

PLASMID PCF10-MEDIATED *ENTEROCOCCUS FAECALIS*
HETEROGENOUS TOWER-LIKE BIOFILM STRUCTURES INFLUENCE
BIOLOGICAL PROPERTIES OF THE BIOFILMS

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ABSTRACT

Horizontal gene transfer transforms commensal *E. faecalis* into multidrug resistance (MDR) opportunistic pathogens causing diseases such as infective endocarditis (IE), septicemia, and urinary tract infections (UTI) (4,1). *E. faecalis* are among the top three leading causes of hospital-acquired infections and pheromone responsive plasmids (pCF10) are the most extensively characterized conjugative plasmids in *E. faecalis* infection (2,4). *E. faecalis* is a potential future public health concern because of the co-occurrence factors of antibiotic resistance and virulence traits (6)

Plasmid-free commensal *E. faecalis* form homogenous biofilms that have a uniform distribution of the bacterial cell and a fluid-like movement (22). The introduction of the pheromone responsive plasmid pCF10 leads to the formation of heterologous rigid structures within the biofilm (22). In the current work, the timeline of biofilm tower formation was characterized. Tower formation was not observed in the commensal strain. The pCF10-containing bacteria formed a rigid base layer on day 1 and small aggregates on day 1. pCF10-containing biofilm forms heterologous towers on days two and three. Interestingly, mixed biofilms with both plasmid-containing and plasmid-free bacteria developed tower-like structures as early as day 1 and had larger resulting structures by day three. In the mixed population, we hypothesize that the induction of aggregation substance and cell clumping during plasmid transfer may further contribute to structure formation (5,10). Plasmid-free mCherry-labeled bacteria could be observed in the viscous biofilms between heterologous rigid structures; however, the rigid structures were predominantly composed of plasmid-containing cells. Occasionally, mCherry cells were observed in the rigid structures, we hypothesize that these cells represent

transconjugants, where pCF10 was transferred by conjugation to mCherry-plasmid-free OG1RF.

The formation of rigid structures can protect bacteria from antibiotics by reducing the penetration of the antibiotic but binding and sequestration of the antibiotic in the outer layers. Antibiotic resistance increased in the pCF10-containing biofilms as rigid structures were formed. We hypothesize that underflow, like that found in the gastrointestinal tract, the heterologous rigid structures may form protected microenvironments for sensitive regions of the biofilms. In future studies, fluorescently labeled antibiotics will be used to access the formation of protected microenvironments in biofilms underflow.

Previous studies in the laboratory demonstrated that the presence of pCF10 protects *E. faecalis* from hydrogen peroxide oxidative stress. *E. faecalis* produces hydrogen peroxide. Higher levels of hydrogen peroxide can be detected in rigid structures. The presence of pCF10 is known to increase the size of heart vegetations during endocarditis and hydrogen peroxide is a known activator of platelet activation (12,19). In these studies, the presence of pCF10 increased platelet activation in pCF10 containing biofilms. Software toolboxes are currently being developed to quantitate visual observations. The role of hydrogen peroxide is supported in our preliminary experiment revealing catalase treatment reduced platelet activation. Studies are ongoing to mutate the *aroc* and *menB* gene of *E. faecalis*, which contribute to hydrogen peroxide production (35). We will compare platelet activation in knockout (double or single) *E. faecalis* and the wild-type strain. For future studies, several of the preliminary data need to be repeated to further the study. We will repeat quantitative hydrogen peroxide production

in the catalase experiments. We will also finish knocking out the *aroc* and *men B* gene of *E. faecalis* responsible for hydrogen peroxide and then compare platelet activation to the control strain.

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µm	micrometer
AS	aggregation substance
bp	base pairs
kbp	kilobase pairs
C	Celsius
CFU	colony forming unit
diH ₂ O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
L	liter
LB	Lysogeny Broth
M	molar
nmol	nano molar
mg	milligram
min	minutes
hr	hours
ml	milliliter
mM	millimolar
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGN	peptidoglycan
rpm	revolution per minute
SDS	sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TH	Todd Hewitt
BHI	Brain Heart infusion Broth
PAI	pathogenicity island
ESP	enterococcal surface protein
GeIE	gelatinase
Fsr	fecal streptococci regulator
Ebp	endocarditis and biofilm-associated pili
MDR	multidrug resistance
IE	infective endocarditis
pCF10	pheromone responsive plasmids
Ebp	endocarditis and biofilm-associated pili

DIC	disseminated intravascular coagulation
<i>fsr</i>	faecal streptococci regulator
vWF	von Willebrand factor
IgG	immunoglobulin G
A2	Thromboxane 2
U	unites
<i>menB</i>	1,4-dihydroxy-2-naphthoic acid synthase
<i>aroC</i>	chorismate synthase
Ccp	cytochrome c peroxidase
pBVGh	temperature sensitive plasmid

CHAPTER 1

INTRODUCTION

Enterococcus faecalis

More than seventeen different species of *Enterococcus* have been identified. Among them, only *E. faecalis* and *E. faecium* are commonly found in humans (1). *E. faecalis* is responsible for approximately 80% of infection in humans and is among the top three leading causes of hospital-acquired infection or nosocomial infection (2,3). *E. faecalis* is a gram-positive bacterium that resides in the gastrointestinal tracts of humans as a commensal. Through horizontal gene transfer, commensal *E. faecalis* can acquire mobile genetic elements that transform the bacteria to multidrug resistance (MDR) opportunistic pathogens (1,4). These nosocomial pathogens tend to cause nosocomial hospital-acquired infections when they colonize the gastrointestinal tract of hospitalized patients who have been treated with antibiotics. Pathogenic enterococci cause many kinds of diseases such as infective endocarditis (IE), septicemia, and urinary tract infections (UTI) especially, in patients that are immunocompromised or treated with antibiotics (5). Although it has not yet happened, *E. faecalis* co-occurrence factors of antibiotic resistance and virulence traits may have the potential of producing infection outbreaks on a global scale (6).

Major diseases caused by *E. faecalis* depend on platelet activation

While *E. faecium* is more resistant to multidrug, *E. faecalis* is the more common cause of the most serious enterococcal infection called infective endocarditis (5). IE is an inflammation of the inner layer of the heart due to the colonization of the bacteria. Usually, enterococcal bacteria bind damaged heart valves from rheumatic heart disease or surgery. A vegetation then forms which consists of bacteria, fibrin, and adherent platelets. The

vegetation results in a leaky heart valve (5). The infection is frequently found in the older age population and immunocompromised patients, 15-39% of the incidents being nosocomial (5). Although the illness was documented for over 450 years and was well described in 1885, IE is considered an emerging problem now in the 21st century (7). Longer survival of patients with degenerative heart disease, increases in the use of antibiotics, increased incidence of prosthetic heart valves, and advances in medical and surgical treatments are the major reasons for the rise of the infection (7).

Another common illness of *E. faecalis* is bacteremia. *E. faecalis* in the blood triggers inflammation resulting in death from sepsis, which includes platelet activation and subsequent disseminated intravascular coagulation (DIC) (5). From the 1980s to 2008 nosocomial enterococci has jumped from 6th to 2nd most common cause of bacteremia (5). Enterococcal bacteremia is the only gram-positive bacteria associated with high mortality, which ranges from 26% to 46% (5). Unfortunately for immunocompromised patients, the rate can rise to 75% (5).

Role of mobile genetic elements converting commensals to pathogens

Horizontal transfer of mobile genetic elements is crucial in *E. faecalis* pathogenicity because it enables *E. faecalis* to gain genes that encode virulence factors and antibiotic resistance (8). Pheromone-responsive conjugative plasmids (pCF10) are the most extensively characterized conjugative plasmids in *E. faecalis* (4). Secretion of pheromone peptides by plasmid-free recipient cells induces the conjugative transfer of the plasmids. Plasmid-containing donor cells can sense the pheromone and upon induction of conjugation, they express aggregation substance and other proteins on the cell surface that are necessary for plasmid transfer (4). Although acquiring plasmids for cellular function is

unnecessary, a plasmid gives the recipient a new virulence factor and antibiotic tolerance important for survival.

E. faecalis has been a commensal bacterium since its paleozoic origin (9), however, in the last decades, pathogenic strains of *E. faecalis* has been isolated from humans (1). The transition from commensal to pathogenic strain can be traced not only to plasmids such as pheromone responsive plasmids but also to pathogenicity islands found in the *E. faecalis* genome (1). The sequencing of the vancomycin resistance *E. faecalis* isolates (V583) reveals that 26 % of the V583 genome is constructed from mobile genetics elements (1). Some of the mobile elements include remnants of integrated plasmids (1). Unlike V583, OG1RF strains are plasmid-free, have CRISPR cas locus, and they have only a few intrinsic resistances to antibiotics (1, 9). V583 has a pathogenicity island (PAI) that encodes an enterococcal surface protein (ESP) and other virulence factors. Pheromone responsive plasmids can encode cytolysin and aggregation substance (AS), which are unique to *E. faecalis* (1). The ESP and AS are found on the cell wall surface of the pathogenic isolates. AS expression is induced by pheromone uptake during conjugation mating and is considered an important virulence factor of *E. faecalis*. Both ESP and AS promote biofilm formation which is ideal for adherence and persistence on tissues seen in IE (5, 10). Also, biofilm formation in medical devices is an ideal mechanism of transmission in hospitals (5, 10). AS has been shown to contribute to the development of larger vegetations in IE (11).

E. faecalis strains identified CRISPR spacers have an identity to pheromone-responsive plasmids as well as phage (1). In OG1RF, CRISPR Cas is a defensive system against mobile elements of plasmids and phage. Therefore, it is plausible to say CRISPR

influences the movement of mobile elements between strains in *E. faecalis* and explains the significant correlation found between the absence of CRISPR-cas locus and antibiotic resistance in *E. faecalis* (1). Thus, the overuse of antibiotics in the modern hospital setting may have unintentionally transformed commensal *E. faecalis* to pathogenic strain. Antibiotic-resistant *E. faecalis* has a smaller genome optimized for survival in the gut. Over time, pathogenic strains increase the size of their genome as much as 30% by accumulation of insertion elements because of their importance increased virulence and antibiotic resistance for refined transmission in the hospital environment (1).

