 Hour
6 Hour
8 Hour
16 Hour
OG1RF
VA1128
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

Figure 6. Analysis by FESEM of early biofilm growth kinetics of *E. faecalis* OG1RF and VA1128 on cellulose membranes. Cellulose membranes carrying biofilms were harvested at the indicated times and processed for SEM. Biofilm growth and processing for microscopy were done as described in Materials and Methods. All scale bars are set to represent 1um. The black arrows point out projections in the matrix material only seen in VA1128 and Black arrow in panel P points at lysed cells not seen in OG1RF until 24 hours.

of cells that probably represent nascent micro-colonies. These cells were also coated with the "sweater" matrix material seen at the earlier time point. At 8h (figure 6. C, G, K) the density and multi-layered cellular architecture continued to increase but the most striking change was the appearance of the surface of individual cells and of the matrix. For many of the adherent bacterial cells, the "sweater" matrix was no longer evident, and the features of the cell surface, such as the septum, were much more obvious. Extracellular material connecting the cells to one another, and to the substratum was readily apparent, but rather than exhibiting the thick, fuzzy appearance of the "sweater" matrix, this material had a much more defined fibrillar structure consistent with our previous SEM studies, which focused on older biofilms; the fibrillar matrix material was prominent in all samples examined at later time points. While the appearance of all these samples is subject to the unavoidable artifacts of fixation and dehydration, all the samples examined were prepared in exactly the same fashion. Thus, it is likely that the temporal changes in the appearance of the cell surfaces and extracellular material observed in these studies reflect changes in the molecular nature of the matrix during the first few hours of biofilm development. It is also important to note that the "sweater" matrix material has never been observed in planktonically-grown cells analyzed by the same method. Therefore it is not simply a carry-over effect from the inocula used to start the biofilm cultures.

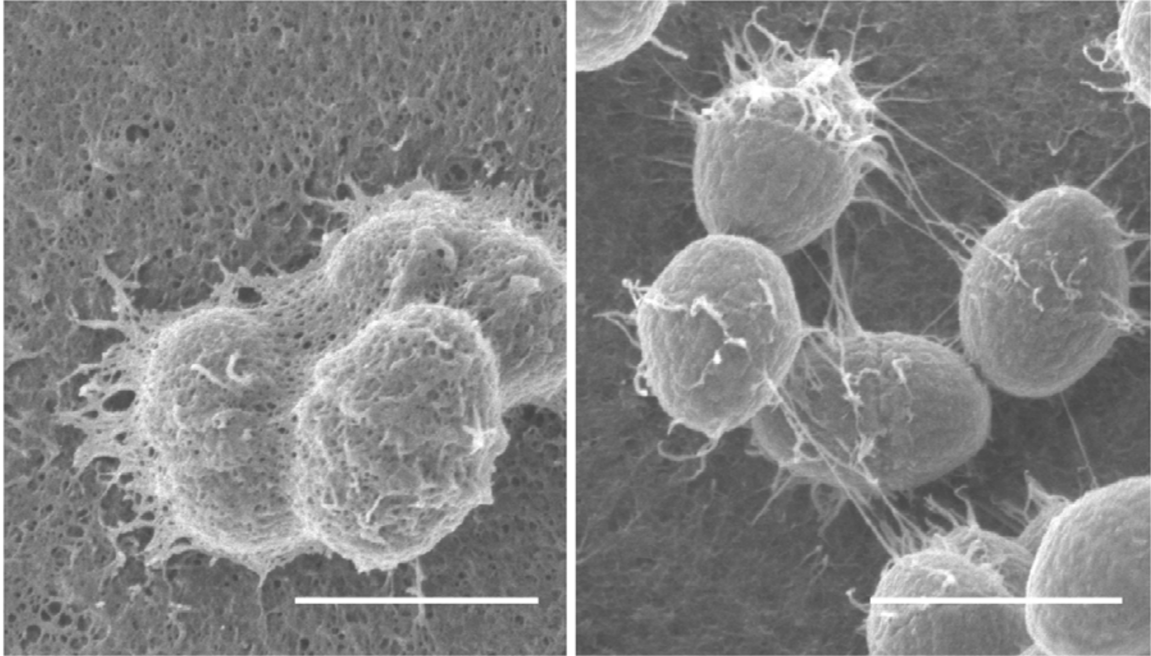


Figure 7 Close up images of the two matrix phenotypes. Show here are images from figures 6 and 8 that have been increased in size to clearly show the two matrix phenotypes seen in enterococcal biofilms. On the left is an image from 2 hours that shows the sweater phenotype and on the left is an image from 24 hours that shows the fibrillar phenotype. In these larger images it is easy to distinguish between the two phenotypes. Both images are of OG1RF and the scale bar indicates 1um.

In the case of strain VA1128, early development of biofilms resembled that of OG1RF in that a "sweater" matrix was abundant at 2h (figure 6 M, Q), with an ultimate transition to a fibrillar matrix (figure 6 P, T). However the kinetics of this transition appeared to be more rapid in this strain, being detectable at 6h, and essentially complete by 8h. Results in figure 7 shows at high magnification the contrast between 2 hour biofilm cells covered in the "sweater" matrix and 24 hour biofilm cells exhibiting the fibrillar matrix material. In addition to the temporal changes related to the matrix, we also observed temporal changes in the cell wall integrity of the biofilm. In OG1 biofilms grown for 24h or longer, we observed cells with damaged walls (figure 8), and similar observations were made in 16h biofilms of VA1128 (figure 6 P, T). However, we never observed any evidence for significant disruptions in cell envelope integrity in OG1RF cells in biofilms grown for less than 24h; these results suggest that cell lysis is not a significant factor in early biofilm development in OG1RF.

Late stage biofilm growth kinetics and appearance of *E. faecalis* strains OG1RF and VA1128. As stated in the materials and methods, for all of the biofilm time points that were harvested after 24 hours, the medium was changed in 24 hour intervals. Results in figure 5 show that there was an increase in CFU between 24 and 96 hours. However it appears that the growth in both strains after 24 hours is in response to the influx of nutrients from the medium change and that growth slows dramatically between 48 and 96 hours. Between 24 and 96 hours the CFU increase by 1.5 logs approximately for OG1RF. This represents approximately 4 doublings whereas there were about 8 between 1 and 16 hours.

Figure 7 shows 24 hour biofilm cells of OG1RF. At 24 hours the long fibers seen in 8 and 16 hour VA1128 biofilms become common in OG1RF as well. The white arrow is pointing to a lysed cell. Lysed cells were not seen at earlier time points in OG1RF like they were in VA1128 and first were detected in OG1RF biofilms grown for 24 hours, indicating the delayed onset of cell lysis in OG1RF biofilms as compared to strain VA1128.

Early biofilm matrix material consists of higher polysaccharide content than later time points but eDNA levels stay constant at early and late time points. OG1RF biofilms were grown for 4 and 24 hours as described in the materials and methods and then the cells were harvested for lectin and eDNA staining. The time points were chosen because 4 hours was within the period between 2 and 6 hours when the "sweater" matrix material was observed in the FESEM analysis. The 24 hour time point was chosen because the "sweater" phenotype was never observed in the SEM images at that time point. The biofilm cells were stained with lectins Concanavalin A (ConA) and Wheat Germ Agglutinin (WGA) which were chosen for their ability to bind many polysaccharides. Figure 9 demonstrates that the binding affinity of ConA (panel A) and WGA (panel B) for the biofilms is higher at 4 hours than they are at 24 hours indicating that there is more polysaccharide present per cell at 4 hours than 24 hours in biofilms formed by either strain. Panel C of figure 9 shows the amount of eDNA/CFU present in 4 and 24 hour biofilms remains constant between the two time points. The change in the amount of polysaccharide between the two time points tested corresponds to the change in the appearance of the matrix suggesting that the "sweater" matrix contains a high percentage of extracellular polysaccharide. The constant level of eDNA supports the

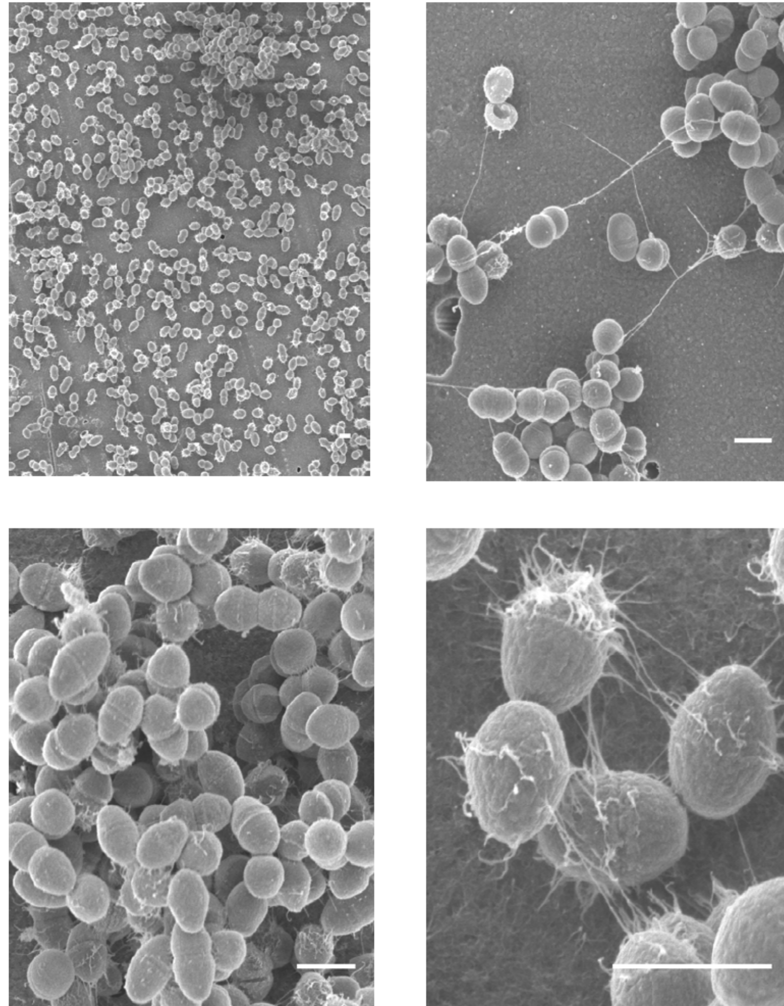


Figure 8 Analysis by FESEM of 24 hour biofilm growth of *E. faecalis* OG1RF on cellulose membranes. Cellulose membranes carrying biofilms were harvested at 24 hours and processed for SEM. Biofilm growth and processing for microscopy were done as described in Materials and Methods. All scale bars indicate 1um.

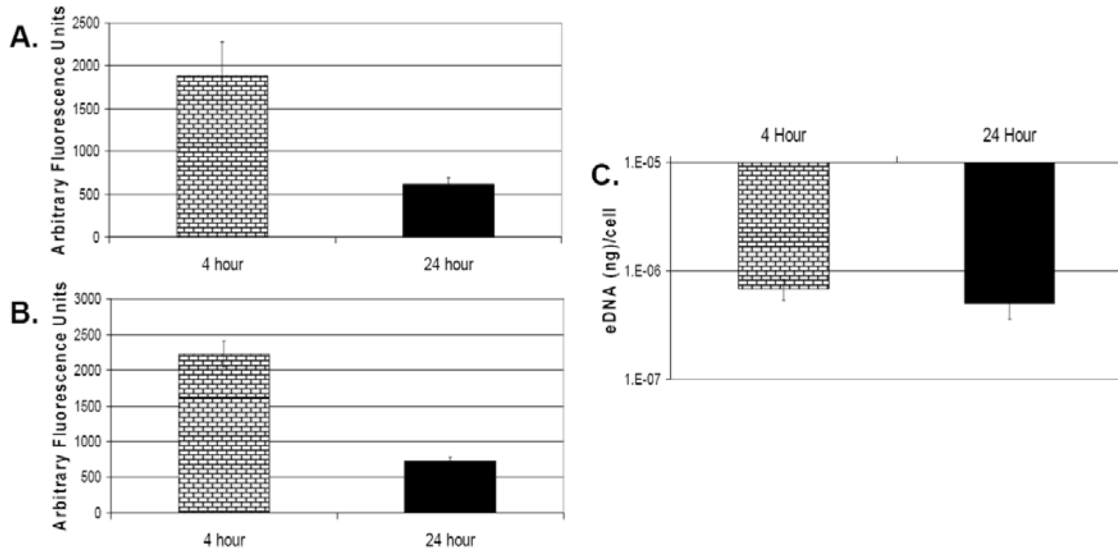


Figure 9. Lectin and eDNA binding of *E. faecalis* biofilms shows differing phenotypes for different matrix materials. Biofilms of OG1RF were grown on cellulose membranes for 4 and 24 hours respectively and harvested according to description in the Materials and Methods section. The lectin and eDNA assay methods are also described in the Materials and Methods Panel A shows the results of binding with Concanavalin A (ConA), Panel B shows the results of binding with Wheat Germ Agglutinin (WGA). Panel C shows the amount of eDNA binding of biofilm supernatant using SYTOX double stranded DNA dye. Error bars represent one standard deviation.

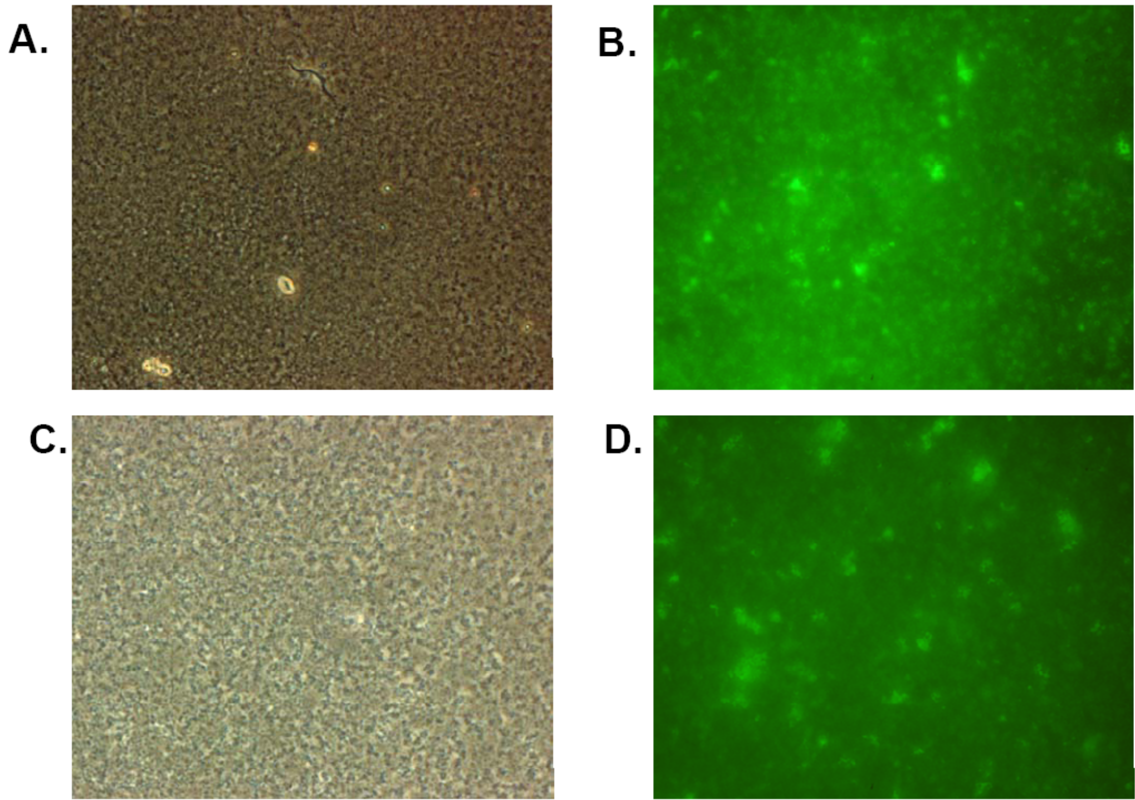
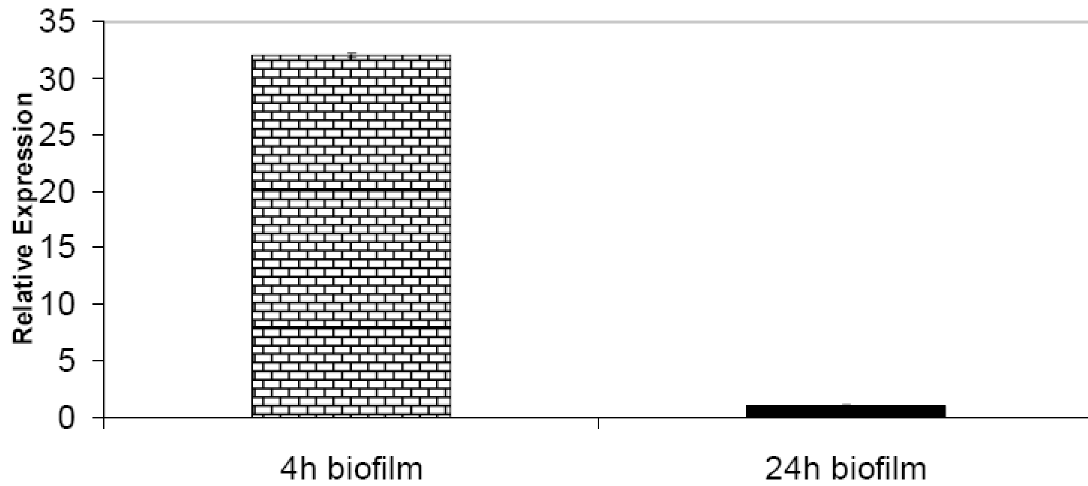


Figure 10. Four and twenty-four hour Concanavanlin A stained OG1RF biofilms. These biofilms were bound with the lectin concanavalin A at either 4 (A and B) or 24 hour (C and D). Panel A and C are phase contrast images at 100X, panels B and D are the matched fluorescence images also at 100X. The 4 and 24 hour fluorescent images are at different exposure settings and should not be directly compared.

assertion that changes in eDNA do not account for differences in the appearance of the matrix as visualized by FESEM. Figure 10 also shows that the lectin staining is due to extracellular polysaccharide and not just staining of the cell wall. However significantly more eDNA was present in all of the biofilms relative to the planktonic cultures tested. The level of eDNA in all of the planktonic cultures was below the level of detection in the assay used. This is an interesting result because eDNA release has been suggested to be a result of cell lysis in *E. faecalis* (96, 97) and we did not observe cell lysis until 24 hours, but the level of eDNA is the same at both early and late time points. This suggests a secondary method of eDNA release in addition to cell lysis.

qPCR shows expression of *epa* locus genes in early biofilm growth. Because the the *epa* operon has been suggested to encode for the biosynthesis of polysaccharide that might play a role in the biofilm matrix (93, 94) I explored the biofilm expression pattern of *epaB* and *epaL* in OG1RF biofilms. As shown in Figure 11, it appears as though the expressions of both *epa* genes are much greater at 4 hours than at 24 hours in a biofilm. *EpaB* expression is 32.5-fold higher at the early time point indicating that this gene cluster is playing a role in early biofilm formation and much earlier than previously studied (94). This suggests that the *epa* polysaccharide may play a role in production of the “sweater” matrix and should be studied further at early time points to elucidate what role this polysaccharide might be playing in the biofilm matrix.



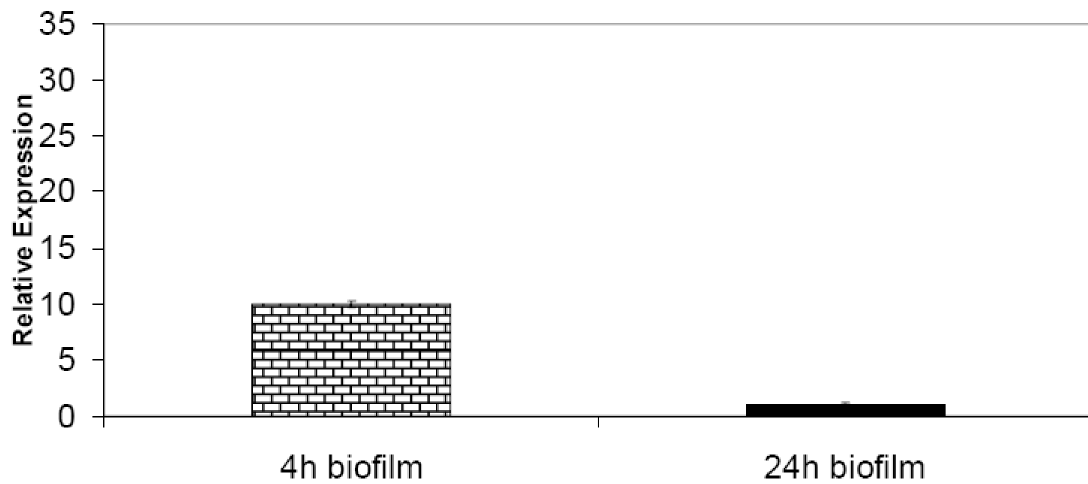


Figure 11. qPCR analysis of *epa* locus gene expression in OG1RF Biofilms. OG1RF biofilms were grown on cellulose membranes for 4 and 24 hours and the cells were harvested for RNA extraction as described in the Materials and Methods. qPCR was performed as described in the Materials and Methods section. Error bars represent one standard deviation.

