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CHARACTERIZATION OF HETEROGENEITY IN BIOFILM PROPERTIES AMONG DIVERSE *ENTEROCOCCUS FAECALIS* STRAINS

by

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To my Pop-Pop, Francis Schaffer, and my Grammie, Barbara Schaffer. I love you both.

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ABSTRACT

Characterization of Heterogeneity in Biofilm Properties Among Diverse *Enterococcus faecalis* Strains:

Scott Daniel Schaffer, Masters of Science, 2021

Thesis directed by: Kristi L. Frank, Ph.D., Assistant Professor, Department of Microbiology and Immunology

It is unclear how biofilms of *Enterococcus faecalis* strains may vary in parameters such as development and composition. We hypothesized that differences in biofilms exist among distinct *E. faecalis* strains. In this work, we evaluated *in vitro* biofilm formation and matrix characteristics of a panel of five genetically diverse *E. faecalis* lab-adapted strains and clinical isolates. All strains formed biofilms similarly when grown in TSB without dextrose (TSB^{-D}), 90% TSB^{-D}, or 90% TSB^{-D} + 10% FBS. However, DMEM with 10% FBS restricted the accumulation of an appreciable amount of biofilm biomass for all strains except VA1128. Treatment of biofilms with dispersal agents indicated that the composition of the *E. faecalis* biofilm matrix is complex. Fluorescence microscopy further demonstrated differences between OG1RF and VA1128 in biofilm development and matrix composition. This work highlights the existence of heterogeneity in biofilm properties among diverse *E. faecalis* strains.

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LIST OF ABBREVIATIONS

AlgL: alginate lyase

AS: aggregation substance

ANOVA: analysis of variance

Ab^R: antibiotic resistance

CBH: bile salt hydrolase

CPS: capsule type

CFU: colony forming units

CF: cystic fibrosis

CYL: cytolysin

DNase: Deoxyribonuclease

DMEM: Dulbecco's Modified Eagle Medium

Esp: enterococcal surface protein

ECM: extracellular matrix

EPS: extracellular polymeric substances

eDNA: extracellular DNA

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

GI: gastrointestinal

GEL: gelatinase

MMP1: matrix metalloprotease-1

MGEs: mobile genetics elements

MLST: multi-locus sequence typing

Mprf: multiple peptide resistance factor

NVE: native valve endocarditis

OD: optical density

OM: otitis media

PAI: pathogenicity island

KPBS: potassium phosphate-buffered saline

SEM: scanning electron microscopy

STs: sequence types

TSB^{-D}: tryptic (trypticase) soy broth without dextrose

CHAPTER 1: Introduction

BIOFILMS

Properties of Biofilms

In 1978, Costerton et al. described the basis of the current definition of a bacterial biofilm, in that a biofilm is a community of cells that is adherent to a surface and encased in a mesh of fibers (35). At the time, they believed the mesh, or what is now known as the biofilm matrix, to only consist of polysaccharides; yet the general biofilm matrix is in fact comprised of much more than only polysaccharides (34; 47; 57). These components are broadly termed as extracellular polymeric substances (EPS) (57). Additionally, biofilms can take the form of mobile aggregates of cells that have formed in the absence of a surface, in which adherence to at least each other and not only a surface, becomes an added element to the biofilm definition (36; 58). To complete the contemporary definition of a biofilm, it is now also known that upon adhesion, bacterial cells begin to display a different gene transcription profile, particularly related to the production of components used by the cells for biofilm formation, as well as an altered growth rate profile, in comparison to their planktonic counterparts (47). These characteristics are notable, as cells that have broken off from a parent community and retain these characteristics can still be considered biofilm cells, i.e., cells with the biofilm phenotype or in a biofilm mode.

To highlight the importance of the biofilm matrix, the hydrated EPS matrix accounts for most of the mass of a biofilm, with the rest of the mass consisting of the actual microorganisms (56; 57). As described by Flemming et al., if a biofilm may be

considered to be a city of bacterial cells, then the matrix serves as the buildings and homes that house the bacteria (56). The matrix consists of an assortment of soluble eDNA, proteins, polysaccharides, and lipids, and insoluble components, which include pili, flagella, and amyloids (57; 58; 98). The specific composition of these components can vary from microbe to microbe (57; 58). These components contribute to the overall stability and architecture of the biofilm, and are important for the adhesion of the biofilm to a surface along with cell-surface proteins, glycolipids, and adhesins (24; 56-58). Furthermore, biofilms display properties distinct from planktonic bacterial cells largely due to this EPS matrix, which are described in detail in an excellent review by Flemming et al. (58). An example of one of these properties, and one of the properties biofilms are most known for in disease, is protection against antibiotics (58). Additionally, as stated in the definition of a biofilm, these microbial communities thrive on interfaces, which include natural body surfaces, such as the epithelium and heart, as well as implanted medical devices, such as urinary catheters and prosthetic heart valves (47). It is easy to think of bacterial cells as individual entities that float around and cause trouble; but in reality, these cells are often found as a community within a biofilm, whether living as commensals in a human's gut, or living as a parasite at a site of an infection in a human being (42). The aforementioned properties of biofilms contribute to the success of bacterial growth and infections that they can cause.