Biofilm formation in *E. faecalis*

Biofilm formation is an important factor for bacteria survival. It has been shown the majority of *E. faecalis* isolated from patients form a biofilm. Biofilm formation is a complex developmental process regulated by quorum sensing as a response to the cell population (12). Quorum sensing can regulate bacterial gene expression to produce biofilm encased with a hydrated matrix of exopolymeric substances, proteins, polysaccharides, and nucleic acids (12). A biofilm is very difficult to eradicate because they attach irreversibly to biotic and abiotic surfaces (12). Furthermore, biofilm formation shields bacteria from the host immune system and allows bacteria to tolerate antibiotics concentration 10-1000 times more than planktonic cells (12).

There are several kinds of environmental conditions reported to affect biofilm formation and studies have investigated many *E. faecalis* biofilm formation under different kinds of environmental conditions. For example, a study by Pillai and collages (13), suggested that the well-defined quorum sensing system fecal streptococci regulator (*fsr*) locus is controlled directly or indirectly by the glucose-dependent transcriptional regulator

which induces downstream genes important for biofilm production. Therefore, it is believed environmental conditions can activate certain genes that contribute to biofilm structure or formation. There are many contributors to biofilm formation a few of them are good indicators of robust biofilm formation. For example, enterococcal surface protein (ESP), gelatinase (GeIE), fecal streptococci regulator (Fsr), and Ebp (endocarditis and biofilm-associated pili) are considered good biomarkers for robust biofilm-forming *E. faecalis* isolates (12).

Toledo-Arana and collages (14) were able to demonstrate 93% of *E. faecalis* Esp-positive isolates form biofilms while none of Esp negative isolates produced robust biofilms. Therefore, Esp presence strongly correlates with biofilm-producing *E. faecalis*. However, Esp is not necessary for biofilm production because GeIE may contribute to biofilm formation in an esp-lacking background (12). GeIE is one of the downstream genes controlled by *fsr* locus thus *fsr* also is associated with biofilm-forming *E. faecalis* isolates. GeIE presence or its enzymatic activity promotes cell aggregation in a microenvironment that results in a three-dimensional biofilm structure (15). Furthermore, Esp presence could be an indicator of strong biofilm-forming isolate because mutating the N-terminal domain region of Esp produced less biofilm than wildtype (12). In a recent study, Esp was described as a novel facultative amyloid-like protein, in which the protein adopts a functional globular folded structure that switches to amyloid conformation depending on the condition of the environment (16). In the study, pH conditions seem to favor amyloid polymerization (16). It is already established that amyloid aggregation contributes to degenerative and autoimmune disorders in humans but in bacteria, it has a functional role in multicellular behavior and biofilm formation (16, 17).

Commensal strains such as OG1RF do not encode ESP.

Furthermore, biofilms are known to have homogeneous and heterogeneous phenotypes. Homogenous biofilms have a relatively uniform distribution of the bacterial cell while heterogeneous biofilms have a sessile microenvironment within the biofilm with a complex structure such as towers, clusters, and plumes (18). The mechanism that underlies these two phenotypes is not well understood, but there are a few reasons for the heterogeneous biofilms structure formation. One of the reasons is that heterogeneous biofilm is a healthier or stronger biofilm because a tower-like structure in biofilm keeps the biofilm well-fed and reproduces by forming water channels that allow nutrients to penetrate the biofilm (18). The *las* quorum-sensing system has been shown to participate in biofilm phenotype differentiation. A study by Davies and colleagues (19) showed that the wild-type *P. aeruginosa* biofilm formed microcolonies composed of a microenvironment separated by a water channel and with virulence. In contrast, mutation of the *las I* gene resulted in *P. aeruginosa* that was avirulent with a much thinner and uniform biofilm appearance.

Platelet activation

Formerly, platelet activation was only thought to be involved in hemostasis, but now platelet activation is considered to play a major role in IE, septicemia, and many other pathogenic processes (20). While this is still considered somewhat controversial, several studies showing a variety of receptors, soluble molecules, and signaling factors direct involvement in antibacterial defense supports the idea (20).

Normally, platelets are found rapidly moving in the blood vessel. Endothelium damage or encountering of foreign microbes activates platelets and they become sticky. Platelet activation can be summarized in three basic processes. In the first step called platelets adhesion, receptors expressed on platelets adhere to exposed ligands due to endothelium damage. In terms of bacteria, specific antibody targets within bacterial are largely undefined but platelet receptors such as GPIIb-IIIa, GPIb, and FcγRIIIa are hypothesized to adhere directly or indirectly to similar endothelium-like ligands found on the surface of bacteria (20). In indirect adhesion, von Willebrand factor (vWF) and fibrinogen play an important role in forming a bridge between platelets and the adhesive structures of bacteria (20). These factors are released/increased because of endothelium damage or foreign particles in the blood. After platelet adhesion, platelet activation occurs. In platelet activation, important positive feedback of degranulation of ADP, ATP, and Thromboxane 2 (A₂) occurs from platelets (21). Also, the activated platelets begin to express glycoproteins and undergo morphological changes, which are necessary for the final steps of platelet aggregation.

Few studies have reported components of the cell wall such as Ebp (endocarditis and biofilm-associated pili) of *E. faecalis* to be a major adhesive to platelets (22). However, the presence of Ebp is not necessary for platelet activation and there are other important surface proteins on the cell wall such as bacteriophage tail protein Pb1A/B that may have a role in platelet activation (23, 24). Pb1A/B is found in *E. faecalis* and has been associated with platelet binding in *Streptococcus mitis* and Pneumococci (24). Additionally, *E. faecalis*' purified peptidoglycan (PGN) could be a potential inducer of platelet activation. Early studies have reported PGN of gram-positive bacterium activates human platelets via

opsonization of anti-PGN immunoglobulin G (IgG) and then binding to activating IgG receptor called FcγRIIa expressed on human platelets but not in murine (25). Activation of FcγRIIa is indicated by the expression of the activated form of integrin αIIbβ3 in platelets, where it has a role in thrombosis and hemostasis (25). Also, the anti-PGN-IgGs can activate the complement cascade leading to the formation of the C5b-9 membrane attack complex to bind to platelets and exposing platelets' procoagulant phosphatidylserine (25). The role of PGN directly activating platelets is controversial. Conflicting works of literature debate the role of Gram-positive PGN with or without lipoteichoic to signal through TLR 2/1 (26, 27, 28, 29). The current consensus in the field is that TLR2/1 recognizes lipopeptide associated with the cell wall PGN in gram-positive bacteria but does not recognize purified PGN. However, PGN does signal through NOD2, which is present on platelets (30). Although the PGN signal is not restricted to gram-positive bacteria but all bacteria with muramyl dipeptide (30, 31). It is proposed platelet activation can occur through TLR2 signaling (20, 32, 33), which may allow cell wall signaling by Gram-positive bacteria in response to wall-associated lipopeptides.

Hydrogen peroxide production

Intracellular production of superoxide is common among aerotolerant prokaryotes, but extracellular superoxide is unique to *E. faecalis* (34, 35). Superoxide is not harmful because its generation is limited but it can be toxic because it can dismutate spontaneously into hydrogen peroxide. There are genes like *aroC* and *menB* that are involved, respectively, in the shikimate pathway of biosynthesis of aromatic amino acid and demethylmenaquinone via chorismate synthase which is essential for superoxide production in *E. faecalis*. It has been demonstrated, independently mutating *aroC* and *menB* in OG1RF

reduces the rate of superoxide production by more than 10-fold (33). The production of hydrogen peroxide has been suggested to be a virulence factor contributing to colorectal and oral cancer, where *E. faecalis* can be found in the oral microbiota community (36, 37).

Project aim

It was recently discovered in the laboratory that the presence of pCF10 in commensal OG1RF increases resistance to exogenous hydrogen peroxide. Furthermore, it remodels pCF10 biofilms, inducing the formation of heterologous rigid structures in the viscous biofilm. The rigid towers stain intensely for hydrogen peroxide and may be associated with increased amounts of hydrogen peroxide. Rigid structure formation is associated with increases in antibiotic resistance. The plasmid pCF10 is present in a majority of virulent *E. faecalis* isolates. pCF10 is associated with an increased size of heart vegetations in IE. Platelet activation is multifactorial (Figure 1) and we hypothesized that hydrogen peroxide and rigid structure formation may contribute to platelet aggregation. The aims of these studies were to (1) determine the timeline of rigid structure formation, (2) determine whether the rigid structures were associated with increased antibiotic resistance, and (3) determine whether the rigid structures were associated with increased platelet activation.

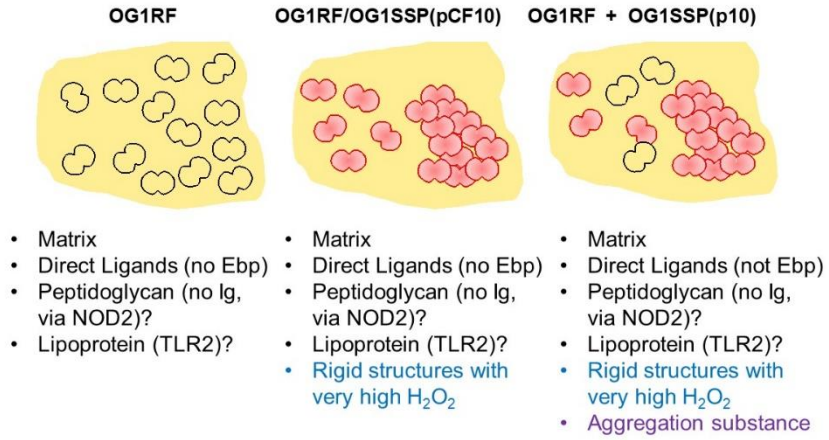


Figure 1. Pheromone responsive plasmid (pCF10) increases the virulence of *E. faecalis* by increasing hydrogen peroxide production and forming rigid structures contributing to platelet activation.

CHAPTER 2

MATERIALS AND METHODS

E. faecalis biofilm preparation

Biofilms of *E. faecalis* OG1RF and OG1RF (pCF10) were grown in 24-well plates on 1.5 microscopic optic cover glass (Fisher Scientific). In the 24 well plates, 2 mL of Todd Hewitt (TH) broth (Appendix) was added to desired wells, and a sterilized cover glass was dropped into the well that contains the media. Then the cover glass was immersed into the bottom of the well using p1000 pipet tips. Freezer stocks of *E. faecalis* were resuspended in 1 mL of TH media, 20 μ l of the media was inoculated into the wells containing 2 mL of media (1:100 dilution). The biofilm was incubated at 37°C for the desired days of biofilm formation and the media was changed twice daily until the day of imaging.