DISCUSSION

I set out to understand the kinetics of biofilm growth in *Enterococcus faecalis*. To do this I evaluated assay methods for biofilm biomass. We also evaluated biofilm growth quantitatively (biomass assay) and qualitatively (scanning electron microscopy (SEM)) in a kinetic fashion. The experiments presented in this study represent the first quantitative and qualitative description of early biofilm grown in the enterococci and from these experiments we have identified two distinct biofilm matrix phenotypes that change over the course of biofilm growth.

I found that strains of *E. faecalis* behave variably in different assays for biofilm biomass (figure 3). I looked at a CFU-based assay and a crystal violet staining assay. It is likely that the crystal violet could stain both cells and the biofilm matrix and could under or over report the number of cells present based on changes to the either component. The CFU assay will report the viable cells present and would potentially underreport biomass if a strain has a higher level of dead cells relative to other strains. However I have shown that at 24 hours OG1RF has very few dead cells or matrix present in the biofilm (data not shown). I felt as though there were fewer caveats associated with the CFU assay and used it as my preferred method of evaluation of biofilm growth in the rest of the paper.

To evaluate the kinetics of biofilm formation in *E. faecalis* I used the CFU based assay and FESEM to examine two strains, OG1RF a common laboratory strain and VA1128, a recent human clinical isolate. Early biofilm growth was accelerated in VA1128 as compared to OG1RF for time points through 6 hours. However by 8 and 16 hours OG1RF appears to have closed any gap in biofilm CFU. In this static assay biofilm

growth stalls between 8 and 16 hours likely due to nutrient depletion and buildup of toxic metabolic by products (figure 4). At later time points the medium was changed at intervals of 24 hours and in the presence of fresh medium each strain was able to achieve about 1 additional log of biofilm CFU by 96 hours (figure 5). OG1RF was equal in CFU to VA1128 at all later time points indicating that the biofilm growth kinetics of these two strains are similar with the exception of the acceleration of VA1128 at very early time points.

CFU data can only give some insight into the kinetics of biofilm growth. The FESEM technique, along with fixation using Alcian blue to stabilize the biofilm matrix, gave me a unique opportunity to look at the cellular and matrix components of the biofilms of these two strains and evaluates how they changed over time. I observed by looking at these two strains that the stages of biofilm formation appear to be population dependent as VA1128 experiences biofilm related changes earlier than does OG1RF however they both experience the same changes and by 24 hours OG1RF and VA1128 have the same appearance when observed by FESEM.

One of the most striking observations from the FESEM images was the apparent change in the matrix material over time, the stark contrast between a 2 hour and a 24 hour OG1RF biofilm cells are shown in figure 8. At very early time points, there is the appearance of a matrix material that looks interwoven and covers the entire cell that it appears on completely obscuring surface features normally observed like the septum. I called this material “sweater” because of the material’s apparent interwoven nature and ability to cover the cells. The “sweater” material was still apparent on some cells at 6 hours but is mostly absent at 8 hours (figure 6). The “sweater” material was not observed

at time points later than 8 hours. The absence is notable since all of the samples were handled in the same way in preparation for FESEM suggesting that the disappearance is not an artifact of the FESEM treatment but rather reflects a temporal change in the composition of the biofilm matrix during development. I speculate that the "sweater" material is either sloughed off, metabolized by the biofilm cells. After the disappearance of the "sweater" matrix material, I see a fibrillar material in the FESEM images (figure 8). The surfaces of the cells are visible in the absence of the "sweater" material. It is possible the fibrillar material was present underneath the "sweater" material in the early biofilm FESEM images but was obscured from view by the interwoven "sweater" material or that the fibrillar material was produced by modification of the "sweater". At 16 hours we observed what appeared to be lysed cells in VA1128. In agreement with the CFU data that suggesting VA1128 progresses through biofilm formation more quickly than does OG1RF the loss of cell wall integrity leading to cell lysis happens more quickly in VA1128 than it does in OG1RF. I did not observe cell lysis in the biofilm until 24 hours in OG1RF. This suggests that cell lysis does not play a significant role in biofilm formation in early biofilm formation of *E. faecalis*; this should be confirmed by other methods.

To my knowledge the "sweater" material has not previously been described, most likely because this is the first in depth examination of early biofilm kinetics in *E. faecalis*. Polysaccharides have been shown to be large components of other biofilm matrices (49, 89). I wondered if the appearance of the "sweater" material was related to the polysaccharide composition of the matrix. To examine this possibility we stained early biofilms (4 hours) and late biofilms (24 hours) of OG1RF with two different broadly

binding lectins, Concanavalin A (ConA) and Wheat Germ Agglutinin (WGA) (figure 9 panels A and B). These lectins had previously been shown to bind to *E. faecalis* (data not shown). The 4 hour biofilm had significantly more polysaccharide present than did the 24 hour sample. This supports the notion that the appearance of the "Sweater" matrix relates to a high level of polysaccharide content; although more studies are needed to confirm this model.

eDNA has also been shown to be a part of the *E. faecalis* biofilm matrix (40, 96, 97). I could not find a reference that looked at levels of eDNA present at very early time points and therefore it was not known if eDNA levels are subject to kinetic changes. To look at this I stained biofilm matrix material from 4 and 24 hour OG1RF biofilms with the double stranded DNA dye, SYTOX green. The amount of eDNA/CFU did not differ between 4 and 24 hours, demonstrating that eDNA was not responsible for the changes seen in the biofilm matrix over time (Figure 9). Cell lysis is the proposed mechanism of eDNA,(96) at later time points (16-24 hours). Our results at 4 hours, indicate that eDNA is already a significant componant of the biofilm. This implies that there is another method of eDNA release other than cell lysis.

The product of the *epa* operon encodes for production of a polysaccharide (94). The genes in this locus have been shown through mutational analysis to play a role in biofilm formation in *E. faecalis* (94). It has been hypothesized that this polysaccharide might play a role in the biofilm matrix. Because the composition of the *epa* polysaccharide has not been completely determined (94), it was hard to address whether or not the *epa* polysaccharide was a part of the sweater matrix directly. However I did look at expression kinetics of genes from the *epa* polysaccharide locus, *epaB* and *epaL*

using qPCR with previously published primers (12, 94). The results of the qPCR (figure 12) show expression levels of both *epa* genes are much higher at 4 hours than they are at 24 hours. The dramatic up regulation demonstrates that the *epa* locus is turned on early in biofilm formation, and essentially turned off at 24 hours when the locus and its product are usually studied. While it is correlatory, the fact that the locus genes are so significantly up regulated may support the assertion that the *epa* polysaccharide is involved in production of the "sweater" matrix at some level. To understand what role if any the *epa* polysaccharide is playing in the sweater matrix it is imperative that the product of this locus be characterized so that the role that this polysaccharide is playing can be determined directly in a biofilm. I suggest that any characterization work be done at early time points (4-6 hours) based on the evidence shown in figure 11 that the *epa* locus is not expressed at later time points. Because the *epa* genes are turned on early in biofilms and are then turned off these genes or a subset could be useful as a reporter system to look for effectors of early biofilm formation.

The experimental results shown here demonstrate that it is important to note that biofilm formation in *E. faecalis* like many other organisms is a dynamic process. We cannot assume that characterizing what is happening at one time point is going to be sufficient to provide an understanding of the process as a whole. As shown here, focusing only on late events in biofilm formation would have missed a key transition in the biofilm matrix of *E. faecalis* that happens in early (2-6 hour) biofilms. It will be important to identify the composition of the "sweater" and fibrillar matrix material as well as the *epa* polysaccharide to better understand if there is a relationship between any of these extracellular matrix components. In addition it will be important to garner a

better understanding of the genetic regulation involved in the switch from “sweater” to fibrillar matrix material and understanding what exogenous signals are involved. Further study of the early events of biofilm formation in *E. faecalis* will hopefully lead to exciting breakthroughs that will assist in the understanding and ultimately in the control of enterococcal biofilms.

CHAPTER 3

Functional Genomics of *Enterococcus faecalis*: Multiple Novel Genetic Determinants for Biofilm Formation in the Core Genome

The ability of *Enterococcus faecalis* to form robust biofilms on host tissues, and on abiotic surfaces such as catheters, likely plays a major role in the pathogenesis of opportunistic antibiotic-resistant *E. faecalis* infections and in transfer of antibiotic resistance genes. I have carried out a comprehensive analysis of genetic determinants for biofilm formation in the core genome of *E. faecalis*. Here we describe 68 genetic loci predicted to be involved in biofilm formation, identified using Recombinase In Vivo Expression Technology (RIVET); most of these genes have not been studied previously. Differential expression of a number of these determinants during biofilm growth was confirmed using quantitative RT-PCR, and genetic complementation studies verified a role in biofilm formation for several candidate genes. Of particular interest was genetic locus EF1809, predicted to encode a regulatory protein of the GntR family. I isolated **5** independent, non-sibling clones containing the putative promoter region for this gene in the RIVET screen; EF1809 also showed the largest increase in expression during biofilm growth of any gene tested. Since an in-frame deletion of EF1809 resulted in a severe biofilm defect that could be complemented by the cloned wild type gene, we have designated EF1809 as *ebrA* (Enterococcal Biofilm Regulator). Most of the novel genetic loci identified in our studies are highly conserved in Gram-positive bacterial pathogens, and may thus comprise a pool of uncharacterized genes involved in biofilm formation that may be useful targets for drug discovery.

INTRODUCTION

Nosocomial infections by multiply-antibiotic-resistant opportunistic pathogens such as *Enterococcus faecalis* have a major impact on morbidity, mortality and health care costs (36, 60, 73, 87). *E. faecalis* is a normal resident of the human intestinal tract, and can

also be cultured from a variety of plants, animals, insects and other environmental sources. Although *E. faecalis* is generally non-pathogenic in healthy humans, it is an extremely hardy and adaptable organism capable of survival and even robust growth under a variety of conditions that would be lethal for many phylogenetically-related bacteria such as pathogenic streptococci (31). Enterococci isolated from clinical sources almost invariably carry a cornucopia of mobile genetic elements, including plasmids, transposons and genomic islands encoding gene products mediating horizontal transfer of high-level antibiotic resistance and virulence (17, 18, 91). Intra- and interspecies horizontal transfer of these elements has a high degree of medical significance, since it accelerates the evolution of increased bacterial virulence and resistance to antimicrobial agents. Both the core genome and the mobile elements of enterococci carry a variety of sensing systems that mediate cell-cell communication and detection of environmental signals; such systems are involved in the remarkable ability of these bacteria to adapt and proliferate in a variety of different ecological niches (25, 27).

Genetic analyses of enterococci have focused primarily on genetic determinants that are found more frequently in clinical isolates than in intestinal strains from healthy individuals (4, 20, 24, 41). These studies have provided important insights into the evolution of enterococcal virulence and resistance by horizontal transfer of mobile elements in the hospital environment. To date, however, there is no single virulence determinant associated with a mobile element that has been demonstrated to be essential for all enterococcal infections. Indeed it could be argued that the *E. faecalis* core genome encodes considerable capacity for adaptation to survival and growth under a variety of conditions, and that this inherent adaptability provides an excellent evolutionary scaffold

for emergence of new clones via acquisition of mobile elements that enhance competitive fitness in immune-compromised patients subjected to extensive antibiotic treatment. We hypothesized that the core genome of *E. faecalis* includes a conserved minimal set of genetic determinants essential for biofilm formation and that disruption of any of these determinants would impair the ability of *E. faecalis* to cause infections that involve a biofilm component. While several loci affecting enterococcal biofilm formation have been identified (41, 42, 45, 68, 92), there has not been a comprehensive interrogation of the entire core genome for such genes. Many previously-identified enterococcal biofilm determinants encode surface proteins involved in adhesion and thus represent a minority of the required functions for biofilm growth.

I set out to test our hypothesis by a systematic search for essential biofilm determinants in the genome of *E. faecalis* OG1RF, a recently-sequenced laboratory strain that lacks plasmids and several other mobile DNA elements encoding antibiotic resistance and virulence genes that were identified previously in the genome of the clinical isolate V583 (11). Because any single approach to this goal has limitations, we simultaneously employed two complementary methods, transposon mutagenesis (53), and Recombinase-In Vivo Expression Technology (RIVET) (16, 59) to identify chromosomally-encoded biofilm determinants. Here we describe the results obtained from the RIVET screen, and from follow-up gene expression analysis and functional complementation studies; the latter experiments confirmed a direct role in biofilm formation for several genes identified in the RIVET screen. For this screen, we employed submerged cellulose coupons, previously shown to support development of robust biofilms with extensive 3-dimensional structures (28); we felt that this system had

the potential to identify biofilm determinants that were not identified in the 96-well polystyrene microtiter dish assay used in the transposon screen. Bioinformatic analyses revealed that a large number of novel potential biofilm determinants identified in our cumulative studies are highly conserved in the core genomes of many important bacterial pathogens. This work highlights the advantages of multiple simultaneous approaches to functional genomic analysis.

MATERIALS AND METHODS

Bacterial strains, culture conditions and plasmids. *E. faecalis* OG1RF planktonic cells were grown in Brain Heart Infusion broth (BHI) (Difco) at 37°C, unless otherwise noted. Mutants harboring nonpolar, in-frame deletions of selected *E. faecalis* ORFs were constructed using a previously described allelic exchange system (50). Each deletion removed the entire predicted ORF, except for the first and last 3 codons. The sequences and genome locations of all primers used to create the deletions described in this paper are listed in table 2. These primers were used to amplify the up and downstream regions, the PCR products from these reactions were fused and inserted into pCJK47 and employed for allelic exchange as previously described (50). Complementation analysis used the genes immediately downstream from the predicted promoters identified in RIVET clones (along with their cognate RBS), cloned and expressed from plasmid pMSP3535 (14). This plasmid allows expression of cloned sequences from a nisin inducible promoter (14). These strains were grown in the presence of nisin, 25ng/mL, and erythromycin (erm) 10μg/mL. All plasmid and chromosomal constructs were

Table 2. Primers used for creation of in-frame, markerless null mutations

ORF Deleted*	Primer Sequence**	Genome Coordinates ***
	A ccc cct gca gtc aac ggt aat atc tcc gaa ttc gc	759659
	B cgt gta gaa agg ggg agc gga atg aat aaa gat atc taa tga aaa aaa caa cag gga ttc	758650
	C gaa tcc ctg ttg ttt ttt tca tta gat atc ttt att cat tcc gtc ccc tct ttc tac acg	758009
EF0798	D ccc cct gca gta agt tat agt ttg gtg cct taf ata gcg	757000
	A ccc cgc ggc cgc caa gat aga cac cgt taa att aca cg	941870
	B ctt gct caa ata gaf cgg cta ttt ttt ttg taa cat ctt gtt cac ctc att c	941479
	C gaa tga ggt gaa caa gat gtt aca aaa aaa ata gcc gct cta ttt gag caa g	943134
EF0984	D ccc cgg tac cca tga atg tga aac tcc ctt ttg c	943543
	A ccc ccc ccc cat ggg ttg aac ctg aga aat tac gtt tgc c	1754338
	B gtt agg gta aag gag aaa gtt atg gaa aat tat cag ata cgc cat att cgg aat agt tag	1753329
	C cta act att ccg aat atg gcg tat ctg ata att ttc cat aac ttt ctc ctt tac ctt tac cct aac	1752616
EF1809	D ccc ccc ccc tgc aga ttc cag agc tgt tta gag gaa gcc ag	1751607

* In all cases Primers A and B were used together and Primers C and D were used together to make the two halves of the deletion construct

** All sequences listed 5' to 3'

*** Coordiates are derived from the *E. faecalis* V583 genome

confirmed by sequencing. Biofilms were grown on submerged sterilized cellulose coupons (28) (SpectraPor RC, Spectrum Laboratories) at either 30 or 37°C for the specified amount of time in TSB-gluc (Difco) in 3mls in a plastic 6 well dish (Costar 3516) unless otherwise noted. Coupons containing biofilms were washed 3 times in phosphate buffered saline (PBS), sonicated 2 times (VirSonic 475, VirTis) on a setting of "2" and vortexed for 30 seconds twice in a plastic tube containing 500µl of fresh PBS in order to harvest biofilm cells.

Construction of a resolvase reporter fusion vector, pJMA61. A *Sal*I fragment bearing a transcriptional terminator (to prevent transcriptional read-through from upstream sequences), a unique *Bgl*II site (to insert genomic DNA), and the gene encoding TnpR resolvase preceded by a modified RBS (to enable efficient translation in *E. faecalis*) was excised from the staphylococcal vector, pES95 (59), and cloned into the broad-host-range shuttle vector, pAM401 (103), to produce the resolvase reporter fusion vector pJMA61 (figure 12).

Construction of an *E. faecalis* RIVET host strain, CK103. A 2.2-kb *BamH*I fragment containing the Ω-Km2 cassette (75) was cloned into the *BamH*I site of pRR51 (78) to insert the kanamycin resistance determinant between 2 copies of the *res* sequence, creating pRRΩ. Subsequently, pRRΩ was digested at the unique *Pac*I site (external to the *res*-Kan-*res* cassette) and *Pac*I-digested *E. faecalis* genomic DNA was inserted. One recombinant clone (pRRΩ12b) bearing a 0.8-kb *Pac*I fragment of genomic DNA, including the entire EF2078 ORF, annotated as a hypothetical protein, plus portions of the upstream and downstream ORFs, was found to efficiently integrate into the EF2078

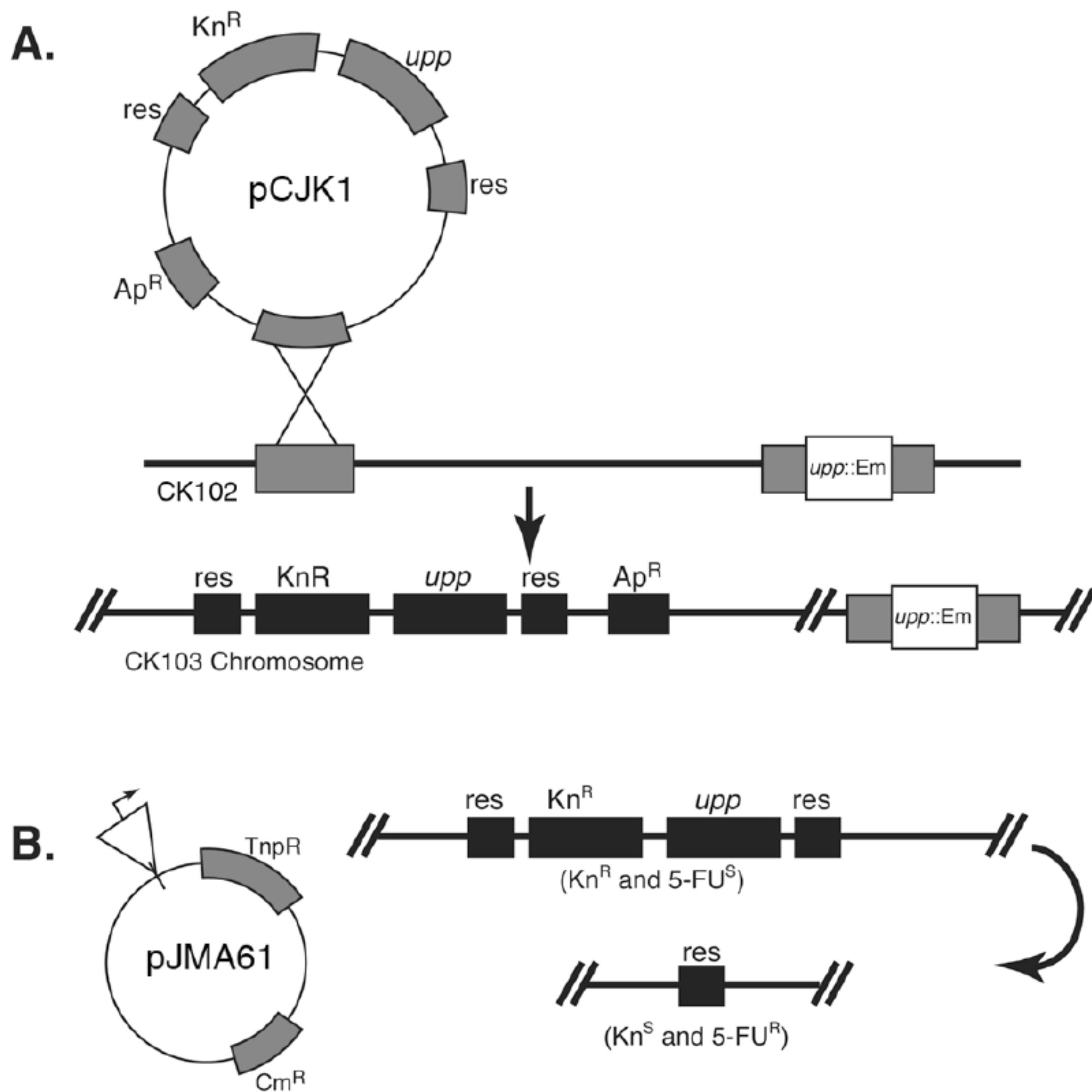


Figure 12. RIVET Plasmid and Strains. A. The RIVET host strain CK103 was created via Campbell insertion of pCJK1 containing the *res* cassettes flanking selectable and counter-selectable genes into CK103 chromosome. B. An OG1RF genomic library was created in the resolvase fusion reporter vector, pJMA61. This vector contains a promoterless copy of the resolvase gene, *tnpR* adjacent to the cloning site. When *tnpR* transcription is activated, the resulting resolvase protein catalyzes site-specific recombination between the duplicated res sites (depicted on the right hand side of the figure), leaving a heritable genetic change in the host strain that can be screened for at a later time. The phenotype of the host strain before and after TnpR-mediated excision is indicated in parentheses.

locus of the OG1RF chromosome via Campbell-type insertion (pRRΩ12b cannot replicate in *E. faecalis*), and the resulting integrants did not display any noticeable defects in planktonic or biofilm growth. To introduce a counterselectable marker into pRRΩ12b, the P-*upp* cassette from pCJK2 (52) was amplified and inserted into the unique *Sph*I site with primer-encoded *Sph*I restriction sites, placing the counterselectable marker adjacent to the kan resistance determinant between the *res* sites, thereby creating pCJK1. The resulting plasmid was introduced into *E. faecalis* CK102 (52) by electroporation with selection for kan resistance, generating strain CK103. CK102 carries the *upp1*::*erm* allele, rendering the cells resistant to the toxic base analog, 5-fluorouracil (5FU) (52). The recombinant strain CK103 was verified by PCR to contain pCJK1 integrated into the EF2078 locus, and found to be phenotypically sensitive to 5FU, as expected due to production of functional *upp* gene product from the integrated P-*upp* cassette.