Biofilm imaging

Biofilms were gently washed 3 times with 1 ml of sterile PBS. After the last wash, the buffer was left in the wells and forceps were used to invert the glass coverslip onto a 10-well multitest slide (MP Biochemicals) containing 1 μ l drop of Syto9 (green fluorescent DNA stain; diluted per manufacturer's instructions). The slide must be carefully centered on the well and care must be taken not to slide the coverslip to create surface tension. The coverslip was sealed, with care being taken not to press down on the coverslip. Imaging was done on Leica SP5 Microscope equipped with a TCS confocal system using a 63X objective and 1024x1024 resolution for 2D images. For 4D images, a 20 μ m thick section of biofilm was imaged in 0.5 μ m increments at a resolution of 520x520 (40 total images). Imaging mode was xyzt and speed was set to minimum, so

each 3D image was completed in approximately 1 min and was repeated 20 times for a time-lapse image of 20 mins. Syto9 was excited with a 488 nm laser and emission was measured from 495 to 540 nm.

Relative change in CFU after antibiotic treatment

Biofilms of *E. faecalis* individual community of OG1RF, OGSSP (pCF10), and 1:1 mix community were grown in 24-well plates on Fisherbrand[®] microscopic 1.5 optic cover glass. The biofilms were treated with a final dilution of 50 µg/mL of erythromycin (Appendix) and incubated overnight at 37° C. The next day the biofilm cover glass was washed three times with PBS (Appendix) and emersed in a conical tube containing 5ml of TH along with the last wash of PBS. The conical tube was kept on ice and sonicated for 30 seconds. 10 µl of the sonicated solution was added to the well containing 990 µl of TH media. To the well, a 10-fold serial dilution was applied and then 10 µl of the solution was spot plated on TH agar (Appendix). The spot plate was incubated overnight at 37°C. The next day a spot-on agar with 3-30 colonies was counted and then used to calculate the CFU. The CFU was used to calculate relative change in CFU between treated biofilm vs. untreated to analyze the resistance of biofilm to the antibiotic.

Platelet interaction with biofilm

Platelets were provided by Dr. John Kostyak from the Thrombosis Research Center at Lewis Katz School of Medicine. For these experiments, 2-day, 4-day or 5-day stationary phase biofilms were washed 3 times with a complete Tyrode's buffer. Freshly isolated human platelets (10^9 /ml) were stained with 1:2000 dilution of Fischer Scientific cell mask Deep Red. From the dilution, 1 ml was added to the well and incubated at 37°C for 2 hours. After 2 hours of incubation, the biofilm was washed with 1 mL Tyrode's

buffer. The platelet activation was analyzed using laser confocal microscopy (LSCM) using the Argon 488 and DPSS 561.

For aspirin treated platelets, the collected platelets were separated into 2 batches and 1 tube was aspirin-treated.

For catalase inhibition studies 3000U/mL of catalase was added to the well-containing biofilm and platelets. Then the well was incubated for 2 hours and then analyzed by a confocal microscope after staining the biofilm with Syto9. Applying catalase as mentioned above seems to damage biofilm some of the time. Therefore, dilutions were determined, and 2250U/mL is the highest concentration of catalase that can be added to the biofilms.

***menB* knockout in *E. faecalis* chromosomes**

Insertion of menB gene on pBVGH vector to construct pRA3

menB gene knockout from the chromosome of *E. faecalis* was attempted using the procedure described by Blancato et al with minor modification (53). A temperature-sensitive pBVGH plasmid with built-in *S. aureus* promoter (TS-pWV01), few known multiple cloning sites, and *ermC* gene that encodes erythromycin resistance was used. This vector also carries a gene that encodes β -galactosidase, ideal for fast color screening of transformants on TH agar plates supplemented with a final dilution of 0.6 μ l/mL 8 % X-gal (Promega). The insert, *menB*, on the OG1RF chromosome was acquired by PCR amplification. *menB* was amplified using dream Taq DNA polymerase (Fisher Scientific) and *menB* forward and reverse primers that have built-in NcoI and Sac I restriction sites, respectively (Table 2). The vector was double digested with NcoI and Sac I to

insert *menB* on the vector via Fischer Scientific T4 ligation. The new construct (pRA3) was then transferred into the DH5 α competent cell via a standard transformation process. The competent cell was grown on LB agar containing 300 $\mu\text{g}/\text{mL}$ of erythromycin antibiotic for 24-48 hours at 37°C. The transformants were cultured by shaking at 174 rpm at a 37°C water bath in LB media containing 300 $\mu\text{g}/\text{mL}$ of erythromycin. A standard DNA isolation method was used to isolate pRA3 from the cell. pRA3 isolation was confirmed by double digestion with NcoI and Sac I and Southern blotting.

Construction of pRA4 from pRA3

Next, another plasmid was constructed using the previous plasmid construct pRA3 vector. This plasmid was constructed by digesting pRA3 with the Ahd-I restriction enzyme, which only cuts pRA3 once in between *menB*. The digested ends were made blunt by filling the sticky ends with 2mM of dNTPs using Promega Klenow polymerase. The digest was ligated with *neo* gene insert. The new construct was named pRA4 and was transferred into a competent DH5 α cell via a standard transformation process. The transformants were grown on LB agar containing 74 $\mu\text{g}/\text{mL}$ of neomycin antibiotic for 24-48 hours at 37°C incubation. The transformants were inoculated into LB media containing 74 $\mu\text{g}/\text{mL}$ of neomycin antibiotic shaking at 174 rpm at a 37°C water bath for overnight incubation. The pRA4 was isolated from the cell by standard procedures and confirmed through PCR using *menB* forward and reverse primers.

Electroporation of pRA4 into OG1RF

Finally, pRA4 (250-1000ng) was transferred into electrocompetent OG1RF *E. faecalis* cell via electroporation. Plasmid-bacteria cell mixtures were electroporated in 100 μl Eppendorf electroporation cuvettes (catalog # 940001005) using the Bio-Rad gene

pulse electroporator settings of 1,800 V, 25 uF, and 200 Ω . The time constant was 2.4. The post-electroporation mixture was resuspended with TH with 0.25M sucrose and incubated for two hours at 37° C. 100 μ l of electroporation cells were inoculated for a single colony on TH agar containing 50 μ g/mL of erythromycin and 0.6 μ l/mL of X-gal for 24-48 hours at 37°C. The blue transformants that grew on the erythromycin-X-gal-TH agar were selected and patched on separate TH-X-gal-agar plates containing erythromycin (50 μ g/ml), low dose neomycin (70 μ g/mL), and kanamycin (70 μ g/mL), against which the neomycin resistance gene also confers resistance. Then the plates were incubated at 37°C for 24-48 hours. After the chosen blue patch from the plate was inoculated in a 50 ml culture tube with 5mL TH media with low dose neomycin (70 μ g/mL) at a non-permissive water bath degree of 42°C. The non-permissive degree of 42°C halts plasmid replication and induces rare events such as crossing over leading to *menB* knockout from the OG1RF chromosome. After overnight growth, the culture was plated on neomycin, erythromycin, and plain TH agar -X-gal plate. The growth of white transformant on neomycin agar-X-gal but not erythromycin agar-X-gal plate alludes knockout event has occurred. The white *E. faecalis* transformant resistant to neomycin and sensitive to erythromycin was further analyzed by PCR for the gene knockout. The knockout viability was assessed by culturing in TH media containing neomycin.

Standard procedures

Plasmid isolation:

DNA isolation was done using a Promega Wizard® DNA Purification kit. An overnight night 20-25 mL of bacterial cells were centrifuged at top speed for 10 minutes. From the aliquot, the supernatant was discarded, and the bacteria were resuspended with 3 ml of resuspension solution. The cell was lysed open with 3 ml lysis buffer (0.2M NaOH and 1% SDS) and immediately was neutralized with 3ml of neutralization buffer to precipitate cellular debris. The cell was centrifuged at top speed for 10 min to remove the cellular debris. The supernatant was transferred to a fresh microcentrifuge tube, and 5µl of RNase was added to remove RNA from the solution. 2 ml of resin was added to the supernatant and loaded into a separation column and the supernatant was removed via vacuum (Promega Vac-Man® Laboratory Vacuum Manifold). The cell was washed with column wash by vacuuming. Finally, the plasmid was eluted with 50-75 µl of eluting buffer into sterile collecting tubes by centrifugation for 90 seconds at top speed.

Chromosomal DNA purification:

DNA gene purification was done after the isolation of plasmid from bacterial cells. Into 200 µl of plasmid isolate mix, 600 µl of binding buffer solution was mixed. The mixture was then added to Fisher Scientific Gene Jet Purification Column and collection tubes and was centrifuged for 20 seconds at top speed. The filtrate was discarded and 800 µl of Fisher Scientific wash buffer with ethanol was added to the purification column. The solution was centrifuged for 20 sec at top speed. The filtrate was discarded, and the purification column was centrifuged for 2 min with the lid open to evaporate left-over ethanol. The column was transferred to a sterilized collecting tube.

The DNA attached to the silica of the column was eluted with 60-75 elution buffer by centrifugation at top speed for 20 seconds.

Restriction enzyme DNA digestion:

The reaction of the restriction enzyme was set up as follows. The mixtures include 10 μ l of plasmids, 1.2 μ l restriction enzyme (NcoI, Sac I, or Ahd-1), 3 μ l buffer (Cut smart), and 15 μ l of the eluting buffer. The mixture was incubated overnight at 37°C. The protocol was used for ligation and conformation.

DNA ligation:

The reaction of T4 ligase was set up as follows. The mixture includes 13 μ l of pBVGH or pRA3, 3 μ l of purified *menB* or neo gene, 1 μ l T4 DNase ligase, and 2 μ l of ligase buffer. The reaction occurred overnight at room temperature. The ligation mix was electroporated into the electro-competent bacterial cells.

Transformation:

A freezer stock of DH5 α competent cell was thawed on ice. 75 μ l of the thawed cell was mixed with a prepared ligation mix. The mixture was kept on ice for 25 min and then heat shocked in a 42°C of water bath for 1 minute. The heat-shocked cell was returned to the ice for 5 minutes. Then 600 μ l of LB broth (Appendix) was added to the cell and was left to incubate for 1 hour at 37°C. After incubation, the cell was centrifuged at 5000 rpm for 3 minutes and only kept 200 μ l of the aliquot. The aliquot was mixed by pipetting up and down. 200 μ l of the mixture was plated on agar with specific antibiotic selection. The agar plate was incubated at 37°C for about 24-48 hours. The transformants

on the agar plate were confirmed further if they have the ligated DNA by PCR. If PCR confirmed the presence of plasmid in the cell, the plasmid was then isolated.