RIVET Screen for promoters induced during biofilm formation. Genomic DNA from *E. faecalis* OG1RF was partially digested with *Sau3A*I. Fragments of 0.5 to 2.5 kb were extracted from an agarose gel and cloned into *Bgl*II-digested pJMA61 (38) to create a library of *E. faecalis* genomic DNA upstream of the *tnpR* reporter. pJMA61 confers resistance to cm. *E. coli* clones carrying the library were pooled, plasmid DNA was isolated, and introduced into CK103 by electroporation. Transformants were pooled and frozen at -80° C in aliquots with 30% glycerol. For RIVET screening, thawed aliquots were cultured for 8 hours at 30°C in 3 ml TSB-gluc with an initial inoculation of 1:50 in the presence of 2000µg/mL kan and 20 µg/mL cm. After 8 hours the cells were collected by centrifugation and fresh medium was added also containing 2000µg/mL kan and 20 µg/mL cm. This culture was incubated for 8 hours at 30°C. The remaining cells were

washed 3x in TSB containing no antibiotics and then grown as biofilms as described above at 30°C for either 2 hours, 3 days or 5 days in TSB-gluc with 10µg/mL cm. For the 3 day and 5 day screens, the liquid medium was removed and replaced every 24 hours. The cellulose coupons containing the RIVET screen biofilms were removed from the wells after growth and cells were harvested as described above. The resulting suspensions were diluted serially and plated on a medium containing 20µg/mL cm and 75µg/mL 5-FU. The resulting colonies were picked and patched to BHI agar plates containing either 1000 µg/mL kan and 10 µg/mL cm or 75 µg/mL 5-FU and 10 µg/mL cm. Those colonies that did not grow on kan were inoculated in 10ml BHI liquid medium supplemented with 10 µg/mL cm and grown overnight for plasmid isolation using a kit (Qiagen). The inserts in the plasmids were then sequenced using primers that would produce sequence from both strands of the insert; Forward 5'agc gtc gac tct aga gat cca g 3' and Reverse 5' tac ccg tgc gta acc aaa aag tcg 3'.

Bioinformatic analysis. The resulting sequences were compiled and batch BLASTed against the omniome at http://www.jcvi.org/ as well as imported into a sequence analysis program, Sequencher 4.8 (www.genecodes.com), and allowed to "assemble automatically" to a contig carrying the *E. faecalis* V583 genome ORFs. Once the *E. faecalis* OG1RF genome was made available the sequence was added to this contig file to verify the results in OG1RF as well. These results were compared with those from the BLAST analysis and the ORF downstream of the cloned chromosomal insert fragment containing the putative promoter was identified.

qRT-PCR. Biofilms were grown as described above to obtain cells for RNA extraction. Planktonic cells were isolated from the same vessel in which biofilms were grown, and at

the same time points. RNA Protect Reagent (Qiagen) was used to stabilize the RNA until extraction. The RNA was extracted from biofilm and planktonic cells using the RNeasy Kit (Qiagen) following the suggested protocol for bacterial RNA with the following modifications. The cell walls were subjected to enzymatic degradation prior to using the kit at 37°C for 10 min with 50mg/ml lysozyme (Sigma) and 1000u/ml mutanolysin (Sigma) in 10mM Tris made with RNase free water. The resulting total RNA was DNase treated using the Turbo DNA-Free kit (Ambion) following the "rigorous" protocol. The DNase treated RNA was then checked for DNA contamination by PCR using published 16s primers (99) and published *gyrB* primers (12). The RNA was reverse transcribed to cDNA using Superscript III First Strand Synthesis System for RT-PCR kit (Invitrogen). PCR primers were designed for genes of interest using the published genome sequence and Primer 3 software (84). qRT-PCR was undertaken following instructions from BioRad using iQ SYBR Green Supermix and the BioRad iQ5 thermocycler. Data was analyzed with the software supplied with the iQ5 thermocycler.

Biofilm Phenotype Assay. Biofilms for this assay were grown as described above, and biofilm cells harvested by sonication. The resulting harvested cells were serially diluted and plated to an appropriate medium. For complementation experiments both the liquid and agar media contained erythromycin (erm) 10 µg/mL. The markerless null mutants were grown with no antibiotics. All mutants were compared to the isogenic wild-type *E. faecalis* control strain.

RESULTS

Construction of a resolvase reporter system for *E. faecalis*. RIVET screens are designed to identify promoters that are active in a condition of interest, such as during

animal infections, and that drive very low or undetectable levels of transcription during growth in laboratory medium. The use of recombination as a reporter for gene expression has been described by Camilli and co-workers (16, 59). The system relies on the ability of the site-specific TnpR recombinase of Tnγδ to excise DNA that is flanked by its target sequences (*res*), generating a heritable change in genotype. If *res* sites flank a counterselectable marker, expression of resolvase results in loss of the intervening marker and, therefore, a selectable phenotype (see figure 12). While this approach has been well-developed for use in Gram-negative bacteria, several modifications were required for use in *E. faecalis*. My modifications included the introduction of appropriate selectable and counterselectable markers into a cassette flanked by *res* sequences, integration of this cassette into the chromosome of an *E. faecalis* reporter strain, as well as construction of a resolvase fusion vector encoding *tnpR* with a RBS modified for efficient recognition in *E. faecalis* (figure 12). When a pJMA61 derivative containing a cloned genomic fragment encoding an active promoter is propagated in CK103, expression of resolvase and excision of the *res-Kan-upp-res* cassette from the chromosome occurs. This permanent genotypic change results in a selectable phenotype: the cells become resistant to the toxic base analog, 5FU (52), enabling selection to be used to identify clones that have undergone resolution.

RIVET for genetic analysis of biofilm formation. To identify genes specifically activated during biofilm growth, we constructed a library of *E. faecalis* genomic DNA in the resolvase fusion reporter vector, pJMA61, introduced the resulting library into the RIVET host strain, CK103, and cultured the library in liquid medium containing high levels of kan and normal levels of cm. This served to eliminate most of the clones

containing promoters expressed during planktonic growth. I then inoculated the surviving bacteria into cultures containing submerged cellulose membranes as a surface for biofilm growth. After various time periods, the membranes were removed, rinsed to dislodge loosely-attached bacteria, and the biofilm cells removed by sonication, and plated on agar medium containing 5-FU. Colonies appearing on these plates were tested for kan sensitivity, and the inserts in the plasmid DNA from approximately 300 kan^S, $5\text{-}FU^R$ clones were sequenced and analyzed as described in Materials and Methods to identify the cloned genomic regions. I expected that expression of genes important for biofilm development would occur in a temporal fashion, and that some important genes might be transiently expressed. Genes that were either transiently expressed or expressed at a relatively low level would still undergo a permanent and heritable genetic change, and the clones containing these promoters should still be present in libraries harvested at any time point following the activation of their expression. Thus, the time point from which a given clone was isolated does not provide a precise indication of when the relevant promoter was activated or of duration of its activation. However in cells harvested from biofilms grown for prolonged periods the representation of the transiently-expressed promoters in the resulting library of RIVET clones might be very low. To increase the representation of such promoters in our library, I isolated clones from biofilms grown for various lengths of time, chosen arbitrarily. Approximately 30% of the clones analyzed were from 72 h biofilms, 50% from120h biofilms and 15% from 2h biofilms. It should also be noted that clones containing a genomic fragment with a biofilm active promoter and a transcription termination signal would not allow for read-through into the TnpR recombinase and would not be represented in the results. The vast

Annotation of Genes Identified in RIVET Screen	
Category	**Number of Genes**
DNA Binding Proteins	8
Protein Synthesis	6
Transporters	10
Cell Wall/Metabolism	13
Stress Response/DNA Repair	8
Hypotheticals	17
Other	6
Total Genes Identified	**68**

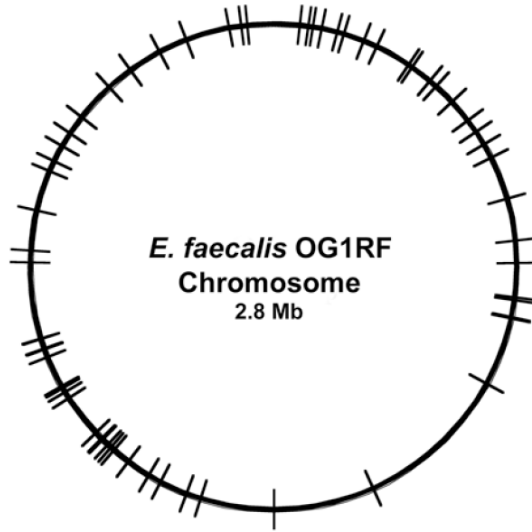


Figure 12. Genome Distribution of Determinants Identified in RIVET Screen. The list on the left indicates the number of putative biofilm-related genes identified by RIVET in various categories based on the TIGR gene annotation for the genome of *E. faecalis* V583 (46) and on the recently published genome sequence of OG1RF (6). In the circular map on the right, the location of each gene in the chromosome is indicated with a line. Further analysis of selected genes is presented in other sections of this paper.

Table 3. Genes Identified in RIVET Screen

TIGR ID	SwissProt ID	TIGR Annotation	Total Hits*	Non Sibling Clones	Screen Identified[a]
EF0038	NA	Glutamate 5-kinase	5	2	120h
EF0059	Q839U1	UDP-N-acetylglucosamine pyrophosphorylase	8	1	2h, 72h, 120h
EF0064	NA	Conserved hypothetical protein	1	1	120h
EF0082	Q839S4	Major facilitator family transporter	1	1	2h
EF0086	NA	Conserved domain protein	1	1	120h
EF0105	Q839Q5	Ornithine carbamoyltransferase	1	1	72h
EF0117	Q839P5	GntR family transcriptional regulator	1	1	72h
EF0203	Q839G6	Cyclopropane-fatty-acyl-phospholipid synthase	1	1	120h
EF0246	Q839C9	Amino acid ABC transporter	2	1	2h, 120h
EF0300	Q838Y3	Putative membrane protein	1	1	120h
EF0365	NA	Conserved hypothetical protein	11	2	2h, 120h
EF0394	Q93LK3	SalB	1	1	Tn
EF0402	Q838N7	Na+/H+ antiporter	1	1	2h
EF0415	NA	Conserved hypothetical protein	1	1	72h
EF0417	NA	Conserved hypothetical protein	5	2	2h, 72h, 120h
EF0457	Q838J0	PTS system, IIB component	4	2	72h, 120h
EF0675	Q837Z8	Glycine betaine ABC transporter	1	1	2h
EF0676	Q837Z7	Arginine repressor	1	1	Tn
EF0699	NA	Conserved hypothetical protein	2	1	72h, 120h
EF0721	Q837V7	ATP-dependent DNA helicase PcrA	1	1	Tn
EF0722	Q837V6	DNA ligase, NAD-dependent	1	1	120h
EF0762	Q837R9	Excinuclease ABC, subunit B	1	1	2h
EF0790	Q837P4	ABC transporter, ATP-binding	1	1	120h
EF0798	NA	Hypothetical protein	9	1	2h, 72h, 120h
EF0799	P37710	Autolysin	4	NA	Tn
EF0873	Q837G8	Cro/CI family transcriptional regulator	1	1	120h
EF0910	Q837D3	Peptide ABC transporter	1	1	72h
EF0977	Q836W8	N utilization substance protein B	4	2	72h, 120h
EF0983	Q836W2	ArgR family transcriptional regulator	2	NA	Tn
EF0984	Q836W1	DNA repair protein RecN	1	1	120h
EF0999	Q836V4	Conserved hypothetical protein	1	NA	Tn
EF1014	NA	Hypothetical protein	1	1	72h
EF1017	Q836U0	PTS system, IIB component	2	2	2h, 72h
EF1081	NA	Conserved hypothetical protein	3	1	72h, 120h
EF1090	Q836M1	ebpR	4	NA	Tn
EF1091	Q836M0	ebpA	2	NA	Tn
EF1092	Q836L9	ebpB	1	NA	Tn
EF1093	Q836L8	ebpC	1	NA	Tn
EF1094	Q836L7	srtC	1	NA	Tn
EF1306	Q835R9	HrcA	2	NA	Tn

EF1308	Q835R7	dnaK	1	NA	Tn
EF1310	Q835R5	dnaJ	1	NA	Tn
EF1348	Q835M9	Glucan 1,6-alpha-glucosidase	1	1	72h
EF1591	Q834Q1	AraC family transcriptional regulator	3	1	2h, 72h
EF1592	Q834Q0	ABC transporter, ATP-binding	1	1	72h
EF1718	Q834E0	Dihydroorotase	1	1	Tn
EF1727	P36920	ebsA protein	3	1	2h, 120h
EF1755	Q834B4	Phosphate ABC transporter	3	1	72h, 120h
EF1809	Q833W6	ebrA	14	5	72h, 120h
EF1826	Q833V0	Alcohol dehydrogenase	5	3	72h, 120h
EF1918	NA	Conserved hypothetical protein	8	2	2h, 72h, 120h
EF1949	NA	Conserved hypothetical protein	2	1	2h, 120h
EF1955	Q833J4	Sigma-54 dependent DNA-binding response regulator	1	1	72h
EF1962	Q833J0	Triosephosphate isomerase	1	1	120h
EF1974	Q833H8	GTP pyrophosphokinase	1	1	120h
EF1978	Q833H5	DNA-3-methyladenine glycosylase	5	1	2h, 120h
EF2050	Q833B4	ABC transporter	2	1	2h, 120h
EF2178	Q832P3	Putative membrane protein	1	1	72h
EF2193	Q832N0	dTDP-4-dehydrorhamnose 3,5-epimerase	1	1	120h
EF2203	Q832M2	TetR family transcriptional regulator	1	1	120h
EF2207	Q832L9	DNA-binding protein, Fis family	6	1	2h, 120h
EF2352	NA	GTP-binding protein LepA	1	1	2h
EF2353	NA	GNAT family acetyltransferase	1	1	120h
EF2371	Q831X4	Asparaginyl-tRNA synthetase	1	1	72h
EF2372	NA	Aspartate aminotransferase	1	1	120h
EF2380	Q9RPP2	Membrane-associated zinc metalloprotease	1	1	120h
EF2382	Q831W5	Glucose 1-dehydrogenase	1	1	120h
EF2432	NA	Metallo-beta-lactamase superfamily protein	1	1	120h
EF2570	NA	Aldehyde oxidoreductase	5	3	72h, 120h
EF2590	NA	Conserved hypothetical protein	1	1	120h
EF2668	Q830V1	Magnesium transporter	2	2	72h, 120h
EF2744	Q830N4	M42 family peptidase	5	2	2h, 72h, 120h
EF2767	Q830L3	Transcriptional regulator	1	1	120h
EF2858	Q830D0	Threonyl-tRNA synthetase	1	1	120h
EF2889	Q830A7	2-hydroxy-3-oxopropionate reductase	1	1	2h
EF2924	NA	Metallo-beta-lactamase superfamily protein	1	1	72h
EF3008	NA	Conserved hypothetical protein	1	1	120h
EF3056	Q82ZJ9	srtA	1	1	Tn
EF3064	Q82ZJ2	Polyribonucleotide nucleotidyltransferase	1	1	120h
EF3124	0	Polypeptide deformylase	1	1	120h
EF3177	NA	Conserved hypothetical protein	2	2	2h, 120h
EF3258	NA	Conserved hypothetical protein	2	2	72h, 120h
EF3282	Q82YZ7	clpC	3	2	72h, 120h

EF3290	Q82YZ0	Sensor histidine kinase	1	1	2h

* Represents how many times the insert was isolated from clones in the RIVET screen or how many times the transposon inserted into the ORF in the transposon screen

** This column shows the similarity in the sequence surrounding the listed ORF using the Region Compare function on the ITGR website. * indicates only the listed ORF has sequence similarity to other ORFs in other organisms. ** indicates that one other ORF adjacent to the listed ORF has sequence similarity to other ORFs in other organisms. *** indicates that two or more ORFs adjacent to and including the listed ORF have sequence similarity ORFs in other organisms.

majority of clones contained one or more ORFs (or partial ORFs) and associated 5' UTR regions oriented such that transcription from a promoter within the UTR would read into the flanking vector sequences and result in *tnpR* expression. Exceptions to this pattern will be discussed below.

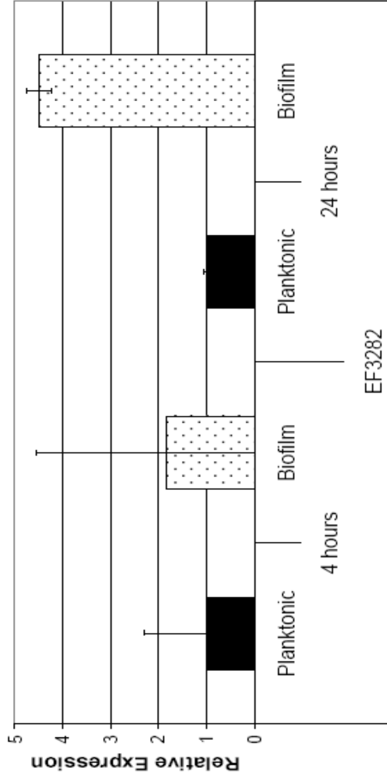
I was able to identify 68 unique cloned regions containing putative biofilm-induced promoters from the screen described above (table 3). As illustrated in figure 12, the adjacent genes whose expression is driven by these promoters are distributed around the entire genome, and encode proteins with a variety of predicted functions. The genes in this group include those previously implicated in biofilm formation (1, 6, 9, 12, 15, 34, 43, 44, 47, 48, 54, 57, 58, 64-66, 68, 70, 72, 74, 88, 100), as well as numerous genes not known to affect formation of biofilms and in many cases were never analyzed functionally. The previously reported biofilm genes of the enterococci are not represented in this group. This is not surprising, given that most previously identified determinants likely mediate adherence, and would therefore need to be expressed in planktonic cells to allow initiation of surface growth. Such genes would have been selected against in our screen during the planktonic growth of the inocula used for biofilm formation. Several of the loci were represented in multiple unique clones suggesting that the screen was approaching saturation under the sampling conditions we used. Table 4 shows the **15** genes that were identified in non-sibling clones more than twice. The most frequently-identified locus contained EF1809, a putative transcription regulator of the GntR family (35), and isolated in **5** unique clones. Further analysis of EF1809 is described below.

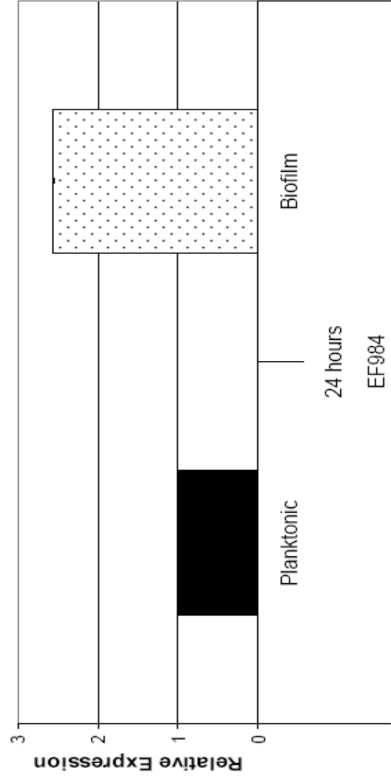
Table 4. Non-sibling clones isolated more than twice

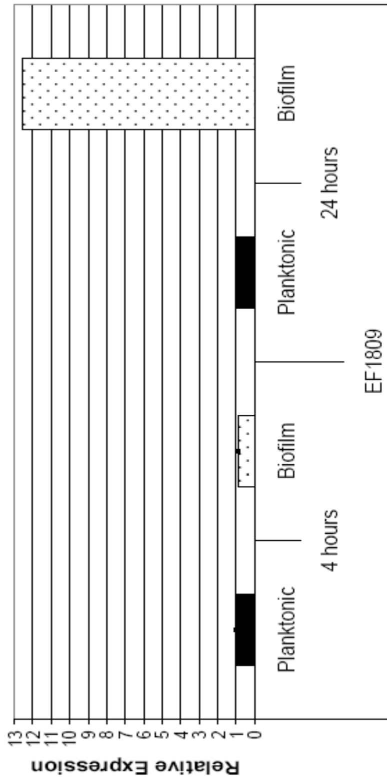
TIGR Identifier	Annotation	Number of Unique Clones
EF0038	glutamate 5-kinase	5
EF0059	UDP-N-acetylglucosamine pyrophosphorylase	8
EF0365	conserved hypothetical protein	11
EF0417	conserved hypothetical protein	5
EF0457	PTS system, IIB component	4
EF0798	hypothetical protein	9
EF0977	N utilization substance protein B	4
EF1081	conserved hypothetical protein	3
EF1591	transcriptional regulator, AraC family	3
EF1727	ebsA protein	3
EF1755	phosphate ABC transporter, ATP-binding protein	3
EF1809	transcriptional regulator, GntR family	14
EF1826	alcohol dehydrogenase, zinc-containing	5
EF1918	conserved hypothetical protein	8
EF1978	DNA-3-methyladenine glycosylase	5
EF2207	DNA-binding protein, Fis family	6
EF2570	aldehyde oxidoreductase, putative	5
EF2744	peptidase, M42 family	5

Confirmation of the RIVET Screen Results. Several genes were chosen for initial follow-up studies confirmation based on several criteria. EF3282 was chosen because homologues of this gene have been implicated in biofilm formation in other organisms. EF798 and EF984 were chosen because they are physically linked to genes identified in the complementary biofilm transposon screen and EF1809 and EF2207 were chosen for further examination because they were identified in the RIVET screen more than three times. These genes were identified from many different regions of the genome, and likely encoded diverse biological functions.

Quantitative RT-PCR was used to confirm that selected cloned genomic fragments identified in the RIVET screen contained promoters that were differentially activated during biofilm growth relative to planktonic growth. For this analysis RNA was harvested from *E. faecalis* OG1RF cells after 4 or 24 hours of planktonic or biofilm growth under the same conditions employed in the RIVET screen. The 24 hour time point was chosen for these conformational studies based on FESEM data that showed that 24 hour biofilms are very similar from those at 72 and 120 hours and the 4 hour time point represents and early point in biofilm development in which there are enough cells present to harvest for RNA isolation. The results of this experiment are shown in figure 14. Expression levels were examined for four different loci, EF1809, EF2207, EF3282 and EF984. In all cases there was a higher level of expression seen in the 24 hour biofilm sample compared to the 24 hour planktonic sample. This was not the case at 4 hours. Only EF3282 showed a modest expression increase and that difference was not statistically significant. The 4 hour time point showed more variability in gene expression and most of the RIVET genes were likely


Relative Expression
0
1
2
3
4
5
Planktonic
Biofilm
4 hours
Planktonic
Biofilm
24 hours
EF3282


Relative Expression
0
1
2
3
Planktonic
Biofilm
24 hours
EF984


Relative Expression
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Planktonic
Biofilm
4 hours
Planktonic
Biofilm
24 hours
EF1809

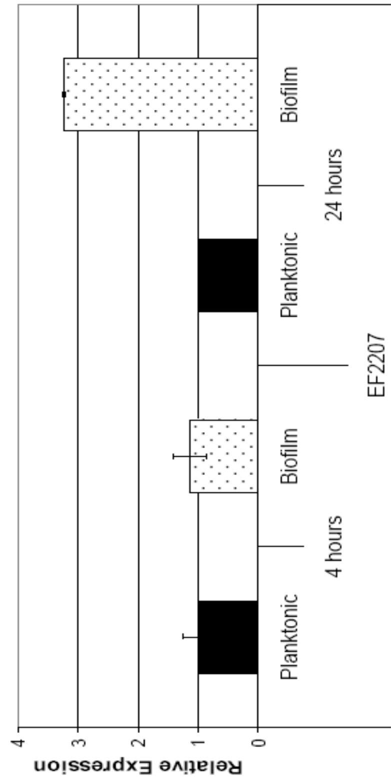

Relative Expression
0
1
2
3
4
Planktonic
Biofilm
4 hours
Planktonic
Biofilm
24 hours
EF2207

Figure 14. Confirmation of Differential Expression of RIVET-Identified Genes During Biofilm Growth. Transcription of four genes identified in the RIVET screen was analyzed using qRT-PCR analysis of RNA extracted from either planktonic cells or biofilm cells after 4 (exception being EF984) and 24 hours of growth. All graphs show the fold expression change on the y axis normalized to that of the reference gene, *gyrB* (7). This gene was shown to be expressed at the same level in biofilm and planktonic cells in *E. faecalis* strain OG1RF, data not shown. The level of expression of the gene of interest relative to *gyrB* in the planktonic sample was set at one in all cases and is shown in black on the x axis along with the biofilm sample shown in white with black dots . A. Expression of EF1809. B. Expression of EF3282. C. Expression of EF2207. D. Expression of EF984. Error bars represent 1 standard deviation. Asterisk indicates a P value of 0.001.

turned on after 4 hours due to the long duration of the RIVET screen. In the case of EF1809 the biofilm expression level was 12.5 times that of the planktonic expression level although it was not differentially expressed at 4 hours. The high level of expression of this locus in biofilms is consistent with the frequent isolation of EF1809-containing clones in the RIVET screen. The fact that differential expression was observed for all four randomly-selected clones, suggests that the isolation of *E. faecalis* loci in the RIVET screen is predictive of increased expression of those loci during biofilm growth.

I have begun to assess the functional role of the RIVET-identified loci in biofilm formation, by constructing in-frame markerless deletions in selected genes and examining the mutants for defects in biofilm formation using a CFU-based accumulation assay. The mutant and wild-type cells were grown as biofilms for 24h under the same conditions used in the RIVET screen. As shown in figure 15, null mutations in 3/4 RIVET-identified genes, EF 1809, EF 798 and EF984 but not EF3282, all resulted in major reductions in the CFU of 24h biofilms relative to the wild type. In all cases tested with biofilm defects, these defects were complemented by nisin-induced expression of the cloned wild-type gene. Thus the RIVET screen has successfully identified genetic determinants that are specifically upregulated in biofilm growth and that play a significant role in biofilm formation.

RIVET Screen Results are Complementary to those of a Transposon Screen for biofilm determinants in *E. faecalis* I recently described a new random transposon mutagenesis system for use in Gram-positive organisms and demonstrated its utility to investigate biofilm formation

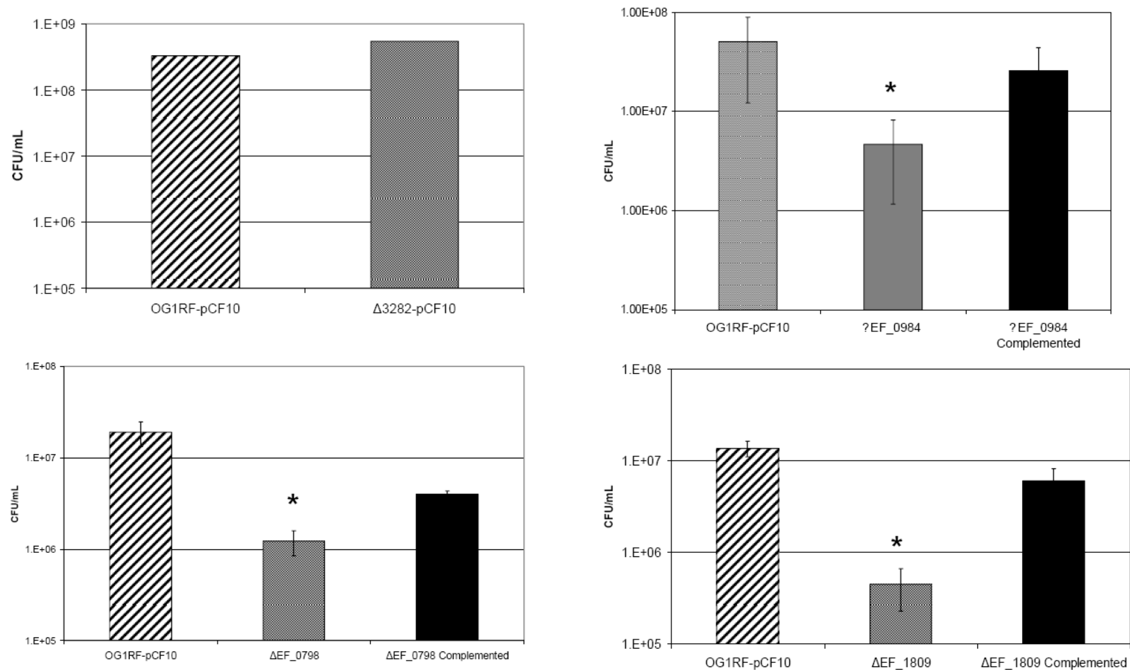


Figure 15. Biofilm Defects of *E. faecalis* Strains carrying Null Mutations in RIVET-identified Genes. Isogenic strains carrying non-polar, null mutations in either EF1809, EF798, EF984 or EF3282 were examined for biofilm formation on submerged cellulose membranes. The populations of mutant cells in biofilms after 24h was compared with those obtained with either wild-type, or with the null strain expressing the cloned wild-type gene in *trans* as described in Materials and Methods. This assay was repeated on five separate occasions. In planktonic growth, neither the null strains nor the complemented strains showed a defect as compared to wild-type (data not shown). The strain designations are indicated on the x axis, CFU/mL are shown on the y axis. Error bars represent 1 standard deviation. P values are as listed: EF1809, 0.01; EF0798, 0.01; EF0984, 0.01.

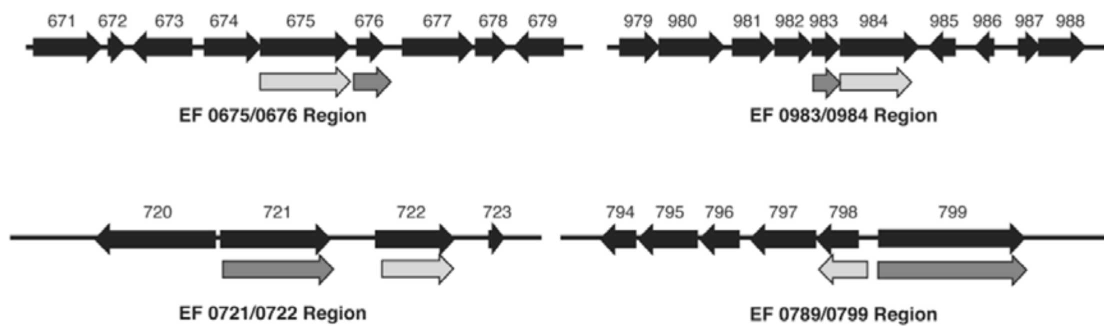


Figure 16. RIVET and Transposon Screens for Biofilm Determinants Identify Unique, but Linked Genes. Linear maps of four regions (black arrows) where the RIVET screen and transposon screens identified adjacent genes are depicted in the upper part of the figure. Annotation of the genes is shown in the lower part of the figure; RIVET-identified genes are in light grey and transposon-identified genes are in dark grey.

(53). Comparison of the data sets for the two screens was noteworthy in that each screen identified unique genes, but in several cases these genes were closely linked, and possibly in shared regulatory networks as well. Figure 16 shows maps of pairs of adjacent genes where one gene was identified in the transposon screen and the other was identified in the RIVET screen. The annotation of the predicted proteins, and the linkage and organization of these pairs of genes indicates that at least some of these pairs may represent a regulator and its target, and suggests experimental approaches to examine these relationships further. Interactions between these gene pairs is supported in some cases by their molecular organization, and by the fact that in two cases tested thus far, inactivation of one of the putatively linked genes (EF 799 identified by transposon mutagenesis (53), and EF984 identified in the RIVET screen (figure 16) produced similar phenotypic defects in biofilm formation to those observed following disruption of the other predicted linked gene (EF798 (figure 16), and EF983 (53). Further analysis is required to verify the predicted regulatory interactions, but the data suggest that the physical linkages could reflect functional linkage.

Comprehensive genomic screening reveals multiple novel regulatory genes related to *E. faecalis* biofilm formation and extensive conservation of putative novel biofilm determinants in Gram-positive pathogens. An important conclusion from our cumulative studies is that the *E. faecalis* core genome contains many predicted transcription factors, heretofore not investigated, that appear to be important in biofilm formation. Table 5 lists 8 predicted DNA binding proteins implicated in biofilm formation based on our screens. The *E. faecalis hrcA* (EF1306) gene and other neighboring genes is highly similar to a conserved stress response operon that also plays

Table 5. Predicted DNA binding proteins identified in the RIVET screen

Gene Desgnator	Family Annotation	Screen Identified	Confirmed Biofilm Phenotype*
EF0117	GntR family	RIVET	ND
EF0676	ArgR family	Transposon	YES
EF0873	Cro/CI family	RIVET	ND
EF0983	ArgR family	Transposon	YES
EF1306	HrcA	Transposon	YES
EF1591	AraC family	RIVET	ND
EF1809	GntR family	RIVET	YES
EF2203	TetR family	RIVET	ND

* ND = Not Yet Determined

a role in biofilm development in the Streptococci (54). The other 7 putative regulators (or orthologs in other organisms) listed in table 5 have not been analyzed prior to our studies. This list of predicted transcription factors is probably an underestimate since our complete collection of candidate biofilm genes (table 3) includes many possible regulatory proteins (such as EF798; figure 16) whose functions and biochemical activities are not known. Interestingly a majority of previously uncharacterized genes identified in our studies are highly conserved among a large group of low G+C gram positive bacteria. This suggests the possibility that a large number of conserved biofilm determinants remain to be identified and functionally analyzed in many important pathogenic bacteria (table 3).

DISCUSSION

The work presented in this paper demonstrates the utility of a RIVET screen for identification of novel genetic determinants for biofilm formation in the Gram-positive nosocomial pathogen *E. faecalis*. In this screen 68 candidate biofilm genes were identified; these genetic determinants include those annotated to have functions previously associated with biofilm formation (stress response and polysaccharide metabolism) as well as many that are completely novel. We identified 17 conserved hypothetical genes and 7 genes predicted to encode DNA binding proteins not previously linked to biofilm formation. As noted above, the current results may be relevant to elucidation of the genetic basis for biofilm formation in other organisms (table 3). This RIVET screen and a recent transposon mutagenesis study also undertaken by our group (53) both focused on genetic determinants in the core genome of *E. faecalis*. While the former study required the use of a microtiter plate biofilm assay, the RIVET study

enabled us to use a submerged coupon system previously shown to support robust formation of *E. faecalis* biofilms with distinctive 3-dimensional architecture and abundant production of extracellular matrix (29). We suspect that this may account for the fact that many more predicted biofilm determinants were identified in RIVET screen. While it is certainly likely that additional determinants could be identified by changing the conditions for biofilm growth used in the screens, the cumulative set of genes already identified may represent a large percentage of the genes involved in biofilm formation in *E. faecalis*. This work thus comprises the first comprehensive investigation of determinants of biofilm formation in the core genome of *E. faecalis* or closely related organisms.

Comprehensive validation of the RIVET screen would require confirmation of differential expression during biofilm growth, and biofilm defects of non-polar mutants for every genetic locus identified. This was beyond the scope of the current study, but it was important to sample a few representative loci to justify longer-term follow-up studies. In an attempt to provide an unbiased sample of predicted biofilm loci we examined a partially overlapping subset of candidate genes, widely distributed around the genome and having a diverse predicted functions. The results presented in figures 13 and 14 suggest that isolation of a particular locus in the RIVET screen is predictive of involvement of that locus in biofilm formation. Current and future studies will extend these analyses to genes predicted to function in the same pathway or process as the genes selected initially, and to other loci. EF1809, encoding a predicted DNA binding protein of the GntR family (35) was most frequently identified in non-sibling clones by the RIVET screen. It has also been confirmed to be differentially expressed via qRT-PCR

Table 6. RIVET clones that do not follow convention

TIGR ID	SwissProt ID	Annotation	Total Hits	Rivet Clone Orientation Relative to ORF
EF0790	Q837P4	ABC transporter, ATP-binding/permease protein	1	antisense internal
EF0873	Q837G8	transcriptional regulator, Cro/CI family	1	antisense internal
EF0910	Q837D3	peptide ABC transporter, permease protein	1	antisense including intergenic region
EF2590	NA	conserved hypothetical protein	1	sense internal
EF2858	Q830D0	threonyl-tRNA synthetase	1	antisense internal
EF2924	NA	metallo-beta-lactamase superfamily protein	1	antisense internal
EF3064	Q82ZJ2	polyribonucleotide nucleotidyltransferase	1	sense internal

(figure 14) and shown to be necessary for the ability of *E. faecalis* to form a wild-type biofilm (figure 15). The GntR family of transcriptional regulators consists of a N-terminal helix turn helix region and a C-terminal effector-binding domain (82). The GntR-family includes the following sub-categories: MocR, YtrR, FadR, AraR, HutC, PlmA, DevA and DasR (2, 82). Based on sequence similarity it appears that EF1809 is a member of the HutC/FarR sub-family (2). EF1809 as well as the other members of this group contain a C-terminal UbiC Transcription Regulator Associated (UTRA) domain as their effector binding domain which has been shown experimentally in other family members to modulate the activity of some of these transcription factors in response to small molecules (2). The null mutant in EF1809 was reduced in biofilm formation by about 50-fold as compared with the wild-type strain. From these results we conclude that EF1809 is a novel regulator involved in biofilm formation and propose to designate this gene *ebrA* (enterococcal biofilm regulator). Purification of EbrA will allow us to generate an antibody to this protein which in conjunction with the purified protein would allow for many more biochemical experiments to elucidate the role of this regulator in biofilm formation.

In addition to the 68 genomic regions described above and in table 3, we isolated 8 clones (table 6) containing genomic regions where either: 1) the entire insert was within an ORF, 2) the predicted transcription of all of the ORFs within and flanking the insert was in the opposite direction from the recombinase reporter gene in the recombinant plasmid, or 3) both 1) and 2) were true. It is possible that these clones are artifacts or that they encode small regulatory RNAs or peptides whose expression is increased during biofilm development. This suggests that the RIVET screen has the potential to identify

significant small non-protein-coding genetic determinants that might be missed in other screens, due to transient expression, to the small size of the genetic locus, or to the confounding effects of other overlapping determinants in the same region. We are currently examining selected clones from this group to assess their significance.

Null mutations with biofilm defects (e.g. figure 15) are likely to be reduced in virulence in experimental animal models of infections that involve biofilm formation such as endocarditis (61), and such studies are being undertaken. If this prediction holds, and if the conserved genes in other pathogens have similar functions, these determinants might make useful targets for the development of antimicrobial drugs that could block infections without directly killing the bacterial agent. Such compounds would also be useful tools in more detailed basic studies of biofilm development. The large number of novel genes identified in our studies comprises a significant pool of target candidates for such studies.

CHAPTER 4

Characterization of EbrA: A modulator of carbon metabolism and eDNA release in *Enterococcus faecalis*

EbrA is a regulator of the formation of complete biofilms in *Enterococcus faecalis* that I identified in a screen for genes activated during biofilm growth. The function of EbrA in biofilm formation is unknown. To begin to address that question I set out to identify the regulon of this transcription factor using the proteomic technique, iTRAQ. I present evidence that EbrA is involved in the regulation of carbon metabolism, and it is sensitive to changes in nutrient levels. In addition qPCR analysis of mRNA levels suggests that EbrA is also functioning as a negative regulator of *gelE*, a key player in the current model of eDNA release into the biofilm matrix of this organism. I propose a modified model of eDNA release that includes EbrA as a negative regulator of *gelE* under conditions of nutrient limitation where cell lysis is disadvantageous for the survival of the biofilm culture.

INTRODUCTION

Enterococcus faecalis is a low G+C Gram positive bacterium that can form biofilms on biotic and abiotic surfaces. *E. faecalis* is a commensal organism of the intestinal tracts of both vertebrates and invertebrates and can also be isolated from environmental samples. The ability of *E. faecalis* to form biofilms likely enhances its pathogenicity in nosocomial environments and plays a role in persistence and horizontal gene transfer. Our group used two genetic screens (5, 53) to identify over 80 potential determinants of enterococcal biofilm formation. For one of those 80 genes, EF_1809 (5); a markerless deletion conferred a biofilm defect. Expression of this gene was greatly increased in biofilm compared to planktonic cells. It was annotated by the TIGR database to be a transcriptional regulator and so it was named Enterococcal biofilm regulator (EbrA). As a previously unstudied regulator of biofilm formation we thought it important to further

characterize the role of this regulator in biofilm formation. As a first step we chose to identify the regulon of EbrA.

To investigate the effects of a deletion in *ebrA* on the proteome of biofilm cells I chose to use iTRAQ, a non-gel based method of proteome exploration. It offers some advantages over traditional proteomic methodologies. iTRAQ/LC/MALDI-TOF-tandem mass spectrometry allows accurate identification and quantitation of all proteins within a complex sample, regardless of molecular weight or isoelectric point, (30) therefore facilitating analysis of a diverse set of proteins with various functional roles, and differing abundance (30, 55, 83). iTRAQ is fast becoming the standard in proteome exploration, displacing older technologies like 2-D gels.