Klenow blunt end formation:

Digestion of plasmid pRA3 with Ahd-I generates sticky ends. These ends were made blunt the with following mixtures. The mixture includes 42 µl of eluted pRA3-Ahd-1 digest, 5µl of Promega Klenow buffer, 8µl of 2 mM dNTPs, and 1 µl of Klenow polymerase. The mixture was incubated at 37°C for 30 minutes and then gene cleaned.

Polymerase chain reaction:

PCR was used to amplify the *menB* gene from OG1RF *E. faecalis* template and to confirm *menB* gene insertion on pRA3 and pRA4 vectors. A total of 25 µl mixtures were prepared on ice. The mixtures include 2.5 µl of Fisher Scientific dream Taq buffer (10x), 2 µl of 2 mM dNTPs (10X), 0.25 µl of 17.4 nmol forward *menB* Nco1 primer, 0.25 µl of 19.1 nmol reverse *menB* Sac I primer, 20 µl of eluting buffer, 0.125 µl Fisher Scientific dream Taq DNA polymerase, and 0.5 µl – ng/µl of *E. faecalis* DNA template or plasmid. The reactions were run using an Applied Biosystems Thermal Cyclor (version 2.08) under conditions listed in Table 1.

Table 1. Polymerase Chain reaction conditions.

Step #	Temperature	Time
1	94°C	1 min
2	94°C	30 sec
3	63°C	30 sec
4	72°C	2 min 30 sec
5	72°C	5 sec
6	4°C	∞

Agarose gel electrophoresis

PCR product was visualized using 1% agarose gel electrophoresis. 0.6g of agarose was dissolved in 60 mL of 1x TAE buffer (Appendix) by microwaving in small intervals. The dissolved solution was cooled and poured into the Fisherbiotech horizontal gel electrophoresis system with an appropriate comb. After the gel solidified, the comb was removed and the wells of the gel on the casting tray were positioned proximal to the anion charge. TAE buffer was added to cover the surface of the gel. A total of 25 μ l of PCR products along with a 1-kb full-scale DNA ladder was loaded into the wells. The ladder was used to compare the size of PCR product bands. If the product was from restriction enzymes, digesting a Promega blue/orange 6x loading dye was mixed with the reaction before loading into the gel. BRL Life Technologies, INC power supply system was used to run the gel. An initial 120 volts was applied until the products entered the gel. Once the product enters the gel the voltage is increased to 130 volts until the dye gets toward the end of the gel. The gel was removed and immersed into a 1x TAE buffer that contains 3 μ l of ethidium bromide bath for at least 20 minutes. Gels were imaged using the Fisher Scientific XR system.

***E. faecalis* crude cell wall preparation for platelet activation**

E. faecalis crude cell wall was prepared as described by Navarre et al with minor modification (38). Each strain of *E. faecalis* was cultured overnight for no longer than 15hr in 25 mL Fisher Scientific BHI broth (Appendix) at 37°C. The next day 25 mL of the media was inoculated into 250 mL BHI broth which was grown until reaching the mid-log phase (OD 660_{nm} = 0.5) at 37°C in a water bath. The cells were recovered by centrifugation (15 min at 8000xg) and then were resuspended in 6 mL Tris-HCL (50 mM

Tris-HCL). The resuspended cells were broken using the zirconia/silica beads for 1 minute, 5 times at 4800xg (resuspended cells were divided into 4 tubes, each holding 1.5 g of zirconia/silica beads & 1.5 mL of the resuspended cells). The cells were cooled on ice between each pulse. Zirconia/silica beads were removed by low-speed centrifugation a total of 4.4 mL of the supernatant was kept and sedimented by ultracentrifugation (100,000 X g for 30 min). Then the cells were suspended in 20 mL of 1% SDS solution, boiled for 30 min, and washed 5 times by centrifugation with diH₂O (30,000 x g). Finally, the cells were dried in a speed vac and weighed. Finally, the crude cell wall effect on platelet activation was analyzed by an aggregometer in the Thrombosis Research Center at Lewis Katz School of Medicine.

***E. faecalis* peptidoglycan preparation**

E. faecalis peptidoglycan was purified as described by Zhang et al (39) which was adapted from Navarre et al (30). We made some minor modifications to the producer. *E. faecalis* was cultured overnight for no longer than 15hr in 25 mL BHI media at 37°C. The next day the 25 mL of the media was inoculated to 250 mL BHI media and was grown to mid-log phase (OD_{660nm} = 0.5) in the water bath at 37°C. Then the cells were separated into four 50 mL conical tubes and centrifuge for 10 min at 4000 rpm at 4°C. After resuspending the cell in 100 mL 4% SDS, the cells were boiled for 30 min, centrifuged, and stored at -20°C. The next day the cells were thawed and were washed with 45 mL diH₂O 6 times at 30,000xg. After resuspending the cell in 6 mL diH₂O, the cells were broken by bead beating using zirconia/silica beads for 1 minute 5 time each at 4800xg (resuspended cells were divided into 4 tubes, each holding 1.5 g of zirconia/silica beads & 1.5 mL of the resuspended cells). Then the zirconia/silica beads were removed by low-

speed centrifugation and a total of 4.4 mL of the supernatant was kept. After centrifugation (30,000 x g for 15 min), the cells were resuspended in several solutions (4.4 mL buffer (50 mM Tris-HCL, 10 mM MgCl_2 pH 7.5), 500 μl Alpha-amylase to final concentration of 100 $\mu\text{g}/\text{mL}$, 50 μl DNase I to final concentration 10 $\mu\text{g}/\text{mL}$, & 50 μl RNase A to final concentration of 50 $\mu\text{g}/\text{mL}$) and were incubated in water bath for 2 hours at 37°C. Next residual proteins were digested with CaCl_2 (final concentration of 10 mM) and trypsin (final concentration of 100 $\mu\text{g}/\text{mL}$) and then incubated overnight at 37°C. The next day, the cell was washed in several solutions (diH₂O two times, 8M LiCl, 100 mM EDTA, di H₂O two times & acetone) and were resuspended in 3 mL diH₂O. After drying in speed vac, the dry mass was weighed and incubated with ice-cold HF (48% to 51 %) for 96 hours at 4°C. After 96 hours, the cells were centrifuged, washed with diH₂O, and resuspended the cells with 1.5 di H₂O. Finally, the purified peptidoglycans were stored at -20° C, dried in a speed vac, and weighed the dry mass. The effect of purified peptidoglycan from both strains on platelet activation was analyzed by an aggregometer in the Thrombosis Research Center at Lewis Katz School of Medicine.

CHAPTER 3

RESULTS

The formation of the heterologous rigid tower structures in *E. faecalis* biofilm

Biofilm formation is an important virulence factor for bacteria to thrive in a host. The majority of *E. faecalis* isolated from patients form biofilms and are present in urinary tract infections and endocarditis. Biofilms can have multiple types of material properties from viscous fluid-like movement to rigid fixed structures. In a previous study heterologous rigid structures were not observed in viscous commensal strain OG1RF, while OG1RF (pCF10) formed heterologous rigid structures. The initial observations were made on 5–7 day old stationary phase biofilms. After 7 days *E. faecalis* would form a homogenous rigid base structure with viscous biofilm still above it, but no heterologous towers, while pCF10-containing OG1RF formed rigid structures. However, *E. faecalis*, in the gastrointestinal tract may not be under starvation and many studies of *E. faecalis* biofilms are done on growing biofilms.

To characterize rigid structure formation in biofilms growing biofilms, biofilms were grown in 24 well plates with the addition of fresh medium twice a day, in the morning and evening. OG1RF (Rif^R, Fus) and OG1SSP (Spec^R, Strep^R) are spontaneous antibiotic mutant derivatives of the commensal type of strain OG1 and have been shown to behave identically in multiple studies including animal models. In preliminary studies, both OG1RF (pCF10) and OG1SPP (pCF10) were shown to form rigid structures with no discernible differences. After 1-3 days of growth, biofilms were stained with Syto9 green-fluorescent stain, and imaged using LSCM (Figure 2). For sake of consistency with mixed species experiments, OG1RF was used as the commensal strain and OG1SSP (pCF10) was used as the plasmid-containing strain (designated p10). OG1RF biofilms do

not develop the tower-like structures. In contrast, the individual pCF10-containing biofilms developed tower-like structures usually by day 2, but always by day three (Figure 2). 4D imaging (3D imaging over time) revealed OG1RF biofilms remained viscous consistent with our previous observations of 2-day biofilms (40) and a homogenous thin rigid base layer would begin to form between days 3-4 (1-5 μm). The pCF10-containing biofilms had heterologous rigid tower structures and the regions of the biofilm without towers had a thin rigid base and viscous biofilm above the base. The viscous biofilm layer in pCF10-containing biofilms was often thinner (5-10 μm) as compared to the 20-25 μm thick viscous layer in OG1RF biofilms. By day 3, the rigid-base layer would thicken in some of the biofilms (Figure 3, p10 day 3 3D biofilm).

To determine the dynamics of a mixed population of plasmid-free recipient cells and plasmid-containing donor cells, OG1RF::mCherry, containing a markerless mCherry fluorescent protein gene (kindly provided by G. Dunny, U of MN) was mixed with OG1SSP(pCF10). These biofilms developed heterologous rigid structures like pCF10-containing biofilms (p10, Fig. 2). However, the tower-like structures developed earlier in day 1 and thinner areas lacked the rigid lower layer of biofilm but, instead, had a thin 1-3 μm layer of viscous biofilm between the towers, which was not as easily visible in the 3D LSCM images. An additional observation worth noting is that tower-like structures were more frequent in the mixed donor-recipient biofilm and overall biofilms appeared to be thicker and more robust. Further studies at lower magnification are needed to confirm this observation. mCherry labeled OG1RF cells were found predominantly in the thin viscous layer between the towers (Fig. 3H). However, occasionally, mCherry cells could be seen incorporated into rigid structures (Fig. 3 I and J). Since the towers are associated with

increased hydrogen peroxide staining (previous unpublished observations) and pCF10 protects from hydrogen peroxide (previous unpublished observations), these mCherry plasmid-free cells may have obtained pCF10 by conjugation prior to forming rigid structures (Fig. 3I) or perhaps after the towers formed (Fig. 3J). Mating pairs of pCF10 may be relatively stable after formation (G. Dunny, personal communication and unpublished observations), which would explain the mixed OG1RF::mCherry (red) and OG1SSP (green) staining patterns. Differential plating to determine plasmid transfer events (TetR pCF10 and FusR OG1SSP for transconjugants) and imaging using fluorescently labeled pCF10 can be used to confirm plasmid transfer events.