In this report I describe the preliminary characterization of the role of the EbrA regulator in biofilm formation in *E. faecalis*. Results of the iTRAQ experiments were coupled with phenotypic tests of carbon metabolism in biofilms and qPCR analysis of genes of interest. These studies revealed that EbrA is involved in responding to changes in nutrient levels. In addition these experiments have allowed refinement of the current model of eDNA release into the biofilm matrix to include EbrA as a negative regulator of *gelE* expression to promote biofilm survival and maintenance. This model currently only includes an activating transcription factor, so this is the first mention of a repressor in the system. This study highlights the complexity of biofilm formation and the close link between cellular metabolism and survival of biofilm cultures.

MATERIALS AND METHODS

Bacterial Strains and Growth: *E. faecalis* strain OG1RF (26), has been described. The Δ*ebrA* strain is an in-frame, deletion of the EF1809 ORF, in an OG1RF background. Construction of this mutant strain was made using methods described in (50) and described in detail in Chapter 3. Bacterial strains were stored at -80°C in Brain Heart Infusion broth (BHI; prepared according to the manufacturer's instructions) supplemented with 50% glycerol. Unless otherwise indicated, all culture media were from Difco. Culture media were as follows: TSB, prepared according to the manufacturer's instructions, without additional exogenous carbohydrate added, BHI, prepared according to the manufacturer's instructions. M9-YE, a medium containing M9 salts, yeast extract and case amino acids (26) was used in the carbon source assays. The standard recipe for this medium uses glucose at a final concentration of 0.4%. In some experiments glycerol or galactose were substituted for the glucose in this medium and were used at the concentration indicated in the experiment. All bacterial strains were grown at 37°C without any form of aeration unless otherwise noted for a specific assay. All biofilms were grown as described in (5) and briefly below.

Biofilm assays: Cells were grown on 1 cm^2 cellulose membranes (SpectraPor, Spectrum Labs) for CFU assays or 10 cm^2 cellulose membranes (SpectraPor, Spectrum Labs) for qPCR assays and protein extraction membranes were submerged in TSB-gluc or M9-YE after being inoculated with a 1:100 dilution of an overnight culture. In the case of any comparison experiments, overnight cultures were measured and adjusted so that the OD600 values were normalized before inoculating. The biofilms were harvested as previously described (5, 28). After harvesting cells at the indicated time point, cells were

serially diluted in potassium phosphate buffered saline and plated to enumerate the CFU/cm^2 or used directly for RNA or protein extraction.

iTRAQ and Protein Extraction: For identification and relative quantitation of proteins affected by the deletion of *ebrA*, iTRAQ reagents (Applied Biosystems) will be used in conjunction with liquid chromatography (LC) and mass spectrometry (MS). The isobaric reagents label all primary amines to yield labeled peptides. In tandem mass spectrometry (MS/MS) they produce strong, diagnostic, low-mass signature ions that allow for quantitation of two to eight different samples simultaneously. Protein was extracted from biofilm cells of both Δ*ebrA* and OG1RF grown as described above on cellulose biofilms in TSB-gluc for 24 hours. After harvesting, the cells were pelleted and flash frozen in a dry ice and 95% ethanol bath and then resuspended in lysozyme buffer (3) containing 30mg/ml lysozyme and incubated at 37C for 15 min. After the incubation the cells were centrifuged and washed twice with KPBS. At this point the cell pellet was resuspended in 500ul 7M urea, 2 M thiourea, 4% CHAPS and 1x HALT protease inhibitor (Pierce). These protein samples were subjected to ultracentrifugation at 50K for 1 hour at room temperature to eliminate particulate material from the samples. Extracted proteins from wild type and mutant cells were subjected to buffer exchange using a YM3 Microcon (Millipore), normalized for concentration of protein, reduced, alkylated, trypsin digested independently in parallel and labeled with iTRAQ reagents 115 and 117 respectively. The labeled peptides from both samples were pooled and subjected to 2D LC-MALDI-MS/MS using Tempo™ LC MALDI spotting system, and 4800 MALDI TOF/TOF™ analyzer at the Center for Mass Spectrometry at the University of Minnesota. This was done twice for confirmation of the results.

Proteomic Data Analysis: Protein identification and quantitation was carried out using ProteinPilot™ 2.0 software (Applied Biosystems). This software compares the fragmentation pattern of the spectra obtained by MS/MS to the theoretical fragmentation pattern of proteins in a protein database to identify the proteins. For relative quantization, ProteinPilot™ calculates the iTRAQ ratio for each reagent pair, and determines an average iTRAQ ratio for each protein. This ratio was used to identify proteins that are up or down regulated as a result of the deletion of Δ*ebrA* in biofilms. Data analysis was first conducted by exporting all data sets from Protein Pilot to Microsoft Excel (Office 2003). To be classified as significantly altered in expression, a protein and its representative peptides must have met the following criteria: (1) the protein had to demonstrate a database match with a enterococcal protein; (2) the protein had to be represented by at least two peptides of differing amino acid sequence at the 95% confidence level, (3) the test to determine if the ratio of protein expression equals 1 had to have a p-value less than 0.05, (4) the protein needed to have an error factor as calculated by Protein Pilot of less than 2, (5) the fold change needed to be greater than or equal to 1.2 or less than or equal to 0.8. (6) Finally to be included in table 7 the protein needed to match the criteria above for the two separate runs of iTRAQ that was performed on these samples for confirmation of the data.

Biolog Phenotypic MicroArray: OG1RF and Δ*ebrA* were sent to Biolog for analysis with their Phenotypic MicroArray® (PM) system. This array uses a 96 well plate based system to test for the ability to grow utilizing or in the presence of various chemicals and nutrient sources. PMs use Biolog's redox chemistry, employing cell respiration as a reporter of cell growth. If the phenotype is strongly "positive" in a well, the cells respire

actively, reducing a tetrazolium dye and forming a strong color. Incubation and recording of phenotypic data is performed by the OmniLog® instrument which captures a digital image of the MicroArray several times each hour and stores the quantitative color change values into computer files. The mutant and wild type strain were grown at 37°C in BUG-B medium (Biolog) and run on plates 1-20 which include plates that test carbon sources, nitrogen sources, osmotic growth and control and chemical sensitivity (www.biolog.com).

Microarray analysis: RNA was isolated as described in (5) from 24 hour OG1RF and Δ*ebrA* biofilms for four independent experiment. cDNA was made and labeled from the RNA using the Super Script III Direct cDNA labeling System (Invitrogen) using dyes Alexa 555 and Alexa 647 (both from Invitrogen) according to manufacturers instructions. All experiments were done as “dye swaps” so each strain was labeled with each dye and 1 experiment refers to a pair of dye swapped slides that are from an independent biological replicate of the original RNA. This experiment was done in quadruplicate.

The slides used in this experiment are oligo spotted arrays that represent 3229 ORFs from *E. faecalis* V583, the only strain sequenced at the time of printing printed onto GAPSII slides (Corning). Each ORF is represented by a 70 mer printed on the slide 4 times. The slides are prepared for the cDNA hybridization following the manufacturer’s instructions.

Hybridization of the cDNA to the microarray slides used and overnight hybridization (12-19 hours) in the MAUI 12-bay hybridization station. Mixing program FL on the instrument was used to move the hybridization solutions over the slides. There were 4 washes: wash 1 and 2, 2x SSC and 0.5x SDS, wash 3, 1x SSC, wash 4 0.1x SSC.

The slides were then dried in the centrifuge at 680 rpm for 8-10 min. This experiment was conducted at the Microarray facility at the University of Minnesota.

The slides were scanned with the ScanArray (Perkin Elmer) using Scan Array Express software (Perkin Elmer). These scanned images were imported into Bluefuse (BlueGnome) for alignment and quantification. Bluefuse was used to normalize the dye swaps using a global lowess. These files were then imported into Expressionist software (GeneData). In Expressionist genes were filtered on the following criteria: 1) Average spot intensity greater than 1000, confidence greater than 0.3, fold change greater than 1.4 or less than -1.4 and that the result was confirmed in more than one experiment.

qPCR: The RNA isolation and creation of cDNA was performed as described in (5). The qPCR was done as described in (5). The primers for *gyrB* (reference gene) were previously published in (94). All primers are listed 5' to 3'. The primers used for *gelE* are as follows, *gelE* Forward: CTTTTTGGGATGGAAAAGCA and *gelE* Reverse: CCGGCAGTATGTTCCGTTAC. The primers used for *sprE* are as follows, *sprE* Forward: GTAGTGACTGTCGGCAAACAAA, *sprE* Reverse: CCTGGATAGCCTGATATTGTGAC. The primers used for *ebrA* are as follows, *ebrA* Forward: ATAGAGGTTCCATCTATCGTCGTC and *ebrA* Reverse: ATATACGGGTATGTGGTTTTCGAC.

Planktonic Growth Curves: OG1RF and Δ*ebrA* were grown in M9-YE +0.05% glucose overnight and then washed and added to 150ul of MY-YE supplemented with either, 0.1 or 0.4% glucose, galactose or glycerol at a final starting OD600 value of 0.01. Cell were allowed to grow in a 96-well plate at 37C and were read on a spectrophotometer (Turner

Biosystems Modulus Microplate Multimode Reader 9300-010) to generate a growth curve.

RESULTS

Proteomic analysis using iTRAQ identified proteins that are differentially expressed between biofilms formed from Δ*ebrA* and OG1RF. Biofilms were grown for 24 hours using both Δ*ebrA* and OG1RF. Protein was extracted from the biofilm cells and subjected to analysis using iTRAQ. In this study we were able to identify a total of 607 proteins using iTRAQ. Thirty-one of these proteins were differentially expressed between the mutant and wild-type in biofilms as shown in table 7. The proteins that were included in this table meet very rigorous standards described in the materials and methods section and have been confirmed by two iTRAQ runs. It should be noted that 25 of the 31 proteins were increased in abundance in the mutant strain where as 6 of the 31 were decreased in abundance in the mutant strain. Of the 25 proteins that are increased in the mutant 7 were involved in some way in carbon catabolism. These results suggest that Δ*ebrA* is normally involved in the regulation, either directly or indirectly, of carbon metabolism in this organism.

Metabolic profiling of Δ*ebrA* compared to OG1RF in planktonic cells shows an decreased ability to grow utilizing N-acetylgalactosamine as a carbon source. OG1RF and Δ*ebrA* were sent to Biolog for analysis with their Phenotypic MicroArray® (PM) system. The mutant and wild type strain were run on plates 1-20 which include plates that test carbon sources, nitrogen sources, osmotic growth and control and chemical sensitivity (www.biolog.com). These strains were tested in the 96-well format used by BIOLOG which means that the information gathered was gathered on planktonic

Accession	Protein Function	Functional Category	Fold Change*	Total Peptides**	Peptides at 95% Confidence**
gi|229549472	Acetolactate synthase	Branched chain amino acid biosynthesis	2.1	29	13
gi|229549479	PTS family component IIB	PTS system	2.1	4	3
gi|229549473	Myo-inositol catabolism protein IolB	Myo-inositol catabolism	2.1	15	8
gi|229549471	5-dehydro-2-deoxygluconokinase	Inositol metabolism	1.9	27	16
gi|29342766	Phosphocarrier protein HPr	PTS system	1.6	12	4
gi|29342723	Hypothetical protein EF_0665	Hypothetical protein	1.6	9	3
gi|81436936	Dihydroorotase	Pyrimidine biosynthesis	1.5	10	4
gi|88953298	UPF0337 protein EF_1180	Protein binding	1.4	24	9
gi|229549882	Possible NAD(P)H dehydrogenase (quinone)	Sterol metabolism	1.4	4	2
gi|29343804	Lipoprotein, putative	Lipoprotein	1.4	17	9
gi|29345145	Dps family protein	Stress response, DNA protection	1.4	40	9
gi|29342399	Aminopeptidase C	Protease	1.4	29	9
gi|73920123	tRNA-specific 2-thiouridylase mnmA	2-thiolation of uridine of tRNA	1.4	10	6
gi|29342284	Basic membrane protein family	Membrane protein	1.3	11	5
gi|3122149	Glycerol kinase	Glycerol Metabolism	1.3	65	19
gi|29343968	Universal stress protein family	Stress response	1.3	21	6
gi|29343803	Conserved hypothetical protein	Hypothetical protein	1.3	10	4
gi|29344180	Hypothetical protein EF_2209	Hypothetical protein	1.3	13	6
gi|29345167	Pheromone cAD1 precursor lipoprotein	Conjugation	1.3	25	8
gi|29343553	Glyceraldehyde 3-phosphate dehydrogenase	Glycolysis	1.3	26	11
gi|73621756	50S ribosomal protein L31 type B	Ribosomal protein	1.2	29	10
gi|29343585	Hypothetical protein EF_1560	Hypothetical protein	1.2	18	9
gi|29342719	Oligoendopeptidase F, putative	Peptidase	1.2	18	8
gi|75541398	Large-conductance mechanosensitive channel	Membrane protein	1.2	13	6
gi|229549990	NADH oxidase	Oxidation of NADH to water	1.2	69	21
gi|29343758	UTP-glucose-1-phosphate uridylyltransferase	Galactose metabolism	0.8	6	4
gi|29344049	Endocarditis specific antigen	Endocarditis	0.8	26	13
gi|81437070	CTP synthase	Pyrimidine biosynthesis	0.8	24	8
gi|61216040	30S ribosomal protein S21	Ribosomal protein	0.7	5	3
gi|29343635	Formate acetyltransferase	Pyruvate fermentation	0.7	83	26
gi|229550191	Flotillin	Integral membrane protein	0.7	64	22

Table 7. iTRAQ-identified proteins differentially expressed between Δ*ebrA* biofilms and OG1RF biofilms cells.

While this is obviously not as useful as if the information gathered had come from biofilm cells there was still one difference noted between the strains. Δ*ebrA* had a decreased ability to grow planktonically utilizing N-acetylgalactosamine, which indicates a potential defect in galactose metabolism or the PTS-system.

The addition of glucose to the growth medium of Δ*ebrA* can rescue the growth defect normally seen in this mutant when grown as a biofilm. The growth medium normally used for biofilm formation in the cellulose biofilm assay is TSB made without the addition of any exogenous carbon source. TSB is has a low amount of fermentable sugars, which are the preferred carbon sources for the enterococci. Because of this fact other researchers routinely add glucose to this medium at either 0.25 or 0.5% final concentration when growing enterococcal biofilms (51). However our group has previously shown that with our lab strain we have seen the largest ratio of biofilm biomass to planktonic biomass in TSB (51). *ebrA* was identified as being involved in biofilms based on a growth defect seen in TSB (5). Because the results of the iTRAQ screen suggested that *ebrA* was involved in regulation of carbon metabolism I thought it would be interesting to see how the Δ*ebrA* strain preformed in the presence of additional glucose. Figure 17 shows that when the biofilm growth medium is supplemented with glucose at a final concentration of 0.5% and the biofilms are allowed to grow for 24 hours there is a complete rescue of the Δ*ebrA* strain growth defect seen in TSB. The glucose and non-glucose biofilms were started from the same overnight starter culture and grown in parallel. Taken together with the data from the iTRAQ screen this supports the hypothesis that *ebrA* is involved in regulating carbon metabolism in biofilms.

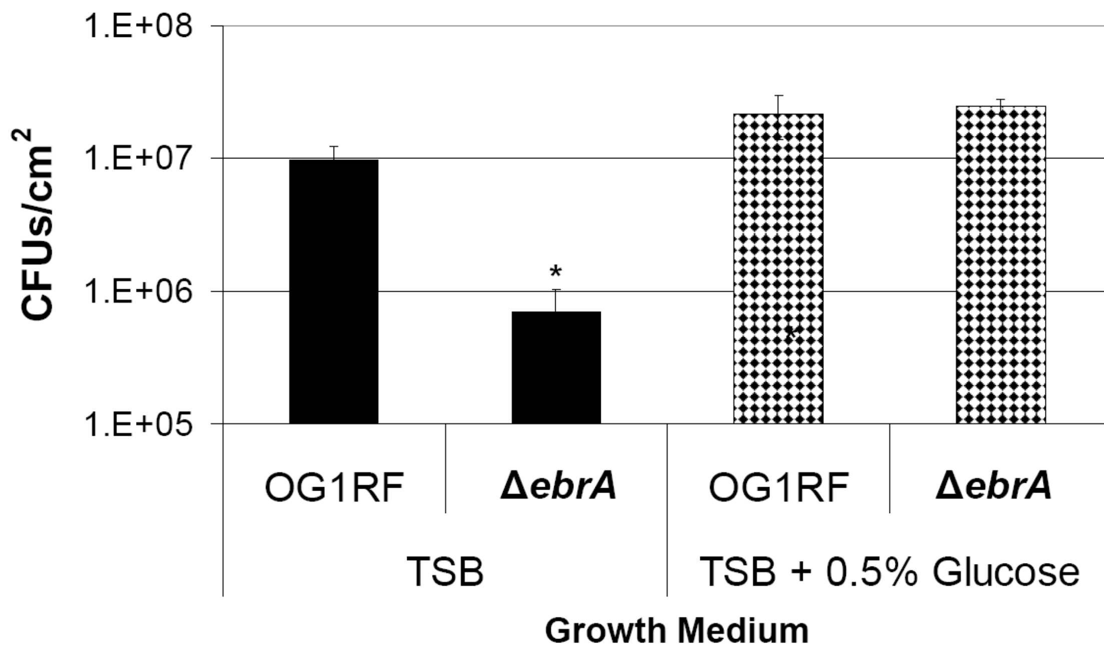


Figure 17. Exogenously added glucose rescues Δ*ebrA* growth defect. Biofilms of both the mutant strain and wild-type were grown for 24 hours on cellulose membranes as described in the materials and methods sections. The base medium, TSB, was the same for each experiment the only difference was the addition of a final concentration of 0.5% glucose in one set of biofilms. To keep all other variables the same a corresponding amount of sterile water was added to the non-glucose biofilms. Membrane adherent CFU were enumerated and expressed as CFU/membrane. Error bars represent 1 standard deviation. Asterisk represents a p-value of 0.05.

Other carbon sources rescue Δ*ebrA* biofilm defect, demonstrating that this phenotype is not glucose specific. TSB supports biofilm and planktonic growth of OG1RF without the addition of glucose as shown in figure 17 for biofilms and for planktonic growth (data not shown). To confirm that the rescue of the biofilm defect seen in figure 17 was attributable to the addition of glucose and to see if the addition of other carbon sources could produce the same rescue phenotype a growth medium was needed that did not support biofilm or planktonic growth without the addition of an exogenous carbon source. For these experiments I used M9-YE medium (26), which contains a small amount of yeast extract but without the addition of a carbon source supports little to no planktonic growth of OG1RF (data not shown) and therefore does not support biofilm growth. For the experiments shown in figure 18, glucose, galactose and glycerol, were added to the base M9-YE medium at the final concentrations 0.4%, 0.2% and 0.1%. Glucose and galactose are simple sugars brought into the cell via the PTS system. Glycerol is brought into the cell via diffusion and is not a simple sugar but does serve as a carbon source for OG1RF (10). The planktonic growth of Δ*ebrA* was tested in comparison to OG1RF in M9-YE medium with each of the carbon sources at each of the concentrations used for the biofilm study and there was no difference in growth (figure 19).

When grown as a biofilm in the presence of either glucose or galactose Δ*ebrA* showed no biofilm defect until the final concentration was limited to 0.1%. In glycerol, which is not actively transported, the concentration needed to be decreased to 0.2% before a statistically significant defect in biofilm formation was seen. These results show that this phenotype is not glucose specific and suggests that *ebrA* is involved in the

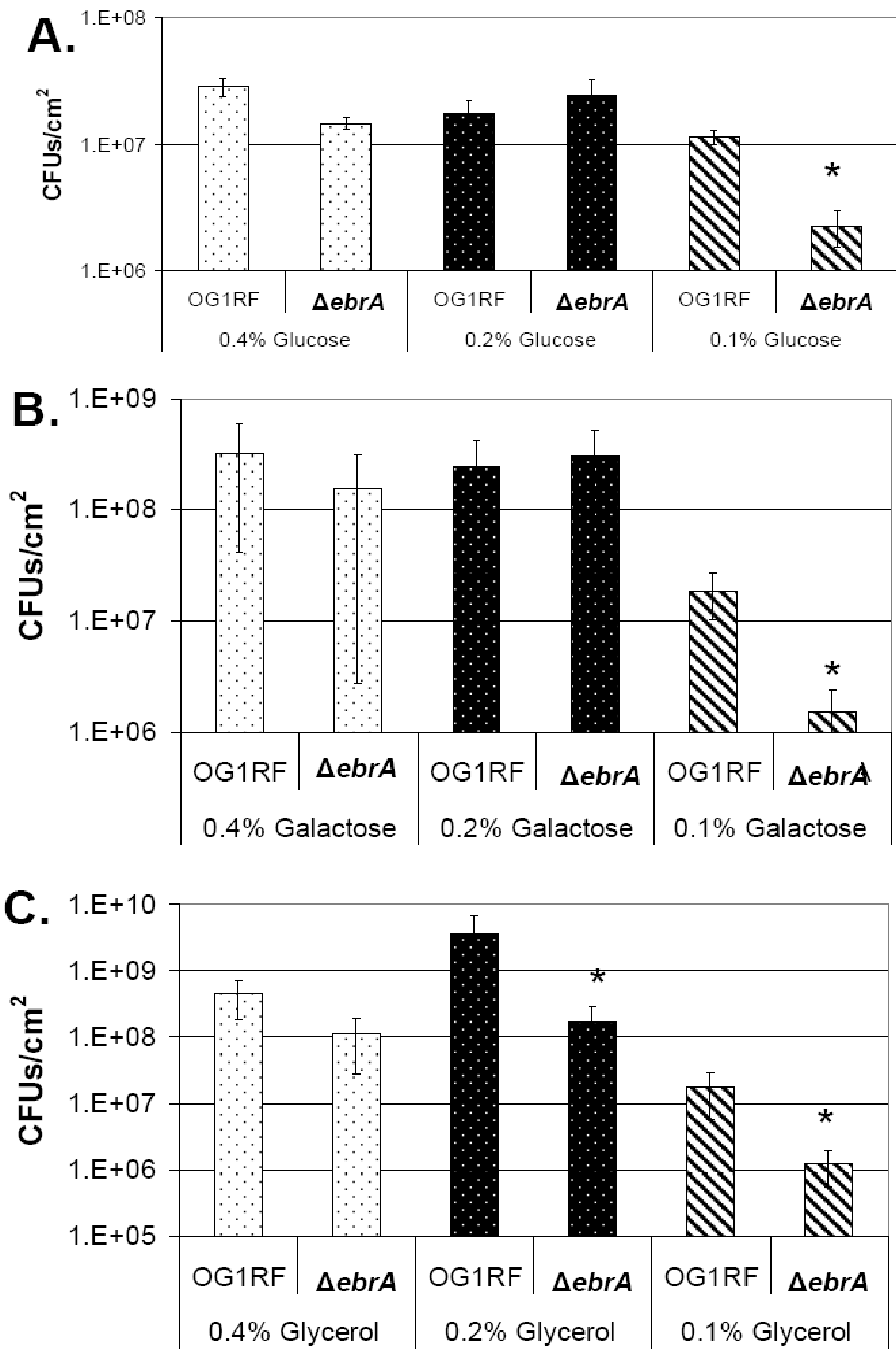
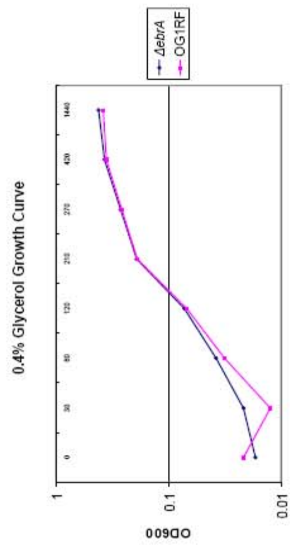
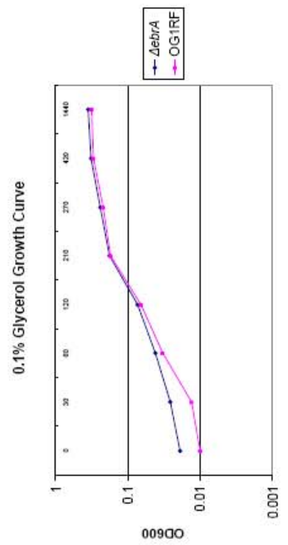
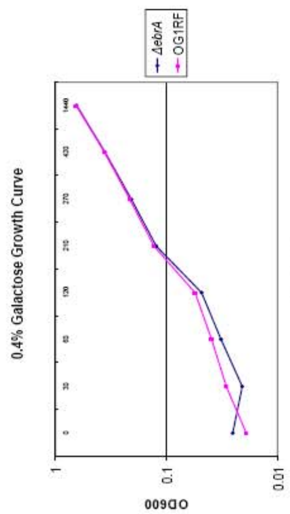

A.
1.E+08
1.E+07
1.E+06
CFUs/cm2
*
OG1RF
ΔebrA
OG1RF
ΔebrA
OG1RF
ΔebrA
0.4% Glucose
0.2% Glucose
0.1% Glucose
B.
1.E+09
1.E+08
1.E+07
1.E+06
CFUs/cm2
*
OG1RF
ΔebrA
OG1RF
ΔebrA
OG1RF
ΔebrA
0.4% Galactose
0.2% Galactose
0.1% Galactose
C.
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
CFUs/cm2
*
*
OG1RF
ΔebrA
OG1RF
ΔebrA
OG1RF
ΔebrA
0.4% Glycerol
0.2% Glycerol
0.1% Glycerol

Figure 18. High levels of exogenous carbon rescue Δ*ebrA* biofilm growth defect. All of the strains were grown as biofilms on cellulose membranes for 24 hours. These biofilms were grown in a base medium of M9YE with the addition of the carbon source at the concentration indicated in the graph. The cells were harvested as described in the materials and methods. Membrane adherent CFU were enumerated and expressed as CFU/mL. Error bars represent 1 standard deviation. Asterisk represents a p value of 0.05 or less.


0.4% Glycerol Growth Curve
OD600
1
0.1
0.01
0
30
60
120
210
270
420
1440
Time in Min
ΔebrA
OG1RF


0.1% Glycerol Growth Curve
OD600
1
0.1
0.01
0.001
0
30
60
120
210
270
420
1440
Time in Min
ΔebrA
OG1RF


0.4% Galactose Growth Curve
OD600
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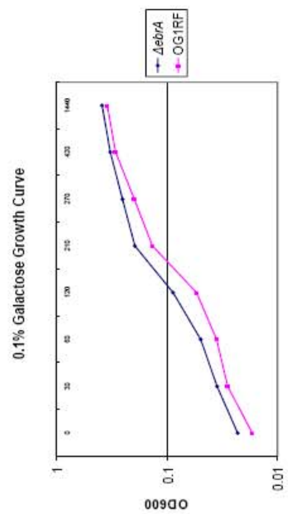
Time in Min
ΔebrA
OG1RF


0.1% Galactose Growth Curve
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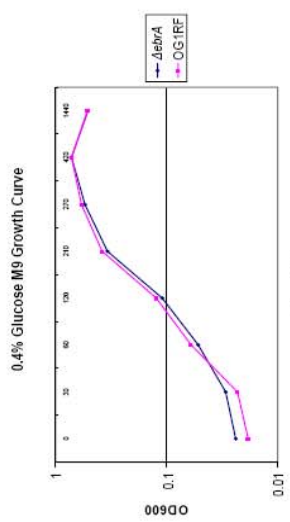
Time in Min
ΔebrA
OG1RF


0.4% Glucose M9 Growth Curve
OD600
1
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210
270
420
1440
Time in Min
ΔebrA
OG1RF

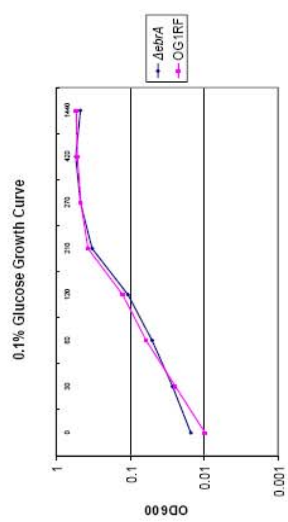

0.1% Glucose Growth Curve
OD600
1
0.1
0.01
0.001
0
30
60
120
210
270
420
1440
Time in Min
ΔebrA
OG1RF

Figure 19. Planktonic growth of Δ*ebrA* is equivalent to OG1RF in M9-YE medium supplemented with three different carbon sources. 96-well plates containing bacteria were incubated at 37C. All strains were measured in biological triplicate using eight technical replicates.

sensing of nutrient limitation in biofilms which allow the wild-type strain to adapt to form more robust biofilms.

Planktonic growth curve data show no Δ*ebrA* carbon source phenotype. When OG1RF and Δ*ebrA* are grown as planktonic cells in the M9-YE base medium supplemented with either 0.1 or 0.4% glucose, galactose or glycerol there is no growth defect in the deletion strain as compared to the wild-type strain. This indicates that the phenotype seen in the deletion strain is biofilm specific. This can be clearly seen in figure 19.

***ebrA* transcriptional levels to not change in response to levels of carbon in the growth environment**. To see whether or not *ebrA* mRNA levels would change in response to the level of carbon in the growth medium we grew biofilms of OG1RF for 24 hours in either TSB or TSB with 0.5% final concentration of glucose or glycerol. Regardless of the carbon source or the amount of carbon present the level of *ebrA* expression remained unchanged indicating that *ebrA* expression is not dependent on carbon level (data not shown).

Transcriptional analysis of Δ*ebrA* compared to OG1RF in 24 hour biofilms gives different results than iTRAQ because of different preparation of biofilm cells. Transcriptional profiling of mutant and wild-type biofilm cells using a printed 70 mer oligonucleotide array yielded 21 genes that have differing levels of transcription (table 8). Eleven of these genes are increased in expression in the mutant and 10 of the genes are decreased in expression in the mutant. The iTRAQ samples were prepared using mutanolysin and lysozyme to digest the cell wall before lysing the cells. This would eliminate cell wall anchored proteins and all secreted proteins. The cells used to isolate

Table 8. Transcriptional Differences in gene expression

TIGR IDs	Annotation	Fold Change OG1RF/ΔebrA
EF0990	cell division protein	-3.384094755
EF1480	conserved hypothetical protein	-2.718622565
EF3241	abortive phage resistance protein	-2.532714225
EFA0074	conserved domain protein	-2.490660025
EF0153	cell wall surface anchor family protein	-2.032520325
EF1818	gelE	-1.926782274
EF3329	DNA-binding response regulator	-1.906577693
EF0259	S4 RNA-binding domain protein	-1.796138303
EF0044	serine O-acetyltransferase	-1.651073198
EF0862	permease protein ABC transporter	-1.646429306
EF3237	DNA-directed RNA polymerase, beta-prime subunit	-1.42993327
EF0809	membrane protein	-1.415177782
EF3083	ABC transporter	1.432
EF2363	hypothetical protein	1.44675
EF2623	cadmium-translocating P-type ATPase	1.499125
EF3287	hypothetical protein	1.565
EF1993	holin	1.6235
EF0465	transcriptional regulator	1.739625
EF1389	NAD-dependent formate dehydrogenase	1.8145
EF1599	Cro/CI family regulator	2.0315
EFB0020	conserved domain protein	2.036
EF2106	conserved domain protein	2.1865

RNA for the microarray were treated in the same way. Not surprisingly, there was no overlap in the lists identified from the two screens since there are quite a few genes that potentially encode for cell wall anchored or secreted proteins on this gene list. One of the most interesting is EF1818 or *gelE*, a gene encoding for a secreted gelatinase that has been implicated in the mechanism for eDNA release into the matrix. It was not expected that *gelE* would be identified in our iTRAQ screen based on how the protein samples were prepared. As a secreted protein this would have been eliminated from the iTRAQ protein sample pool.

***gelE* but not *sprE* is increased in expression in Δ*ebrA* biofilms**. Previous work on *ebrA* expression (5) demonstrated that *ebrA* is only expressed between 4 and 24 hours of biofilm growth. I hypothesized that *ebrA* might regulate the expression of *gelE* or other proteins involved in eDNA release into the matrix. To examine this we used RT-PCR comparing cDNA made from RNA samples from Δ*ebrA* and OG1RF biofilms grown for 24 hours in TSB-gluc. As shown in Figure 20 there is a statistically significant difference in the expression level of *gelE* in Δ*ebrA* biofilms implying that *ebrA* is a negative regulator of *gelE* under these biofilm conditions. *sprE* which has been shown to be cotranscribed with *gelE* in planktonic cells (77) does not have a difference in expression levels indicating that under the conditions tested *sprE* is not cotranscribed with *gelE* under all conditions or that EbrA controls the expression of *gelE* indirectly.

Expression kinetics of *ebrA* and *gelE* in biofilms are inversely related to each other. When the expression levels of *ebrA* and *gelE* were examined kinetically in biofilms using qPCR at 4 and 24 hours the results showed that *gelE* expression was higher at 4 hours than 24 and *ebrA* expression was higher at 24 hours than at 4 hours (figure 21). These

results are correlatory but do support the assertion that EbrA is involved in negatively regulating *gelE* expression since as *ebrA* expression increased over time; *gelE* expression decreased.

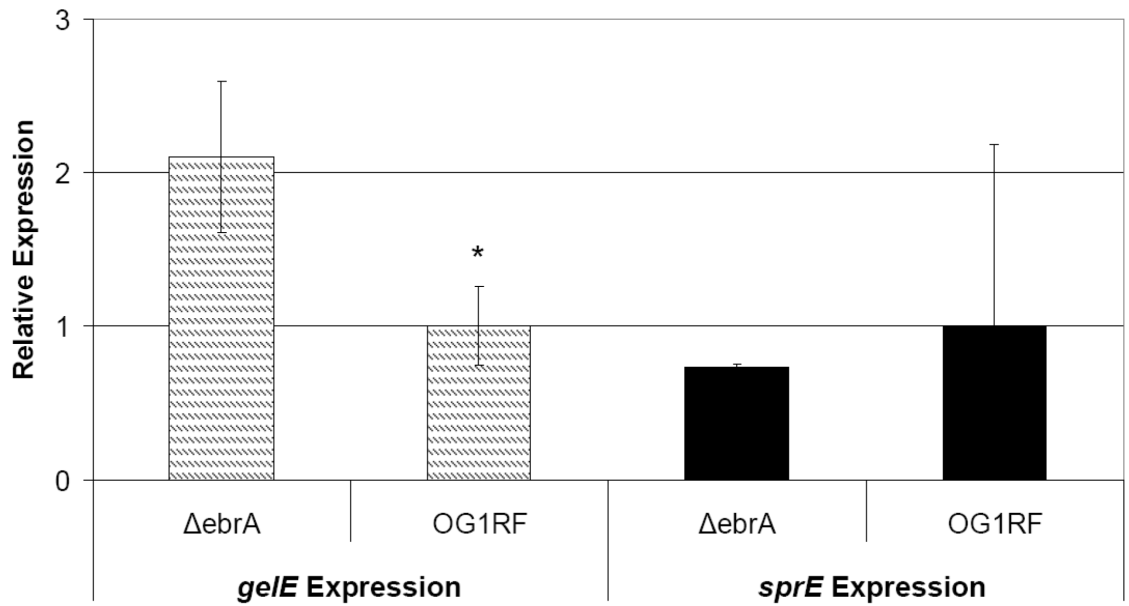
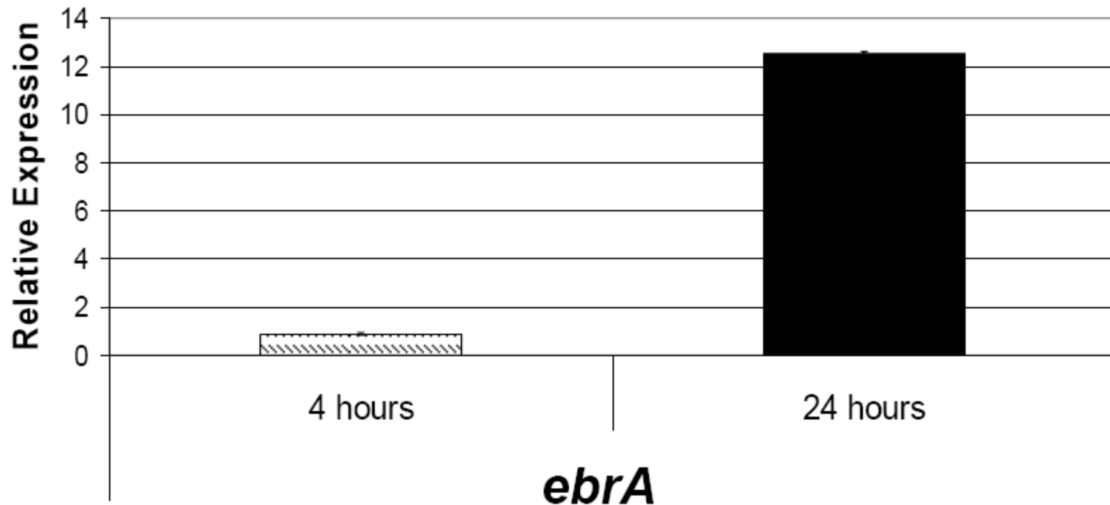


Figure 20. qPCR analysis of *gelE* and *sprE* expression levels in biofilms of Δ*ebrA* vs. OG1RF. OG1RF and Δ*ebrA* biofilms were grown on cellulose membranes for 24 hours and the cells were harvested for RNA extraction as described in the Materials and Methods. qPCR was performed as described in the Materials and Methods section. Error bars represent one standard deviation. Asterisk is for a p-value of 0.05.



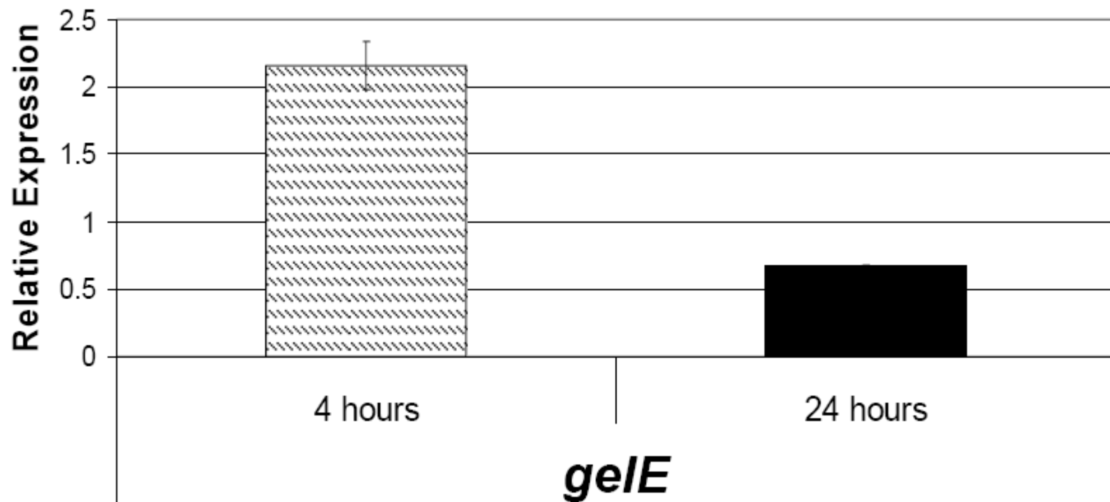


Figure 21. *ebrA* and *gelE* kinetic expression patterns are inverse to each other. OG1RF biofilms were grown on cellulose membranes for either 4 or 24 hours and the cells were harvested for RNA extraction as described in the Materials and Methods. qPCR was performed as described in the Materials and Methods section. The values in this graph are expressed as the biofilm/planktonic ratio of expression at each time point listed. Error bars represent one standard deviation.

The microarray analysis also showed that in the absence of *ebrA* there was an increase in *gelE* expression.

DISCUSSION

EbrA (EF1809) was identified using a RIVET screen designed to discover genes that are turned on in biofilms (5). It was shown that a deletion in this ORF had a biofilm defect when grown on cellulose membranes in TSB and additionally it was confirmed via qPCR that this ORF was differentially expressed between biofilm and planktonic cells (5). This gene was annotated as a GntR family transcriptional regulator.

Because it was annotated as a transcriptional regulator and it had a biofilm defect I was interested in identifying the regulon of this regulator. I chose to use iTRAQ to examine the proteome of EbrA by comparing OG1RF biofilm protein samples to that of protein samples from *ΔebrA* biofilms grown under identical conditions. My iTRAQ analysis identified 607 total proteins and we were able to categorize 31 proteins that were differentially expressed between the two samples (table 7). Twenty-five proteins were increased in abundance in the mutant strain and six were decreased in abundance in the mutant strain. Of the twenty-five proteins of increased abundance in the mutant strain, seven were involved in carbon catabolism. This suggests that EbrA is normally involved in responding to nutrient availability and tuning the metabolic rate accordingly by directly or indirectly repressing those proteins.

TSB was used for all of the experiments used to identify and characterize EbrA. TSB is low in concentration of fermentable sugars, the preferred carbon source for *E. faecalis*. In figure 17, I examined what effect the addition of exogenous glucose would have on biofilm formation in the Δ*ebrA* strain. The biofilm defect was completely

rescued by the addition of exogenous glucose to TSB. This result demonstrates that glucose concentration can affect the ability of EbrA to function normally in the wild type strain. To examine the role of exogenous carbon and the response of the Δ*ebrA* strain we moved to M9-YE. If no carbon source is added to M9-YE (0.4% final concentration glucose is normally added), this medium supports little to no planktonic or biofilm growth. I utilized this base medium and supplemented it with 3 different carbon sources; glucose, galactose and glycerol. Glucose and galactose are both simple sugars that are brought into the cell actively using the PTS system where as glycerol is not a sugar and it is brought into the cell via diffusion but can still be utilized by *E. faecalis* as a carbon source (10). The data generated from the BIOLOG phenotypic microarray with the Δ*ebrA* strain suggested that under planktonic conditions there was a loss of the ability of the mutant to grow on N-acetylgalactosamine. This suggested that there may be a defect in the ability of the mutant to utilize galactose or in the PTS system. To get an idea of what concentration of carbon is needed to rescue the Δ*ebrA* strain biofilm defect we looked at 3 different final concentrations of the 3 different carbon sources, 0.4%, 0.2% and 0.1% (the moles of carbon molecules are equivalent for the three carbon sources) in the M9-YE base medium. Planktonic cells of the mutant strain have no growth defect in M9 supplemented with either glucose, galactose or glycerol at any of the concentrations listed as compared to wild-type (figure 19). As shown in figure 18 in all three carbon sources a biofilm defect was seen at 0.1%. However in the presence of glucose and galactose the biofilm defect was rescued at 0.2%. This was not the case in glycerol, most likely because diffusion is not as efficient as the PTS system at bringing carbon sources into the cell leaving the intracellular levels of glycerol lower than those of glucose and

galactose at 0.2% starting medium concentration. Because the biofilm defect could be rescued by all three carbon sources the phenotype is not limited to glucose or PTS sugars but is probably mediated by total carbon concentration in the environment.