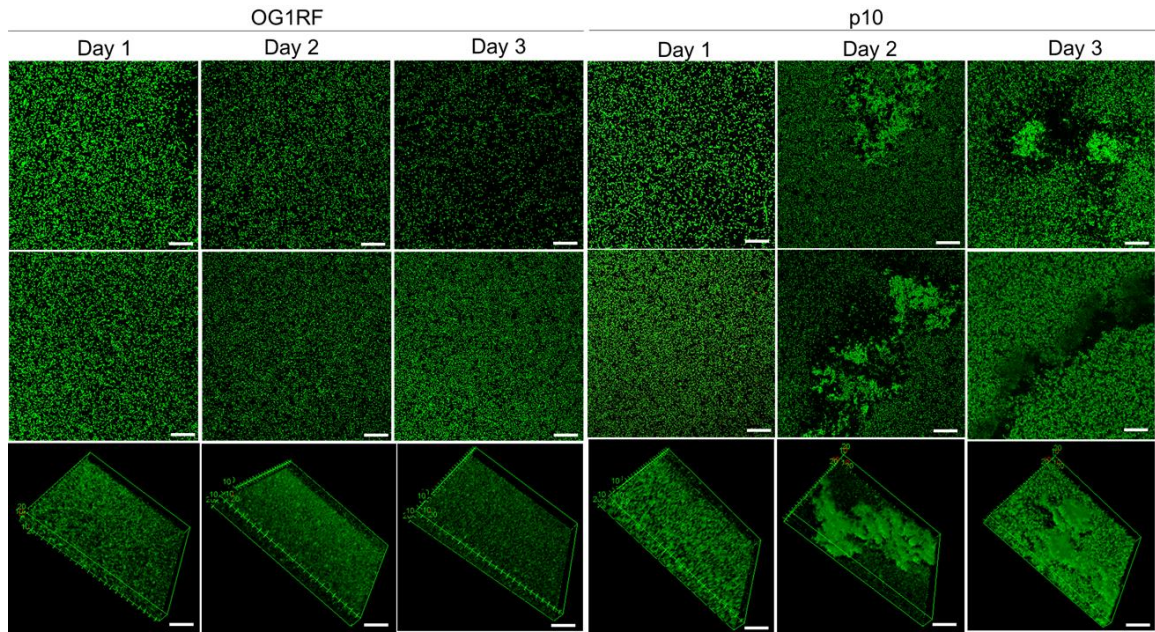


Figure 2. The presence of pCF10 promotes the formation of heterologous rigid structures in *E. faecalis* biofilms. Biofilms of *E. faecalis* OG1RF or OG1SSP (pCF10) were grown on glass coverslips in Todd Hewitt (TH) media with twice-daily media changes. Single species biofilms were stained with Syto9 green and imaged by LSCM. Scale bar is 20 μm .

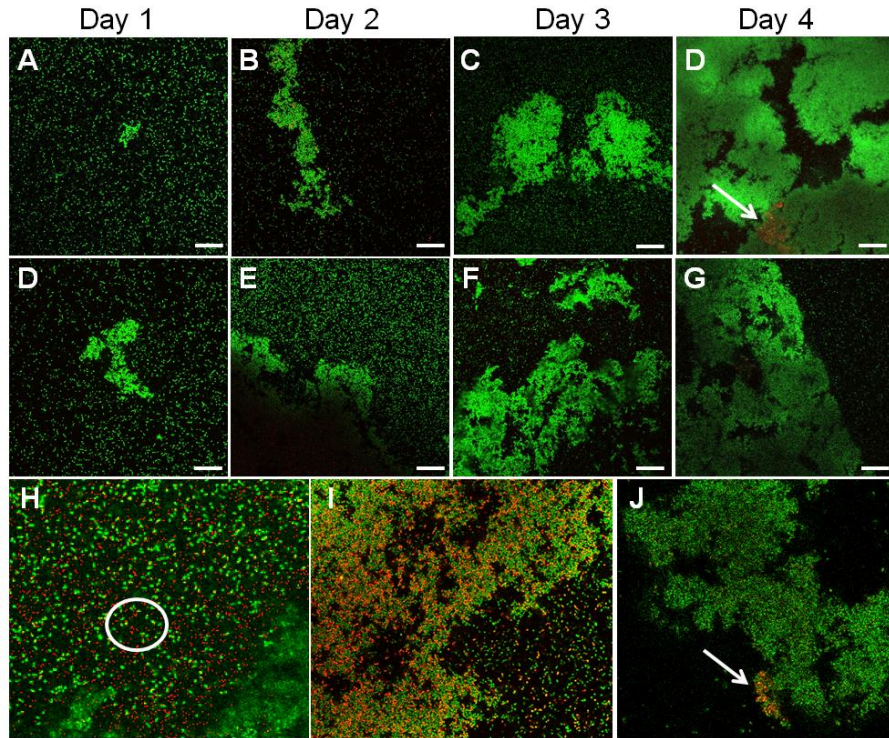


Figure 3. Organization of mixed plasmid-free and plasmid-containing *E. faecalis* biofilms. *E. faecalis* OG1RF::mCherry and OG1SSP(pCF10) cultures were mixed 1:1 immediately prior to inoculation and biofilms were grown on glass coverslips in Todd Hewitt (TH) media with twice daily media changes. Biofilms were stained with Syto 9 (green) and imaged on the green channel (Syto9) and red channel OG1RF::mCherry (red). Green and red overlays are shown. Experiments are representative of at least 3 independent experiments. Scale bar is 20 μm .

Heterologous rigid structures correlate with the increase in antibiotic resistance

Rigid structures in biofilms have been associated with antibiotic resistance (41,42, 43). In *P. aeruginosa* tower-like structures play an important role in tolerating biocides such as sodium dodecyl sulfate (SDS, 0.2%). Mutation of *P. aeruginosa*'s gene (*las I*) responsible for tower formation made the bacteria vulnerable since SDS was able to detach the biofilm from the surface of attachment within five minutes (23). In our study, we examined whether the formation of tower-like structures increases antibiotic resistance by treating individual species and mixed species of *E. faecalis* with erythromycin. Biofilms, with and without pCF10, were treated with 100X the MIC of erythromycin for 24 hrs. Treatment on day 0 measured inhibition of biofilm formation by inhibiting the growth of planktonic cells. Treatment on day 1 of biofilm growth was before rigid structure formation and on day 2 it was after rigid structures began to form. The CFU per biofilm was calculated by serial dilution plating and the results were plotted as a reduction in the ratio of treated CFU/untreated CFU (Figure 4A). Ratios <1 reflect inhibition of growth of the antibiotic treated biofilms, whereas ratios of = or >1 reflect the continued growth of the antibiotic treated population (Figure 4).

Antibiotic resistance was not observed in commensal OG1RF biofilms. However, significant antibiotic resistance was observed on day two of pCF10-containing biofilms, sometimes showing higher growth than the untreated controls, and trends toward resistance in mixed-species biofilms. These data suggest that there is a correlation between tower development and antibiotic resistance as structures begin to form. There are differences in the size and arrangement of heterologous structure formation between independent biological experiments and replicates within the same independent experiment, so the

variation in levels of protection may be due to differences in structure formation. Imaging of biofilms was not a useful technique. Consistent with its bacteriostatic mechanism of action, erythromycin does not kill *E. faecalis*, so Live/Dead staining showed no differences in the killing. Because even viscous OG1RF *E. faecalis* biofilms have thinner and thicker regions (Figure 2), the reduced growth was difficult to assess in an unbiased manner and changes in heterologous structures across an entire biofilm were even more difficult to access without introducing bias.

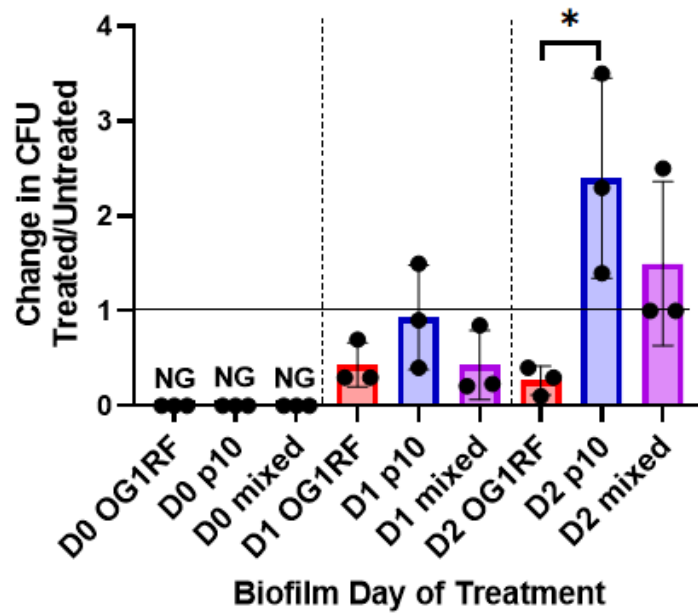


Figure 4. Antibiotic resistance is increased in Day 2 biofilms. Biofilms of *E. faecalis* OG1RF or OG1SSP (pCF10) were grown on glass coverslips in Todd Hewitt (TH) media with twice-daily media changes. erythromycin (100X MIC) was added to the biofilms (Day 0, prior to biofilm formation, Day 1 fluid biofilm, or Day 2 biofilm with rigid structures). The biofilms were incubated an additional 24 hrs, washed, and dispersed by sonication. Colony-forming units (CFU) were determined by serial dilution. Results are reported as a change in CFU Treated/Untreated. Each dot represents a biological replicate. NG indicates no growth (lower limit of detection of 100 CFU/ml).

Activation of human platelets by *E. faecalis* biofilm

Platelet activation is an important virulence factor of *E. faecalis* in endocarditis and septicemia (25, 17). To examine platelet activation by *E. faecalis* biofilms, *E. faecalis* biofilms with and without pCF10 were grown on a glass coverslip for 4 to 5 days. After biofilm development, 1×10^9 human platelets isolated from healthy donors labeled with CellMask orange were incubated for 2 hrs with biofilm. These biofilms were grown under stationary biofilm conditions without medium changes. Under these conditions, pCF10-containing biofilms form rigid structures more slowly. Rigid bases and small aggregate beginning to form around Day 2 and towers form around 4 - 5 days after incubation without medium changes. On days 4 and 5 biofilm after 2 hours of incubation, unassociated platelets were removed by gentle washing, and biofilms were stained with Styo9 and platelet activation was determined using LSCM. Both the commensal OG1RF and the pathogenic pCF10 biofilms activated human platelets (Figure 5). However, platelet aggregation was stronger in pCF10-containing strains than OG1RF (Figure 5B). Platelets in contact with OG1RF biofilms went from inactivated approximately $1 \mu\text{m}$ cells (the same size as the bacteria) to enlarged cells and some small aggregates suggesting early activation. However, in pCF10-containing biofilms larger platelet aggregates could be observed, suggesting stronger activation of platelets (Figure 5B).

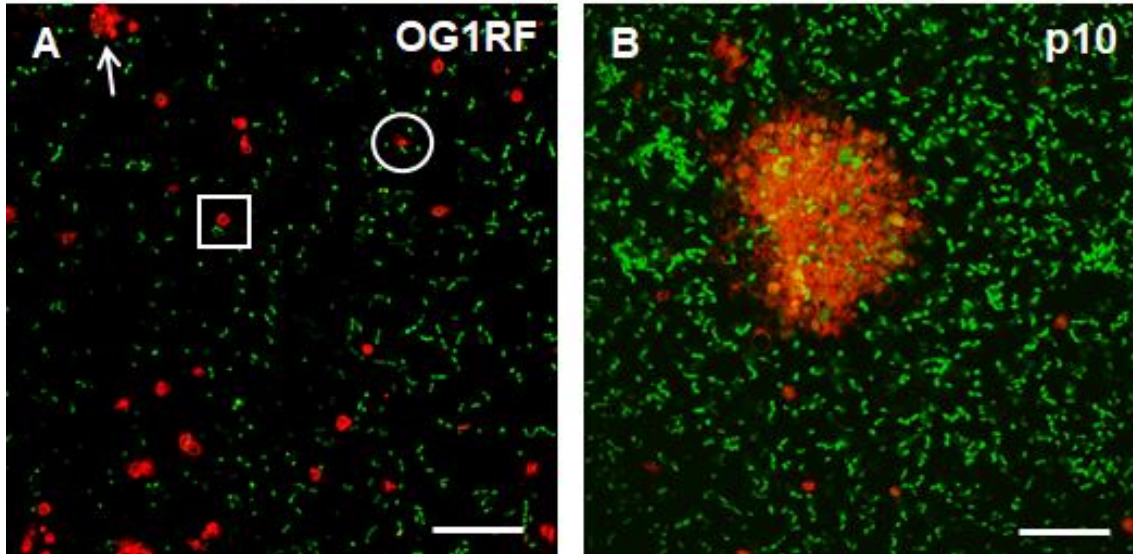


Figure 5. Stationary phase 2-day biofilms of pCF10-containing *E. faecalis* show increased activation of platelets. Human platelets from healthy donors were labeled with Cell Mask orange (red) and added to 2-day stationary phase *E. faecalis* biofilms and allowed to incubate for 2 hr. After two hours, platelets not associated with the biofilms were removed by gentle washing. Biofilms were stained with Syto9 (green) and imaged using LSCM. Unactivated platelets are approximately 1 μm , the same size as *E. faecalis* (circle), in early activation the platelets enlarge (square) and in later activation the platelets aggregate (arrow). The micron bar is 20 μm . These results are representative of at least three independent experiments.

To confirm the platelet enlargement was due to platelet aggregation, human platelets were pretreated with aspirin, a major anticoagulant prior to addition to the biofilms as described above (Figure 5). Aspirin blocks thromboxane A₂, a platelet-produced signal, released early during activation, that amplifies platelet aggregation. In two preliminary experiments, one on Day 4 biofilms and a second on Day 5 biofilms, aspirin attenuated platelet aggregation when incubated with both pCF10-containing and pCF10-free biofilms (Figure 6). Platelet aggregation visually appeared greater in the proximity of the rigid structures. Therefore, we hypothesize that platelet activation was a result of *E. faecalis* biofilms triggering the cascade of platelet activation. These experiments will be repeated with growing biofilms as described in previous sections and analyzed with a toolbox under development to correlate biofilm density and platelet activation.

Hydrogen peroxide is a known activator of platelets (34, 44, 45). To determine if hydrogen peroxide contributes to activation, a preliminary experiment was done to determine if catalase could reduce the amount of hydrogen peroxide present (Figure 7). Catalase (3000 U/mL) was added to the wells of 3D pCF10-containing biofilms. A reduction in platelet activation was observed. Although not observed in this experiment, in subsequent experiments, high levels of catalase were found to disrupt biofilm structures. A series of imaging experiments were done to determine that 2250 U/mL of catalase is the highest level that does not show visible disruption of biofilm structures. This concentration will be used in subsequent experiments.

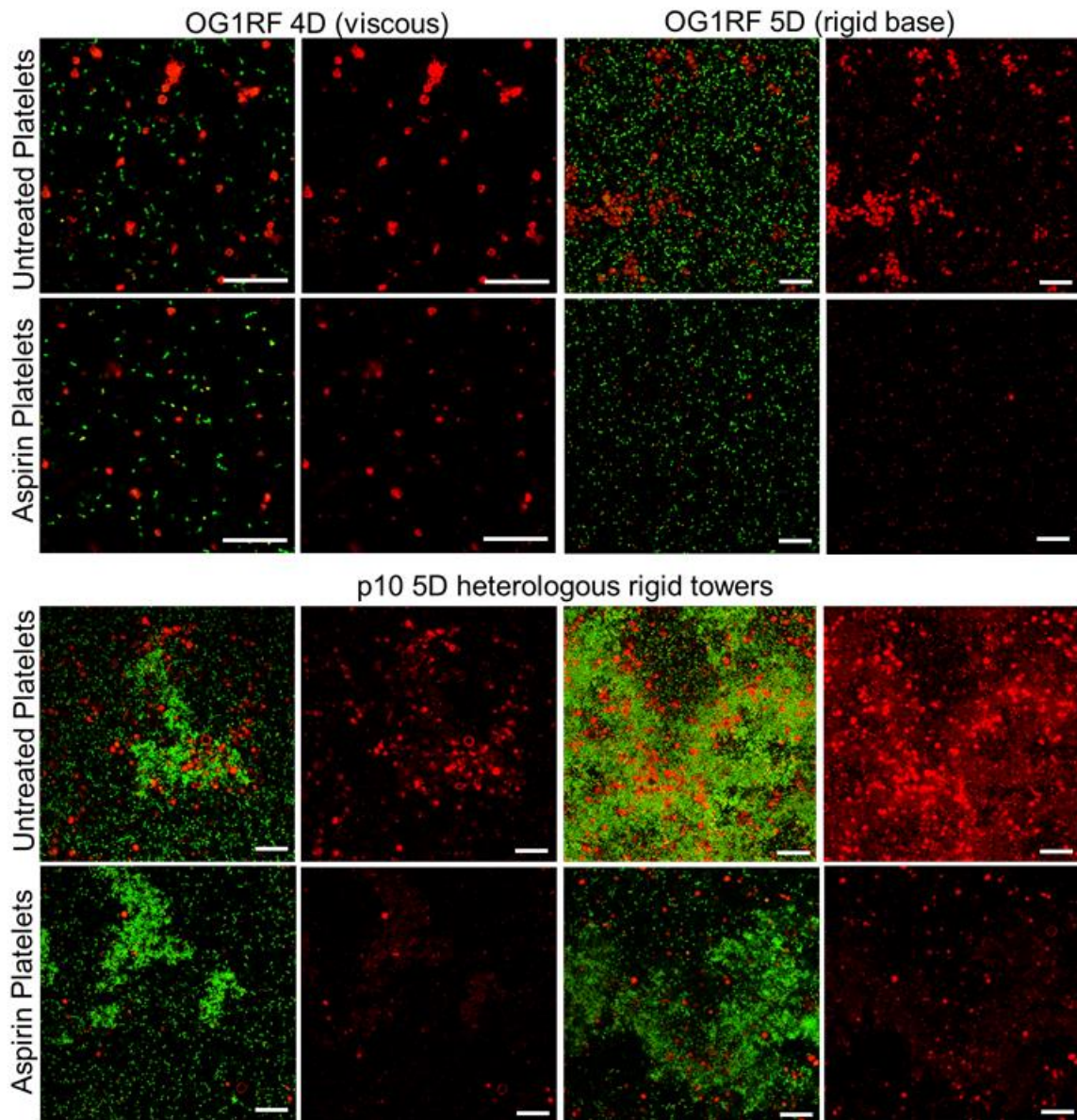


Figure 6. Increased activation of platelets can be inhibited by aspirin. Human platelets from healthy donors were labeled with Cell Mask orange (red) and added to *E. faecalis* biofilms and allowed to incubate for 2 hr. After two hours, platelets not associated with the biofilms were removed by washing. Bacteria were stained with Syto9 (green) and imaged using LSCM. Unactivated platelets are approximately 1 μ m, approximately the same size as *E. faecalis* (circle), in early activation the platelets grow in size (square) and in later activation aggregate (arrow). The micron bar is 20 μ m. These results are representative of 2 independent experiments one on 4 day biofilms and one on 5 day biofilms.