Other GntR family transcriptional regulators that have been studied biochemically have been shown to be transcriptionally autoregulatory and taken together with the results of the experiments shown in figures 16 and 17 we hypothesized that in low carbon conditions we would detect higher levels of *ebrA* transcripts from biofilm cells than we would from biofilms that were grown in higher levels of fermentable carbons. We used qPCR to look at this in 24 hour biofilms grown with and without 0.5% glucose in TSB base medium and 24 hour biofilms grown with and without 0.5% glycerol in TSB base medium. In both cases the expression levels of *ebrA* transcript were indistinguishable from that of the no carbon biofilm sample. This result indicates that the regulation of *ebrA* mRNA expression is not regulated by carbon concentration. Interestingly the Interpro protein database (linked from the TIGR gene page for EF_1809, www.tigr.org) lists DeoR family regulators as the next closest match for this gene. DeoR family regulators are involved in repression of sugar catabolism and are not known to be transcriptionally autoregulatory. This could be a case of miscategorization by the annotation algorithm used by the TIGR database since our data are more consistent with EbrA being a DeoR family regulator.

One drawback of the iTRAQ screen that we used to identify the regulon of EbrA is the treatment that was needed to obtain a protein sample from the enterococcal biofilm cells. We had to treat the cells after harvesting with a mixture of lysozyme and mutanolysin to create protoplasts. The protoplasts were pelleted and the supernatant was

removed. The supernatant, in addition to containing large concentrations of mutanolysin and lysozyme would also contain all of the cell wall anchored proteins and any proteins that were secreted into the medium. To address this drawback we conducted a microarray experiment using chip based spotted oligoarray to compare OG1RF and Δ*ebrA* biofilms. Preliminary analysis suggested that *gelE* expression was increased in the Δ*ebrA* biofilms. *gelE* is a secreted protein and would have been washed away by our pre-treatment of the biofilm cells used for protein extraction and so we would not have expected to find it using our iTRAQ screen. We confirmed the increase in expression seen in our microarray experiment using qPCR (figure 20). We also looked at expression of *sprE* (not implicated in the microarray results), the antagonist to *gelE,* because they have been reported to be cotranscribed under planktonic conditions. In our hands they do not appear to be perfectly cotranscribed because there was no difference in expression of the *sprE* transcript between OG1RF and Δ*ebrA* in biofilms. The results shown in figure 18 suggest that EbrA directly or indirectly represses expression of *gelE* in wild type biofilms. This is supported by the expression kinetics of these two transcripts which are inverse to each other (figure 21). At 4 hours the expression level of *gelE* is high and it gets lower at 24 hours where as the expression level of *ebrA* is low at 4 hours and higher at 24 hours. This expression pattern is what you would expect from a repressor/target gene pair.

GelE has been characterized as part of a larger model of eDNA release for matrix production in enterococcal biofilms (95-97). There is no negative regulator proposed in the current model of eDNA release. We propose based on our findings here that EbrA

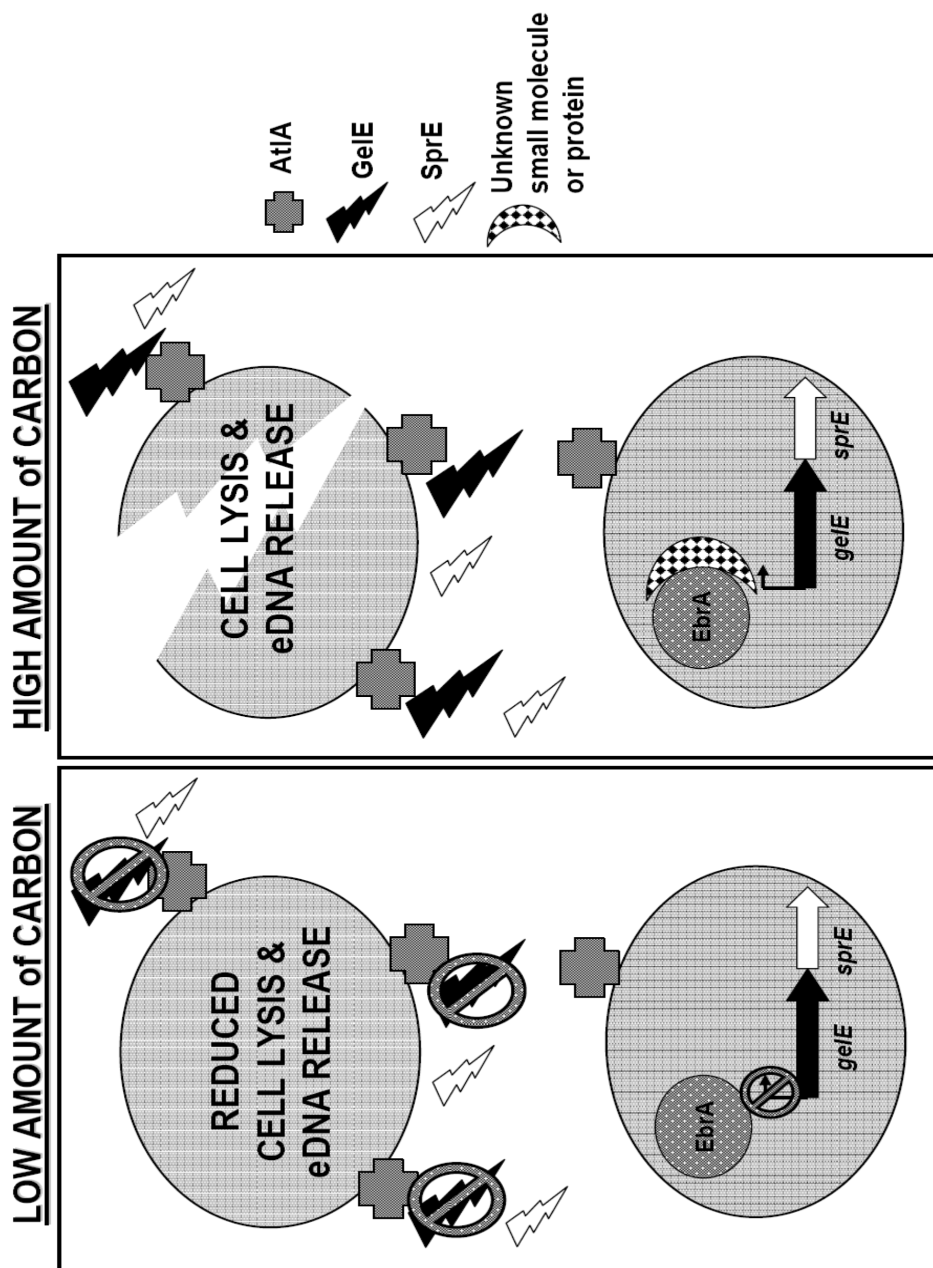


Figure 23. New model for EbrA function. This model builds upon the previously published model of GelE mediated DNA release by providing EbrA a negative regulator of *gelE* but not *sprE*. This will likely help provide a balance between eDNA release and survival of cell in the biofilm at large. We propose that through an as yet undescribed mechanism EbrA senses carbon levels and exerts its regulatory functions under lower than normal carbon conditions in a biofilm setting.

should be added to the current model as a negative regulator. Currently the model states that *gelE* and *sprE* are activated by the Fsr quorum sensing system. GelE and SprE are secreted and are able to compete for binding and processing of AtlA, an autolysin. If GelE cleaves AtlA this leads to cell lysis and eDNA release. SprE binding to AtlA protects the cell. Figure 23 shows our new model of eDNA release which incorporates EbrA as a negative regulator of *gelE*. We have drawn the model to show a direct repression of *gelE* by EbrA for simplicity, however it is possible that the negative regulation of *gelE* by EbrA is indirect and future experiments should be designed to distinguish between the two types of regulation. I hypothesize that biofilm formation under the static conditions that we tested under can reach levels of nutrient depletion where cell lysis is unfavorable for the survival of the biofilm because there are not enough nutrients left to support cell division and growth. In these conditions maintenance of the matrix is less important than maintaining living cells in the biofilm and thus *gelE* is repressed by EbrA. Under conditions where there is enough carbon to support continued growth and development the repression by EbrA is relieved allowing cell lysis to proceed and eDNA to be released into the matrix. This is the first mention of a negative regulator of this system. In 2004 Pillai et al hypothesized based on their own experiments that eventually experiments would show that there was a glucose dependant regulatory mechanism regulating Fsr expression (76). I hypothesize that EbrA constitutes the predicted glucose-dependant regulatory function (76). The foresight is impressive, however we believe the regulation is in response to carbon in general not glucose specifically.

CHAPTER 5

CONCLUSION

CONCLUSIONS

The research described in this thesis was aimed at elucidating the genetic components of biofilm formation from the initial attachment stage all the way through to a mature biofilm. This is an important distinction because most studies to date have not examined biofilms comprehensively at time points earlier than 8-24 hours. To highlight the major conclusions of each data chapter I have listed them below and will comment further on their significance later in this chapter.

Conclusions Chapter 2:

- Biofilm biomass assays can be sensitive to changes in physical properties and as such can give inconsistent results. The CFU-based assay is the most consistent for use in quantitating enterococcal biofilms
- OG1RF and VA1128 both reach biofilm maturity by 24 hours based on evaluation of CFU.
- The biochemical composition of the biofilm matrix appears to change over time. Early time point show a high amount of polysaccharide material which decreases overtime.
- eDNA levels present in the matrix remain the same over time.

Conclusions Chapter 3:

- 68 candidate biofilm genes were identified in a biofilm RIVET screen.
- A partially overlapping set of these candidate genes were shown to either be differentially expressed in RT-PCR experiments or were shown to confer a decreased ability to form biofilms thereby validating the use of RIVET screens to examine biofilm formation.
- EF1809 a putative transcription factor was shown by RT-PCR to be differentially expressed in biofilms. A mutant strain with a deletion in the EF1809 ORF was shown to have a decreased ability to form biofilms as compared with wild-type. Based on this evidence this gene was named Enterococcal Biofilm Regulator (*ebrA*) since it appears to be required for full biofilm formation in *E. faecalis*.

Conclusions Chapter 4:

- EbrA function is sensitive to the concentration of exogenous environmental carbon. Higher concentrations of carbon rescue the biofilm defect seen in low carbon conditions.
- This rescue phenotype is not limited to PTS sugars because glycerol, which uses diffusion to be brought into the cell and is not a sugar can also rescue the biofilm defect.
- EbrA appears to negatively regulate expression of *gelE* which encodes for production of a gelatinase involved in eDNA release.

In my thesis I describe comparative analysis of the increase in biomass and adherent bacterial populations in the early stages of biofilm formation by the laboratory strain OG1RF in relation to several clinical isolates. I compared OG1RF biofilm growth to that of 4 other clinical isolates and determined that a static biofilm assay using flat cellulose sheets as the biofilm substrate gave a consistent measure of biofilm formation. The crystal violet method was variable in its accuracy perhaps due to differences in the cell wall of each bacterial strain which allowed for gross underestimation of the actual number of bacteria present.

I then combined this analysis with high resolution Field Emission Scanning Electron Microscopy (FESEM) analysis of the cell surface and the extracellular matrix of the developing biofilms. In conjunction with the cellulose sheet based viability count data I was able to demonstrate that OG1RF reaches a mature biofilm stage at 24 hours and increases in biomass moderately thereafter (figure 5).

By using the FESEM at numerous stages of biofilm formation we were able to achieve an understanding of the temporal aspects in the biofilm in a way that viable count data cannot elucidate. These studies revealed a dramatic temporal change in the

appearance and biochemical composition of the extracellular matrix that has not been previously reported. At early time points (2-6 hours) OG1RF was encased in a polymer that covered the cell surface topography completely. Because of its apparent interwoven nature called this material the “sweater” matrix. However under the same growth conditions and handling for FESEM preparation by 8-24 hours this material was completely absent from the biofilm cells. Starting in small part at 6 hours and continuing on through 8 and 24 hours a different type of matrix material was seen that was more fibrillar in nature. Long protrusions come from the cell surface, sometimes covering large differences. Because the “Sweater” material was absent the cell surface was again visible in the presence of the fibrillar matrix material.

To gain a better understanding of matrix components biofilms from 4 and 24 hour biofilms were stained with lectins (figure 9). Polysaccharide levels are much higher at 4 hours than they are at 24 hour suggesting that the polysaccharide is a component of the sweater matrix. The *epa* locus, which has been suggested to encode for proteins that produce a polysaccharide product (94), is expressed at a higher level at 4 hours than it is at 24 hours suggesting that the product of the locus might be a component of the “sweater” matrix, however work done with a deletion of the locus still showed what appeared to be “sweater” matrix in FESEM images (Data not shown). It should be noted that the *epa* locus has only been studied to date at 24 hours (94) and this study highlights the importance of further studies of this locus at an earlier time point. It is important to determine the composition of both the “sweater” matrix and the *epa* polysaccharide. Characterization of these structures will help confirm or refute whether or not *epa* is playing a role in the production of the “sweater” matrix.

Biofilms of 4 and 24 hours were also examined for their production of eDNA. The supernatants of each time point were examined with a dye that binds to dsDNA. Interestingly biofilms of 4 and 24 hours produce the same amount of eDNA/cell (figure 9). Our FESEM images do not show cell lysis, the current model for eDNA release (95-97), prior to 24 hours, however we have shown equivalent levels of eDNA release at 4 and 24 hours suggesting that there is another mechanism for eDNA release in *E. faecalis* biofilms.

The data provide potential phenotypes and gene expression patterns that suggest the importance of examining biofilm formation at early time points. The data also suggest that there are two major types of materials in the biofilm matrix that change over time. One of those materials, the "sweater" matrix, has not been previously described. This work highlights the importance kinetic studies of early time points to identify novel phenotypes in biofilms.

When I started my thesis work most of the genetic components of biofilm formation had been identified from clinical isolates and specifically from plasmids or pathogenicity islands that they harbored. OG1RF does not contain any plasmids and it is free of the known pathogenicity islands (11) however it is quite capable of forming biofilms (5, 51). It was clear that the core genome contained all of the genetic components that were both necessary and sufficient to form robust biofilms. To examine the core genome of *E. faecalis* for the genetic components of biofilm formation I used a RIVET screen. RIVET screens allow for the identification of promoters that are turned on under the conditions and duration of the screen.

The biofilm RIVET screen identified 68 genes that contained a promoter region that was expressed during biofilm growth (table 3). I was able to show that when 3 of these genes were knocked out that the resulting isogenic mutants were unable to form biofilms with the wild-type level of biomass, suggesting that these genes are involved in biofilm formation. In addition 4 randomly selected RIVET associated genes were differentially expressed between biofilm and planktonic cells, confirming their biofilm involvement. Additionally the RIVET screen showed remarkable complementarity to a transposon screen that was undertaken in the lab at the same time.

The RIVET screen and transposon screen did not directly overlap and identify the same genes. This is not surprising since the high throughput nature transposon screen only allowed for a screening assay that predominantly tested for initial attachment and many of these surface factors would have had to have been expressed in planktonic cells prior to biofilm formation. These types of genes would not have been identified in the RIVET screen based on my assay conditions. However there were 4 instances where the RIVET and Transposon screens identified genes that were physically adjacent to each other. In one case the transposon gene is a known autolysin that is involved in biofilm formation. I predict that most of these cases will not turn out to be coincidence once the loci are investigated further and characterized. I think that their implication in two biofilm genetic screens indicates their importance for biofilm formation in this organism. The RIVET and transposon screens represent the first comprehensive examination of the chromosomal potential for bioflm formation in *E. faecalis*.

In the previously described RIVET screen I identified and initiated characterization of the role in biofilm formation of a novel transcription factor,

Enterococcal Biofilm Regulator (EbrA). This transcription factor was differentially expressed between biofilm and planktonic cells at 24 hours and a mutant strain in which the open reading frame was deleted was defective in its ability to form biofilms as compared to wild-type.

To determine the regulon of EbrA I utilized methods that examined the proteome and the transcriptome. The bulk of the information was gathered from a mass spectrometry based technique for proteome examination, iTRAQ. This allowed me to compare the proteins present in an OG1RF 24 hour biofilm and a 24 hour biofilm made by an Δ*ebrA* strain (table 7). The iTRAQ results showed that 7 of the proteins that were increased in the deletion strain were involved in carbon metabolism. When the deletion strain was grown in the presence of PTS and non-PTS carbon sources the biofilm biomass defect was rescued at higher concentrations and present at lower concentrations of carbon (figures 16 and 17). From this evidence I determined that EbrA is responsible for modulating the metabolic rate to promote survival of the biofilm population when it undergoes nutrient stress.

As a function of this survival role my experiments suggest that EbrA is also a negative regulator of the cell lysis mechanism that is thought to mediate eDNA release for biofilm matrix production. Using RT-PCR I showed that *gelE*, but not *sprE*, which in planktonic cells is cotranscribed, is negatively regulated by EbrA. In a Δ*ebrA* strain the levels of *gelE* expression were 2-fold decreased as compared to wild-type (Figure 20). Interestingly this not only demonstrates the first evidence for a negative regulator of GelE expression but also shows that *gelE* and *sprE* are not cotranscribed under all conditions as was previously assumed (77).

FUTURE DIRECTIONS

Chapter 2:

What is the composition of the fibrillar and "sweater" matrix?

I clearly demonstrated that there are two different biofilm matrix components (figure 8). The sweater matrix contains polysaccharides as I demonstrated in (figure 9) however I used lectins that had a very relaxed specificity so it is not possible to make more definite conclusions about the molecular composition of the matrix at either 4 or 24 hours.

Lectins of higher specificity could be used for the purpose of characterizing the matrix. This could be useful if the lectins used were conjugated to a fluorescent molecule and the biofilms stained in situ. It would lend much more certainty to the conclusion if it were certain that the lectin we binding to the structure seen by FESEM (although under a confocal microscope they would be hydrated and might look very different). However to get precise molecular confirmation of the composition of the matrix at the indicated time points it will be necessary to undergo acid hydrolysis of the biofilm matrix followed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD) for concrete identification (32).

Is there another mechanism for eDNA release?

Some of the results in my thesis suggest that the current model of eDNA release by cell lysis is not complete. I did not see cell lysis prior to 24 hours however (figure 7) eDNA levels remained steady on a per cell basis between 4 and 24 hours (figure 9). This suggests eDNA is being released through another mechanism at earlier time points.

I think that first of all it will be important to obtain mutants that are defective for the cell lysis based eDNA release mechanism and test them to see if there is eDNA

present in the early (4 hour) biofilm. I would hypothesize that while late eDNA release would be impaired or abolished that there would be eDNA release at early time points. If that hypothesis holds true it would be important to investigate the genetic determinants of the second mechanism.

To elucidate a putative second mechanism of eDNA release I would grow 4 hour biofilms utilizing the transposon library in 96-well plates. At 4 hours I would suggest using a modified version of the assay described in Chapter 2 to look at eDNA to identify transposon mutants that showed a decrease in eDNA levels as compared to wild-type at this time point. In this way, if there is a second mechanism of eDNA release at earlier time points as my data suggest some or all of the genetic components of the system could be identified.

Chapter 3:

Characterize other RIVET-identified genes.

The RIVET screen identified 68 genes and I only had the time to characterize one of the genes. Presumably the other 67 genes also encode for proteins with interesting and important biofilm functions. The preliminary characterization would be to check the ORF for differential expression between biofilm and planktonic cells and also to create in frame deletions and check them for biofilm biomass production. This will be a long process as there are many loci to examine however the work is ongoing in the lab currently with some exciting progress being made on EF0984 demonstrating the significance of the work described in Chapter 4.

Chapter 4:

Identify the direct regulon of EbrA.

The work done to investigate the regulon of EbrA included techniques that could not distinguish between those effects that were directly caused by EbrA and those that were indirectly caused by EbrA. To better understand where in the presumed regulatory cascade EbrA fits it is important to know which of the genes and proteins that are affected by EbrA expression are affected by direct interaction with EbrA. To do this I think it would be most expedient to set up a ChIP/seq type assay. As described in Chapter 4 I have already created a highly sensitive and purified polyclonal antibody for EbrA. This antibody is ready to be used in such an assay. To reduce the costs and complexity I think that the “seq” part of the assay could be accomplished by digesting the crosslinked DNA with a restriction enzyme that was a frequent cutter but was also compatible with a “work horse” cloning vector, perhaps one of the pGEM family vectors (Promega). In this way after the protein bound DNA fragments were selected using the anti body they could be cloned into the cloning vector and electroporated en mass into a cloning strain of *E. coli* and propagated and sequenced from that population.