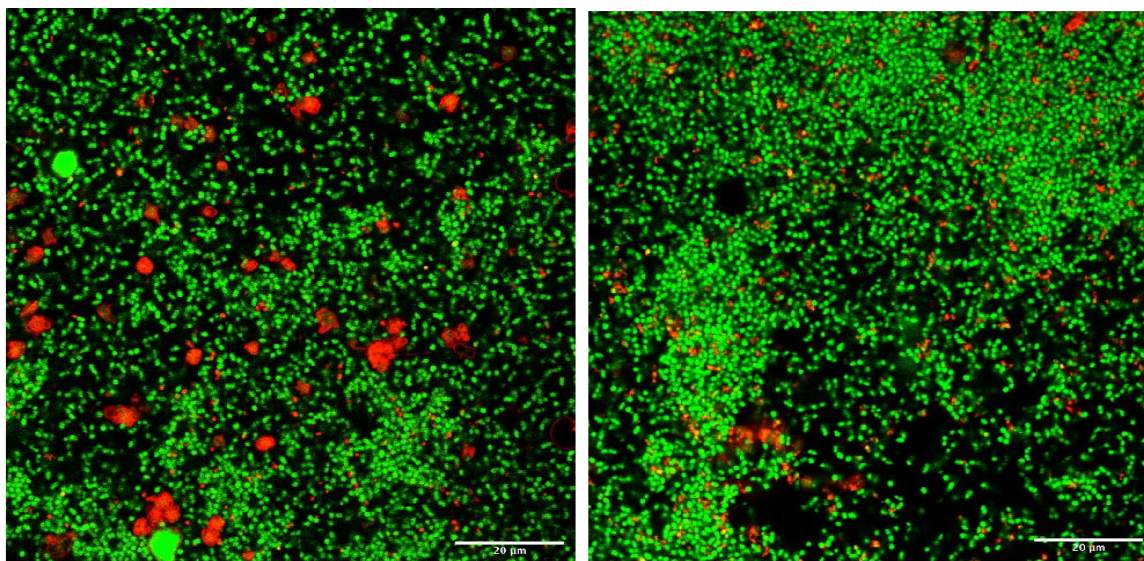


Figure 7. Catalase reduces platelet activation. . Human platelets from healthy donors were labeled with Cell Mask orange (red). Platelets were added to *E. faecalis* biofilms in the absence (A) and presence of 3000U/ml of catalase (B) and allowed to incubate for 2 hr. After two hours, platelets not associated with the biofilms were removed by washing. Bacteria were stained with Syto9 (green) and imaged using LSCM. Micron bar is 20 µm.

Knockout of *menB* and *aroC* gene involved hydrogen peroxide production

Hydrogen peroxide is a known activator of platelets. *E. faecalis* produce hydrogen peroxide. Reduced NAD (NADH) is produced as a byproduct of various sugar fermentations including glycolysis used for energy production by *E. faecalis*. To recycle the NADH to NAD, demethylquinone is coupled with various oxidases producing superoxide as a byproduct. The superoxide is dismutated into hydrogen peroxide. While the gastrointestinal tract is predominantly anaerobic, oxygen gradients are present near the mucosal layers, which may play a role in hydrogen peroxide production in the GI tract. Two pathways are important for the synthesis of demethylmenaquinone and the production of hydrogen peroxide, *aro* for the synthesis of chorismite (also a precursor for phenylalanine, tryptophan, and tyrosine) and *men* for the synthesis of demethylmenaquinone (35). Mutation of *aroE*, *C*, or *A* by plasmid integration reduced production 10-fold and mutation of *menB* reduced production only by 22%, suggesting there may be other pathways for hydrogen peroxide production that utilize chorismite (35). Since *menB* mutants only reduced production by 22% compared to a 99% reduction in the *aro* pathway, it suggests there may be an alternative to produce superoxides for hydrogen peroxide production (35).

To examine the role of hydrogen peroxide production in the activation of platelets and its dependence on oxygen, construction of knockout mutants of 1,4-dihydroxy-2-naphthoic acid synthase (*menB*), chorismate synthase (*aroC*), and a double mutant was initiated. Since the original mutations were made by plasmid integration, the goal was to construct mutants with antibiotic gene insertions by double-crossover to allow the eventual production of a double mutant. Since chorismate is necessary for amino acid

synthesis, *menB* was chosen as the first target because demethylmenaquinone is not as essential for growth since there are redundant metabolic mechanisms in *E. faecalis* to recycle NAD and this would eliminate the known oxygen-dependent pathway. The strategy was to use the temperature-sensitive vector pBVGH for insertional inactivation of the *menB* with an antibiotic cassette through the double cross-over integration. In *E. faecalis*, chromosomal integration requires the insert first be on a plasmid, then the gene will go into by single cross-over and then the plasmid will be excised from the chromosome leaving the integrated antibiotic resistance gene disrupting the gene of interest. The temperature-sensitive plasmid pBVGH (6kb) has a temperature-sensitive origin (TS-pWV01), few known multiple cloning sites, and *ermC* that encodes erythromycin resistance (53). This vector also carries a gene that encodes β -galactosidase that allows for fast screening of transformants on agar plates supplemented with X-Gal (53).

To knockout *menB* on the OG1RF chromosome we first need to acquire *menB* from *E. faecalis* chromosome. Using *E. faecalis* template, PCR amplification was done via dream Taq DNA polymerase and *menB* forward primer containing a 5' NcoI and a reverse primer that had 3' Sac I restriction sites (Table 1, Figure 8). Using the restriction enzyme sites (NcoI and Sac I) on pBVGH, *menB* (0.75 kb) was ligated into pBVGH forming plasmid pRA3. The pRA3 construct was confirmed using NcoI and Sac I double digest. There were two bands on the blot around 0.75kb and close to 6kb which corresponds to *menB* and pBVGH, respectively. We then constructed a plasmid we called pRA4 (Figure 8A). The sequence is known, so unique restriction sites were identified in *menB* and then pRA3 was screened for a unique restriction site

that would cleave pRA3 only once in *menB* and not in the cloning vector (Figure 8B). Next, the digest ends were made blunt by filling the sticky ends with dNTPs using Klenow polymerase. The digest was ligated with the *neo* gene (2kb) allowing the construct to encode neomycin resistance. The new construct, pRA4 is confirmed through PCR using *menB* forward and reverse primers (Figure 8C). The primers amplified strands of DNA that were approximately 3kb (*menB* (0.75kb) + *neo* (2kb)). Therefore, a competent cell that acquires pRA4 will be resistant to neomycin and erythromycin antibiotics.

Finally, pRA4 was electroporated into competent OG1RF and was grown overnight on TH media containing 0.25 sucrose. After, the culture was plated on erythromycin and neomycin-containing agar plate with X-gal at 37° C for 24-48 hours. Blue transformants grew on the erythromycin and neomycin plate indicating the presence of pRA4 in OG1RF. Then a blue transformant from the neomycin plate was inoculated in TH media with sub-inhibitory concentrations of neomycin antibiotic at 42°C, and grown for 24 hrs, which does not allow for plasmid replication forcing the plasmid to integrate into the chromosome by single crossover events (Neo resistant as well as Erm resistant and Blue colony formation on X-gal plates due to the integrated plasmid). Removal of the integrated plasmid by a second crossover event would result in Neo resistant, Erm sensitive, white colonies. Colonies were plated on X-gal containing plates. A few white colonies were isolated that did not grow when patched onto a neomycin or erythromycin plate, suggesting the plasmid had excised, but only the wild-type copy of the gene remained. These white OG1RF strains are analyzed via PCR using *menB* forward and reverse primers. The PCR product showed the presence of the wildtype sized 0.75 kb *menB* product, suggesting the

strains still have an intact *menB* copy. Modifications to the mutation protocol including repeated temperature upshifts during full neomycin selection will be done to optimize the chance of obtaining *menB* mutants.

Table 2. Primers used for amplification of <i>menB</i> and <i>aroC</i> gene in <i>E. faecalis</i> strain OG1RF by PCR.	
<i>menBF1NcoI</i>	5'- CCGCCATGGAGCAAGCAGGCAAAGTAGC-3'
<i>menBR2 sac I</i>	5'-CCGGACCTCCGCCTTCTTGTGCTTCAGCC-3'
<i>arocF1NcoI</i>	5'-CCGCCATGGTCAGAAGAACGTCTC-3'
<i>arocR2SacI</i>	5'-CCGGAGCTCACTGTTTGGATGCCC-3'

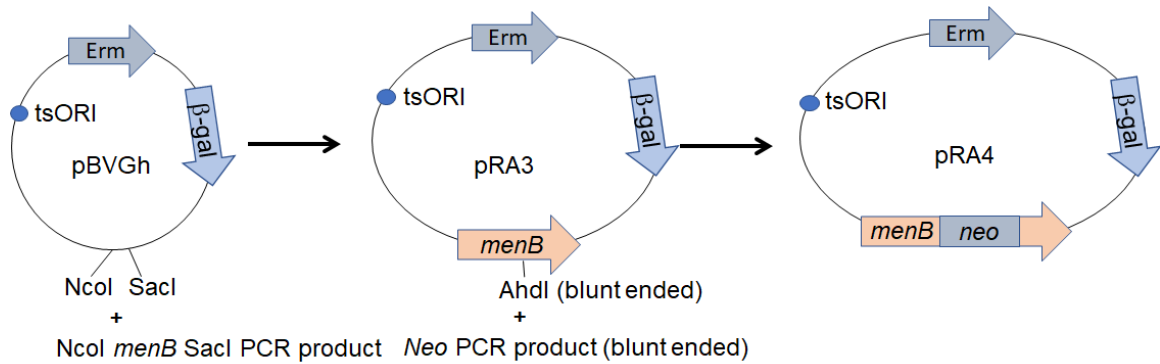


Figure 8. Overview of temperature sensitive cloning vector construction.

CHAPTER 4

DISCUSSION

Although a highly evolved microbiota commensal, over the decades *E. faecalis* has emerged as the top three leading causes of nosocomial infections (3). *E. faecalis* are known to cause major illnesses such as infective endocarditis (IE), septicemia, and urinary tract infections (UTI) (5). For several reasons IE is considered an emerging problem in the 21st century. An increase in life expectancy of patients with degenerative heart disease because of medical advances and the increased frequency of MDR bacteria because of increased use of antibiotics are some of the practices that lead to IE as a major problem in modern medicine (5). Other than IE, bacteremia due to Enterococci has been a major concern because of its high mortality rate of 75% in immunocompromised patients (5). Pheromone responsive plasmids are found in 60-80% of pathogenic enterococcal isolates and recently have been shown to be stably carried in the GI of model animals, suggesting a potential benefit for maintenance in the GI community (46, 47, 48, 49). Pheromone responsive plasmids are modular (constantly rearranging, (50) and the best studied of these plasmids is pCF10 (51). The presence of pCF10 increases the size of heart vegetations in endocarditis (11, 52). In these studies, we have found the presence of pCF10 induces the formation of rigid structures in otherwise viscous biofilm commensal type strain OG1RF. These heterologous structures form in growing biofilms starting around day 2. Rigid structure formation is associated with increased antibiotic resistance and, preliminary studies suggest, increased platelet activation.

The presence of pCF10 induces rigid structure formation in commensal *E. faecalis* biofilms.

In our study, we have discovered new phenotypes of commensal biofilms in the presence of pCF10. Commensal type strain OG1RF/SSP biofilms form relatively homogeneous viscous biofilms, however, OG1RF/SSP biofilms containing pCF10 becomes heterogeneous as the biofilm gets older. In pCF10 containing biofilms in day 1, a rigid base layer begins to develop, tower-like structures begin to form in day two and by day 3 biofilms begin to fill in and the biofilms become difficult to image without disrupting the thick biofilm.

In mixed plasmid-containing donor and plasmid-free recipient biofilms, heterologous rigid tower structures begin to form in Day 1. This may be due to conjugation, which is induced by the secretion of small 7 aa peptide pheromone secreted by the plasmid-free recipient *E. faecalis* cells. The pheromone is taken up by plasmid containing-recipient cells and then the concentration of pheromone surpasses a concentration threshold, conjugation is induced. The induction of conjugation causes the expression of aggregation substance on the plasmid-containing cells leading to the formation of mating aggregates. It is possible these aggregates may act to seed the early formation of towers. Future studies with aggregation substance mutants (kindly provided by G. Dunny, Univ. MN) could be used to determine the role of aggregation substance in an early tower formation.

The viscous portion of the mixed donor and recipient biofilms were thinner (1-3 μm as opposed to 20 μm in OG1RF biofilms). This thinner layer always contained a mixture of mCherry-labeled OG1RF cells (red) and OG1SSP cells (green) (Figure 3J).

The rigid structures were predominantly OG1SSP pCF10-containing cells (green). Rigid structures are associated with higher hydrogen peroxide staining (unpublished observations, Buttaro). The plasmid pCF10-confers resistance to hydrogen peroxide (unpublished observations, Buttaro). Therefore, it might be reasonable the more sensitive plasmid-free OG1RF cells were killed if they were associated with rigid structures. Occasionally, mCherry-labeled OG1RF could be seen associated with the heterologous structures (Figure 3H, I). It is possible the OG1RF cells in these structures obtained pCF10 by conjugation. Transconjugants, OG1RF that have obtained pCF10 by conjugation, would be FusR (OG1RF chromosomal mutation and TetR (*tetM* on pCF10)). Early conjugation events may result in larger mixed rigid structures (Fig. 3H) as opposed to later conjugation events that may have only a small region of mCherry-cells (Fig. 3G). Conjugation events can be detected by differential plating for OG1RF (FusR), OG1SSP(SpecR), and pCF10(TetR). Fluorescently labeled pCF10 could be used to visually document the location of transconjugants.

In day 3 biofilms, the rigid tower structures were bigger and the space between the towers contained a thin mobile phase with a mixture of donor and recipient biofilms rather than an overall thickening of the base layer. Future studies at lower magnification could be done to better compare the overall changes later in biofilm maturation between pCF10-containing biofilms and mixed plasmid-free and plasmid-containing communities.

The presence of rigid structures correlates with an increase in antibiotic resistance.

The formation of rigid, dense structures has been associated with antibiotic resistance in biofilms. A study by Davis and colleagues have suggested loss of biofilm

heterogeneity attenuates *P. aeruginosa* resistance to SDS (23). In our study, we found the homogeneous OG1RF biofilm is more susceptible to the antibiotic (Figure 4). However, the heterogenic pCF10 biofilm acquired resistance to the antibiotic as the biofilm tower structures developed (Figure 4). The formation of the rigid structures may prevent or slow the penetrance of the antibiotics into biofilms by diffusion. Experiments on the penetration of antibiotics into the biofilms could be studying by examining the effect of prolonging antibiotic treatment on killing and the accumulation of fluorescently labeled antibiotics. Finally, we hypothesize that under flow conditions, like those found in the GI tract, the formation of heterologous rigid structures may produce protected microenvironments, shielding sensitive bacteria exposure to antibiotics.

The presence of pCF10 enhances platelet activation by *E. faecalis*

Platelet activation and then aggregation is a well-known virulence factor of both gram-positive and gram-negative bacteria that leads to high mortality via septicemia and contributes to the development of endocarditis. The presence of pCF10 has been associated with increases in the size of heart vegetations in rabbit models of infectious endocarditis. *E. faecalis* cell wall components such as pili Ebp and Pb1A/B, as well as peptidoglycan are a potential virulence factor that induces platelet activation (14, 15, 16, 22). In the current study, crude cell wall and peptidoglycan were purified from both OG1RF and pCF10 planktonic strains, but both crude cell wall and peptidoglycan did not activate platelets significantly. However, we were unable to complete the study with proper peptidoglycan controls. In addition, full characterization of peptidoglycan preparations requires HPLC. So, prior to using HPLC, we wanted to first confirm the preparation activated platelets. Therefore, the current cell wall and peptidoglycan extracts

were only crudely characterized using the Bradford assay, which can be interfered with by remaining traces of SDS used to prepare the crude cell wall and peptidoglycan. For future experiments, the BacTX[®] Assay System to confirm the isolation of peptidoglycan and a commercially prepared peptidoglycan preparation will be used as a positive control. If platelet activation activity is observed, the preparation will be characterized by mass spectrometry techniques.

When human platelets from healthy donors were mixed with *E. faecalis* biofilms, platelet activation was evident (Figure 5A and B). In these studies, we used stationary phase biofilms, where the heterologous rigid structures form more slowly in pCF10-containing cells, beginning around day 4. Plasmid-free stationary phase OG1RF biofilms remain viscous except for a 1-5 μm thick rigid base that begins to form around day 5 in stationary phase biofilms. Visually, the presence of rigid structures in OG1RF biofilms and the presence of pCF10 enhanced platelet activation (Figure 5A and 5B). Methods are being developed to map the rigid structures and determine the bacterial density and platelet activation associated with the structures to quantitate the differences. In preliminary experiments, aspirin treatment, a known inhibitor of platelet aggregation, reduced platelet activation. This is consistent with the observed aggregation being due to platelet activation and not just accumulation of platelets on the rigid structures. Hydrogen peroxide, a known activator of platelets and the rigid structures produce high levels of hydrogen peroxide. The addition of catalase reduced platelet activation. Taken together this preliminary data leads to the hypothesis that heterologous rigid structures in *E. faecalis* biofilms may lead to increased activation of platelets in two ways. First, it would allow for better crosslinking of surface receptor molecules on platelets and increased

concentrations of hydrogen peroxide would allow for stronger signaling. This hypothesis can be tested in future experiments using biofilms formed under more active growth conditions using the experimental protocols established in these studies.

Attempt of *menB* Knockout from *E. faecalis* chromosome

To study the contribution of hydrogen peroxide to the organization of the donor and recipient biofilms as well as its role in the platelet activation experiments were initiated to produce hydrogen peroxide production mutants. Vectors were constructed to knock out *aroC* and *menB* genes that are known to result in hydrogen peroxide production in *E. faecalis* (24). Initial attempts to knockout *menB* were unsuccessful, however, repeated temperature upshifts during neomycin selection can be done to optimize the chance of obtaining *menB* mutants.

Broader implications and future studies

A recent publication has emphasized the importance of hydrogen peroxide for spatial organization in colonic surfaces (54). Hydrogen peroxide but not oxygen seems to govern the spatial organization of microbes at closer proximity (intimate attachment) of the mucosal surface during gut homeostasis (54). Pathogens which can associate with the mucosal surface in the presence of hydrogen peroxide have an advantage over the microbiota. Some of these pathogens have cytochrome c peroxidase (Ccp) allowing them to utilize the hydrogen peroxide for respiration to obtain energy (54). While Ccp is only found in gram-negative bacteria, it is possible that hydrogen peroxide production by *E. faecalis* may be important in its virulence. *E. faecalis* may have intimate attachment to the mucosal layer as it has been shown in germ-free animals to have the ability to colonize the epithelial layer (55). This is consistent with the observation that systemic

infection by the more virulent nosocomial *E. faecalis* strains is preceded by colonization the GI and an intestinal bloom following antibiotic treatment. The presence of pCF10, which increases hydrogen peroxide resistance and allows for the formation of rigid structures associated with high hydrogen peroxide production may be important virulence factors contributing to this colonization and also may provide a selective advantage for the maintenance of pCF10-containing cells in the GI tract *Enterococcal* community as observed in animal models.

Summary

Collectively, we observed *E. faecalis* with pCF10 forms a biofilm with heterologous rigid tower-like structures. This phenotype was associated with increased resistance to antibiotic treatment and increased activation of platelets. In mixed plasmid-containing and plasmid-free populations, plasmid-containing cells were found predominantly rigid structures and a mix of plasmid-free and a plasmid-containing cells were found viscous biofilm between the structures. This newly discovered phenotype of contributes our knowledge of how pCF10 transforms the commensal *E. faecalis* into pathogenic strains and how pCF10 may provide a competitive advantage allowing for the maintenance of pCF10 in the commensal community.

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APPENDIX

Growth Media

LB Broth

20 g Lenox L LB Broth Powder

Dissolved in 1 L with diH₂O

Autoclaved for 45 min. at 121°C

LB Agar

20 g Lenox L LB Broth Powder

20 g Difco™ Agar

Dissolved in 1 L with diH₂O

Autoclaved for 45 min. at 121°C

Todd Hewitt Broth

30 g Bacto™ Todd Hewitt Broth Powder

Dissolved in 1 L with diH₂O

Autoclaved for 45 min. at 121°C

Todd Hewitt Agar

30 g Bacto™ Todd Hewitt Broth Powder

15 g Difco™ Agar

Dissolved in 1 L with diH₂O

Autoclaved for 45 min. at 121°C

Brain Heart infusion Broth

37 g Bacto™ Brain Heart infusion Broth Powder

Dissolved in 1 L with diH₂O

Autoclaved for 45 min. at 121°C

Phosphate Buffered Saline (1x)

In 400 mL diH₂O, dissolve

4g NaCl

0.1g KCl

0.72g Na₂HPO₄

0.12g KH_2PO_4

q.s. to 500 mL with diH_2O

Autoclaved for 45 min. at 121°C

TAE Buffer (50x)

242.2 g FisherChemicals Tris-Base

57.1 mL Fisherchemicals Glacial Acetic Acid

100 mL of 0.5M FisherChemicals EDTA-pH 8.0

q.s. to 1L with diH_2O

TAE Buffer (1x)

10 mL of 50x TAE buffer

500 mL diH_2O

Erythromycin antibiotic (Biofilm treatment)

50 mg 98% erythromycin Powder

Dissolved in 1 mL 10% ethanol

Erythromycin antibiotic (Gene knockout)

500 mg 98% erythromycin Powder

Dissolved in 10 mL 95% ethanol