What is the spatial and temporal distribution of EbrA in biofilm cells?

To address this question it will be important to create a fluorescent protein transcriptional fusion of *ebrA*. I would suggest that this be a transcriptional fusion either in the chromosome or on a plasmid. Preliminary data suggest that increasing the expression of *ebrA* above what is normal is not tolerated well by the cells and so a chromosomal version of this construct might be more stable. Once this fluorescent protein tagged strain is made it could be used in a system that could be monitored in real time using confocal microscopy. It is important to know when and where this gene is expressed to

have a better idea of precisely what role it is playing in the regulation of biofilm formation in this organism.

Does the deletion of *ebrA* lead to a decrease in eDNA in the biofilm?

Data from my thesis work in Chapter 4 suggests that EbrA is negatively regulating the expression of *gelE*. This would presumably lead to a decrease in the amount of GelE produced and ultimately a decrease in the amount of cell lysis and eDNA release. This phenotype should be measurable by two methods. The first method would be indirect. OG1RF and Δ*ebrA* would be grown as biofilms for 4 and 24 hours in TSB. The biofilm cells and planktonic cells from the biofilm vessel would be stained with a "live/dead" stain. The hypothesis would be that at 24 hours the Δ*ebrA* biofilm cells would have fewer dead cells present. The second method would be direct. OG1RF and Δ*ebrA* would be grown as biofilms for 4 and 24 hours in TSB. The biofilm and planktonic supernatants would be collected as described in Chapter 4. The total cell pellets from the biofilm and planktonic cells would be plated for CFU/pellet as described in Chapter 4. The supernatants would be stained with SYTOX green. The hypothesis is that the Δ*ebrA* biofilm sample would contain less eDNA than the OG1RF biofilm sample. Additional versions of these experiments could be carried out with and without added carbon. The hypothesis in those experiments would be that the addition of carbon would restore the wild-type amount of cell death/eDNA release in the Δ*ebrA* strain.

My thesis provided the first kinetic and temporal examination of biofilm formation from its inception to maturity in *E. facalis* and utilizing FESEM was able to identify two novel biofilm matrix phenotypes that change over time. In addition I participated in performed the first comprehensive interrogation of the core genome of *E.*

faecalis for biofilm determinants in by utilizing the RIVET screen in conjunction with a complementary transposon screen. These novel approaches identified over 80 genes (68 from the RIVET screen) most of which had not been known to be involved in biofilm formation previously. Finally I was able to characterize one of those RIVET-identified genes, EbrA, which is involved in modulating the rate of cellular metabolism in biofilms in response to nutrient limitation. This transcription factor is also a negative regulator of *gelE*, which is involved in eDNA release. This is the first evidence for a negative regulator of eDNA release in the current model (95-97).

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APPENDIX I

Proteome comparison of OG1RF 5-day biofilm cells and 1-day planktonic cells using iTRAQ.

This was a pilot study to evaluate iTRAQ as a method and determine its feasibility for use in other experiments like those described in Chapter 4. OG1RF was grown as a biofilm on cellulose sheets as described in (5) for 5 days in which the liquid medium (TSB-gluc) was changed once per 24 hours. At the end of the 5 day period the biofilm cells were harvested using a combination of physical disruption and sonication. Planktonic cells were harvested from the same vessel as the biofilms were grown from the liquid phase making these planktonic cells 24 hour cultures. Protein was isolated from these cells and they were submitted for iTRAQ analysis as described in the materials and methods section of chapter (EbrA). After analyzing the iTRAQ results using the methods described in the methods section of chapter (EbrA) the differences in the proteome of planktonic and biofilm cells were noted in table 9.

The iTRAQ screen identified 490 proteins in total. Of those 490 proteins 32 had a significant fold change (criteria described in the materials and methods section of Chapter (EbrA)). 20 of the proteins with a significant fold change were decreased in the biofilm cells and 12 were increased in the biofilm cells. Interestingly of the proteins that were decreased in the biofilm cells HPr was the most decreased. HPr is involved in signaling and the phosphorelay of the PTS system. This might be an indication that the biofilm cells are using less PTS sugars than are the planktonic cells. Supporting that hypothesis is the fact that one of the proteins that is the most increased in the biofilm cells is the glycerol

Table 9. Proteomic analysis of biofilm and planktonic cells

TIGR IDs	Accession	Annotation	Planktonic/ Biofilm
709	gi|29342766	phosphocarrier protein HPr	4.863
665	gi|29342723	hypothetical protein EF 0665	4.719
2925	gi|29344856	cold-shock domain family protein	3.522
1367	gi|29343396	cold-shock domain family protein	3.376
1623	gi|29343645	microcompartment protein	2.320
1560	gi|29343585	hypothetical protein EF 1560	2.201
2884	gi|29344817	acyl carrier protein	1.954
1151	gi|29343188	cell division protein DivIVA	1.795
1550	gi|29343575	DNA-binding protein HU	1.621
3233	gi|29345145	Dps family protein	1.608
3256	gi|29345167	pheromone cAD1 precursor lipoprotein	1.601
1002	gi|29343045	cell division protein DivIVA	1.504
2784	gi|29344722	conserved hypothetical protein	1.444
177	gi|29342285	basic membrane protein family	1.410
686	gi|29342743	hypothetical protein EF 0686	1.402
1548	gi|29343573	ribosomal protein S1	1.323
2879	gi|29344812	acetyl-CoA carboxylase	1.298
2556	gi|29344510	fumarate reductase flavoprotein subunit precursor	1.252
1796	gi|29343804	lipoprotein	1.249
1936	gi|29343926	hypothetical protein EF_1936	1.248
878	gi|29342923	DNA polymerase I	0.817
634	gi|29342694	decarboxylase	0.814
247	gi|29342347	amino acid ABC transporter	0.811
3257	gi|29345168	oxidoreductase	0.810
178	gi|29342286	ABC transporter	0.806
1684	gi|29343701	DegV family protein	0.800
1700	gi|29343715	signal recognition particle protein	0.793
3037	gi|29344963	glutamyl-aminopeptidase	0.781
3317	gi|29345226	carboxylase	0.768
3320	gi|29345229	citrate lyase	0.747
1927	gi|29343918	glycerol uptake facilitator protein	0.720
2030	gi|29342332	ribosomal protein L36	0.620

uptake facilitator protein. Glycerol is a non-PTS sugar and its increase in abundance in biofilm cells could indicate that non-PTS sugars are being metabolized instead.

Another protein that was decreased in biofilm cells was the DNA binding protein HU. HU has been shown to regulate the expression of 8% of the genes in *E. coli* and among those genes HU regulates the response to acid stress (69). This might indicate that being free floating in the liquid phase exposes the cells to more stress from acid than does being a part of a biofilm. Acidic pH change is a known by product of the fermentation that the enterococci use for energy generation. In addition there are two other stress proteins that are increased in planktonic cells in addition to HU.

This iTRAQ screen compared biofilm and planktonic cells of OG1RF and examined the differences on a protein level. The RIVET screen utilized a promoter trap cloning scheme and identified promoter regions that were turned on during biofilm formation. The RIVET screen would not have identified genes that were decreased in biofilm formation based on the design of the screen. The 20 proteins that were decreased in biofilm formation offer insight into what functions of planktonic cells are either not needed or disadvantageous to biofilm cells. From this analysis it appears as though biofilm cells are not utilizing PTS sugars as much as planktonic cells are based on the significant decrease in HPr, a member of the PTS system and the increase in the Glycerol uptake facilitator protein. Glycerol is not a PTS sugar. Also planktonic cell are perhaps more susceptible to acid stress as that is one of the categories of genes regulated by the DNA-binding protein HU. In addition to HU there are other stress proteins that are more abundant in the planktonic cells.

There was no direct overlap between the results of the RIVET screen and the proteins that were increased in expression in the iTRAQ screen, which is where overlap between the two techniques would have been expected. This demonstrates that screens that rely on population averages like iTRAQ will not detect differences in protein levels that are often present at low levels like regulatory proteins. iTRAQ will be able to detect proteins that are relatively abundant overall, however since iTRAQ can detect proteins that are also decreased in a condition of interest providing a useful complement to a genetic screen like RIVET.

There were two proteins that were identified in the iTRAQ screen as being increased in biofilm cells that have gene loci that are adjacent to genes that were identified in the RIVET screen. EF246 (RIVET) and EF247 (iTRAQ) are annotated as two parts of an ABC transporter system. And EF3257 (iTRAQ) and EF3258 (RIVET) are a pair of oxidoreductase (EF3257) and a conserved hypothetical (EF3258). In this case I would predict that these pairs are either part of an operon in which the promoter is in front of the RIVET-identified gene or that the protein identified by iTRAQ is more abundant than the RIVET-identified gene. The fact that these loci were identified in two screens might mean that they are very important for biofilm formation in this organism. These two loci would make good targets for further study using mutational analysis and RT-PCR.

APPENDIX II

Development of biochemical tools to examine the direct effect of EbrA

Expression of RIVET and Transposon-identifed proteins in *E. coli*. PCR was used to amplify the ORF plus the TIGR predicted ribosome binding site for EF_798 (RIVET), EF_1809 (RIVET), EF_983 (transposon) and EF_676 (transposon). These PCR fragments were then ligated to the pET28b+ expression vector (Novagen). This plasmid is designed to express the protein of interest with the addition of a 6 his tag on the C terminus of the protein. The correct plasmids were then introduced into *E. coli* strain BL21 via electroporation. A protocol to optimize for the expression of these proteins from their *E. coli* hosts was adapted from the manufacturers manual as well as (3). Specific inducer amounts and growth temperatures are given in table 3. In general an overnight culture of BL21 carrying the pET28b+ vector containing the ORFs plus RBS was taken and diluted 1:100 into fresh Luria Bertani broth (LB) kan 50µg/ml and incubated shaking at either 30 or 37°C until an OD600 reading reached 0.7. At this point the inducer IPTG is added to induce expression from the promoter on the plasmid. After growth in the presence of inducer for 4 hours the cells are pelleted and lysed. The cell lysates are run on an SDS-PAGE gel for analysis of protein expression.

Over expression of 2 RIVET and 2 Tn screen-identified genes were induced in *E. coli*. The protein over expression constructs made from the enterococcal biofilm-related ORFs in pET were used to produce the enterococcal proteins in the *E. coli* host strain BL21. However for each strain differing temperatures and inducer concetrations were required for optimal expression of the protein as detected by SDS-PAGE gel. To determine optimal protein expression, cell lysates of BL21 cells grown under different

Table 10. Conditions used for optimal protein over-expression

TIGR ID	Size (dalton)	Concentration IPTG Used for Optimal Protein Expression	Growth Temperature for Optimal Protein Expression (degrees Celcius)
EF1809	28064	10mM	30
EF798	25218	1mM	37
EF983	17153	1mM	30
EF676	16901	1mM	37

inducer concentrations and differing temperatures were compared to lysates that were given no inducer on the SDS-PAGE gels. The specific conditions required for expression of each protein in BL21 are given in table 10.

EbrA Purification: *E. coli* BL21 cell carrying the pET28b+ vector plus the *ebrA* ORF (EF1809) and RBS were grown overnight in LB kan 50µg/ml and diluted 1:100 into fresh LB kan 50µg/ml and incubated shaking at 30°C until an OD600 reading reached 0.7. At this point the inducer IPTG (10mM) was added to induce expression from the promoter on the plasmid. After growth in the presence of inducer for 4 hours the cells were pelleted and lysed via enzymatic degradation with lysozyme (10mg/ml) and sonication. The protein was then purified with the aid of the 6 his tag using the methods described in (3). An adaptation was made in the elution buffer to maintain protein stability in solution. EbrA elution buffer is as follows: 200mM imidiazole, 30mM Tris-HCl pH 7.5, 0.5M NaCl and 10% glycerol. Dialysis was preformed overnight at 4°C in 30mM Tris-HCl pH 7.5, 0.5M NaCl and 10% glycerol to eliminate the imidiazole. This protocol was adapted from (3).

EbrA Antibody production and purification: A rabbit was injected twice 1 month apart with 1mg of purified EbrA. After a small amount of serum was checked for production of antibody the animal was bled completely. The resulting serum was absorbed with cell extract containing proteins from the Δ*ebrA*, and BL21 containing an empty pET28b+ vector to eliminate any possible cross reacting antibodies. This was done using a serum to cell extract ratio of 1:100. The mixture was left to absorb overnight at 4°C (3). The absorbed serum was purified using a column that was bound with purified EbrA. First the serum was precipitated in using an equal volume of

ammonium sulfate to isolate the immunoglobulin fraction. The precipitate was centrifuged to create a pellet which was then dialyzed in PBS at 4°C overnight twice to eliminate the ammonium sulfate. The purification column, which consists of cyanogen bromide activated matrix (Sigma Aldrich) coupled to 2mg EbrA purified protein was prepared according to the manufacturers instructions. The immunoglobulin fraction was run over the prepared column after washing with PBS. The column with immunoglobulin bound was washed with PBS until the protein reading (OD280) on the flow through was less than 0.05mg/ml. The purified EbrA specific antibodies were eluted with 0.5M acetic acid pH 3. The antibody solution was neutralized with 2M Tris and dialyzed in PBS 4°C overnight.

Western blotting: The western blot protocol used here was adapted from (3). The EbrA western blots to test the EbrA antibody were run with a 1:15000 dilution of the primary antibody (whole serum) or a 1:5000 dilution purified antibody (described above). The blots were blocked in a 10% milk solution and a 1:5000 dilution of a HRP-goat anti rabbit IgG (H+L) DS grade (Zymed) secondary antibody was used for detection. The blots were washed in KPBS containing 0.1% Tween 20. The HRP signal was detected with the Super Signal West Pico kit (Pierce) used according to the manufacturers instructions.

Electrophoretic Mobility Shift Assay (EMSA): The DNA probes used in the EMSA were designed to overlap each other and cover the entire length of the intergenic region preceding *ebrA*, probe 6 was the closest to the start codon. The oligos that were annealed together to make the probes are given in table 11. In all gel shifts 775 fmol purified EbrA was used and 31 fmol annealed probe was used. The EMSAs were performed using the

Table 11. Oligonucleotides used in EMSA

Oligo Name	Sequence	Length
P1F	ATA ACT TTC TCC TTT ACC CTA ACT GGT TAT AAC CAC AAA CCA ATA GTA CGG TGG TTT TTT TTA TTT TGT CAA TAA AAA GTA TTA ATA AAT ATC TTC ATA	99
P1R	CCA CCG TAC TAT TGG TTT GTG GTT ATA ACC AGT TAG GGT AAA GGA GAA AGT TAT	99
P2F	TAA CGT TTA CAT TTT TTT ATT TTT CTT TAT TGT AAT ATT TTT TTC	95
P2R	AAA CGT TAT CAT TTC AAG GTT ATA GCG GTA TGA AGA TAT TTA TTA ATA CTT TT	95
P3F	TAA AAA ACC ATC CTT AAA TAT GAT ACC AGT TGC TGG CTT TAT TTT AAA TAG ACA A	100
P3R	TGG TTT TTT ATA AAA AAA TGA TTT CTT GAG GAA AAA AAT ATT ACA ATA AAG AAA A	100
P4F	AAA AAT CAC TTT AGA TTT TTG TCT CAT ACT CAA AA	80
P4R	CTT TTT TGT CTA TTT AAA ATA AAG CCA GCA ACT GG	80
P5F	GAT CCC GAC TTC TTC GTT TCT ATT GTT TGG TTC TGG CAA CAA CTA ACA AGT AAC T	100
P5R	AGT CGG GAT CGA ACT GTT TTT CAG ATT TTG AGT ATG AGA CAA AAA TCT AAA GTG A	100
P6F	ATG ACA ATA TTC ATT	60
P6R	GTT GCC AGA ACC AAA	60

DIG Gel Shift kit, 2nd Generation (Roche) according to manufacturer's instructions. This protocol was adapted from (3).

Generation of stable purified his-tagged EbrA protein leads to the production and purification of a specific EbrA antibody. An antibody was produced in the serum of the rabbit as shown in Figure 23. Figure 23 contains a PAGE gel file showing the antibody serum and the purified antibody which is free of other serum proteins and antibodies as well as a western blot detecting purified EbrA and EbrA from cell extracts both from serum and using the purified antibody. The purified antibody does not react with any other proteins in a cell extract of Δ*ebrA* strain producing EbrA from a plasmid however it does react with the his-tag on PrgX so this antibody should not be used in the presence of non-EbrA his-tagged proteins for the highest accuracy.

EbrA ORF were used to examine whether or not EbrA was able to bind directly to stretches of this DNA. Under the conditions used in this assay it does not appear as though EbrA can bind to any part of the DNA in the intergenic region directly preceding the *ebrA* ORF. This suggests that EbrA is not required for transcriptional activation of the *ebrA* ORF. This data is in agreement with qPCR data described above for the experiment looking at *ebrA* expression levels in biofilms grown with and without additional carbon sources.

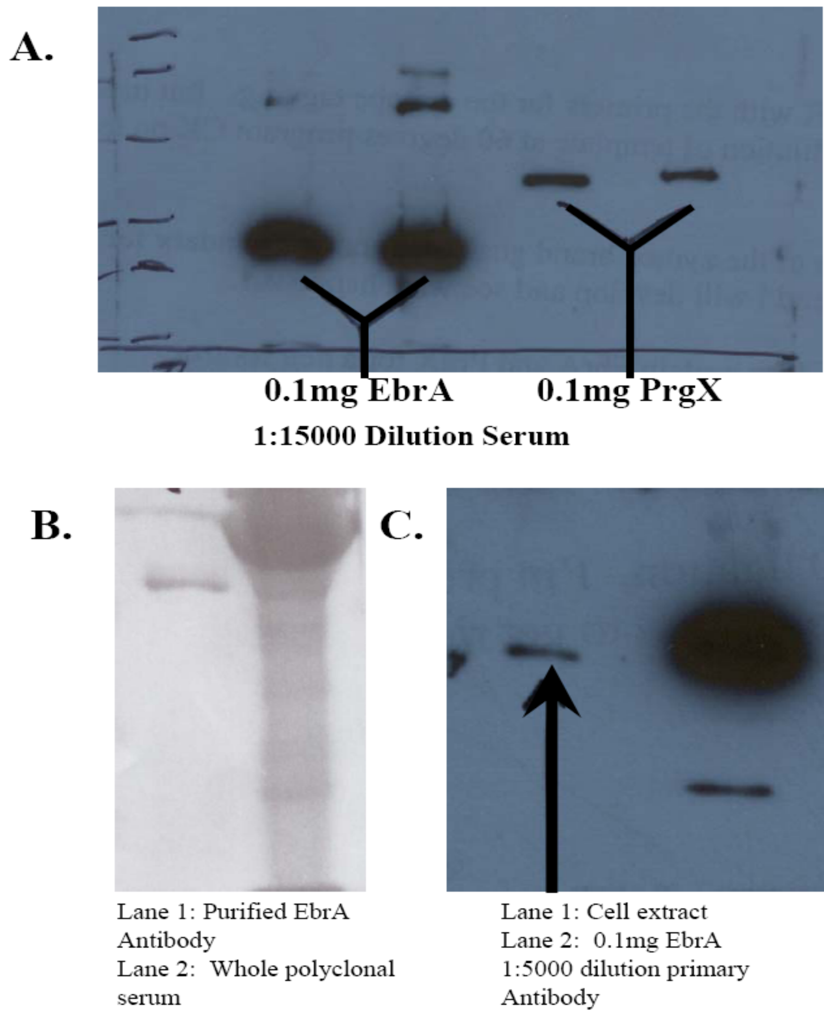

Figure 23. Purification and testing the polyclonal EbrA Antibody. Panel A. shows a western blot using 0.1mg purified EbrA and 0.1mg purified PrgX. The primary antibody used was the whole serum from the rabbit immunized against EbrA. This antibody does recognize the 6-his tag on PrgX as evidensed by the band in the PrgX lanes. Panel B. shows a PAGE gel in which lane 1 is the purified EbrA polyclonal antibody and lane 2 is the whole serum containing the EbrA polyclonal antibody. Panel C. shows the purified polyclonal EbrA antibody used to detect EbrA from a cell extract in lane 1 and purified protein in lane 2. There is a cross reacting band in the purified protein lane that does not show up in the cell extract lane.

EbrA does not appear to bind to DNA in its own intergenic region. EMSAs using purified EbrA and DNA probes that span the intergenic region directly preceding the

I have described here the generation and purification of both his-tagged EbrA protein as well as a polyclonal antibody specific for EbrA as shown in Figure 23. These will become important tools for use in the elucidation of the role of EbrA in biofilm formation in *E. faecalis*. One of the most important future directions described was to identify the direct regulon of EbrA. Chaper 4 described important preliminary work to identify the regulon of EbrA, however the techniques used identified the total regulon which likely contains the set of genes and proteins that are both directly and indirectly subject to regulation by EbrA. To determine the set of genes that are directly regulated by EbrA it will be imperative to utilize the EbrA specific antibody in a ChIP-seq experiment. In addition the purified protein will be useful for further EMSA experiments to determine the DNA binding site of EbrA. The antibody will be an important tool for detection of the EbrA protein via western blotting for a number of different applications. This description of the development of these tools along with the initial descriptions of protocols for their use will be useful in the further characterization of EbrA in the future.