Biofilms and Disease

A number of infectious diseases in humans are known to be associated with biofilms. Examples include native valve endocarditis (NVE), chronic bacterial prostatitis, cystic fibrosis (CF), periodontitis, and otitis media (OM) (22; 68; 72; 92; 97; 171).

Biofilms can also cause disease in humans by forming on medical devices, such as prosthetic heart valves, urinary catheters, contact lenses, intrauterine devices, and central venous catheters (37). Research to understand the mechanisms of how biofilms specifically cause or contribute to the diseases they are linked to is ongoing, and these mechanisms and associations are better understood in some diseases than others, such as in cystic fibrosis (30; 79). Yet with certainty, biofilms are primarily problems in disease due to their recalcitrance to antimicrobial treatment, which contributes to increased difficulty to treat biofilm diseases, making what could be a simple treatment with an antibiotic ineffective (46; 47; 58; 71; 157). This recalcitrance can be specifically described as resistance and tolerance, in which resistance is defined as the ability of a microorganism to grow in the presence of a concentration of an antimicrobial that would normally inhibit growth, and where tolerance is defined as the ability of a microorganism to survive in the presence of a lethal dose of an antimicrobial (71; 101). An example of a biofilm resistance mechanism is the sequestration of antibiotics by components of the biofilm matrix, as depicted by Billings et al. in their description of Psl, a major polysaccharide within the biofilm matrix of *Pseudomonas aeruginosa*, serving as a protective barrier to antibiotics (18). An example of a biofilm tolerance mechanism is the persister state that many cells within a biofilm may be found in, in which antibiotics do not affect these cells due to their less active state, as described by Spoering et al. for *P. aeruginosa* biofilms (151).

Another significant process of how biofilms elicit disease is the protection they provide from the host immune system (47; 120; 138). For example, in 1998, Shiau et al. demonstrated the inhibitory effect of *Staphylococcus aureus* biofilms on macrophage

phagocytosis (147), which has been further examined and better understood since then (139; 164). A third, suggested mechanism of how biofilms cause disease involves the detachment of cells from biofilms formed on medical devices, such as catheters, which could lead to bloodstream or urinary tract infections initiated by these dislodged cells (55). Detachment of biofilm cells from mature biofilms is a commonly accepted step of biofilm formation and development (37; 153). Detached biofilm cells can also be the progenitors for separate biofilms elsewhere in the body. Additional suggested mechanisms of how biofilms contribute to disease include the production of endotoxins by cells within a biofilm and the development of resistant bacteria through increased rates of acquisition of antimicrobial resistance genetic determinants by horizontal gene transfer within biofilms (11; 172).

ENTEROCOCCUS FAECALIS

Brief Summary of Characteristics

Enterococcus faecalis is a member of the *Enterococcus* genus, which comprises Gram-positive lactic acid-producing bacteria that can be found in the gastrointestinal (GI) tract of humans and animals and ubiquitously in the environment (9; 65; 109; 110; 141) . In particular, *E. faecalis* is one of most common enterococci to be found in human feces and is not typically pathogenic; however, it can become pathogenic in immunocompromised individuals or if given the opportunity to grow in an environment where not normally found, such as the surface of an implanted medical device (87; 137; 178). Notably, *E. faecalis* and other enterococci that become pathogenic are often found growing as a biofilm, which is one of the most common associations seen of these organisms in disease (119). Additional characteristics that can contribute to their

prevalence as pathogens include an intrinsic resistance to antibiotics, such as aminoglycosides and β-lactam-based antibiotics, and their propensity to acquire genetic material, which may include resistance determinants (80; 112).

Epidemiology and Disease

As previously stated, enterococci are organisms of a dual nature: they are commensal bacteria of the GI tract of humans, but they can also flourish as opportunistic pathogens. The latter nature is highlighted by the increase in enterococcal infections over the last few decades, of which this increase is associated with an increase in antibiotic resistant enterococci and an increase in hospital-acquired infections (3; 8; 43; 126). These antibiotic-resistant strains have been shown to be abundant on medical devices and common surfaces in hospitals (81; 93). Additionally, patient-to-patient spread has been observed to occur by transmission through the hands of healthcare workers (75; 118). These factors have been linked to an increase in GI colonization by antibiotic-resistant enterococci in hospitalized patients (3; 48; 49). Patients colonized with antibiotic-resistant enterococci are at a greater risk of infection by these bacteria, which can be spurred by neutropenia, transplantation, immunosuppressed conditions, or treatment with vancomycin or third-generation cephalosporins (3).

Of the enterococci, *E. faecalis* is known to more densely colonize the GI tract of humans and to cause greater than 80% of human enterococcal infections, which include endocarditis and urinary tract infections (3; 111; 134). Interestingly, *E. faecium* displays a much higher prevalence of antibiotic resistance than *E. faecalis* despite the greater occurrence of *E. faecalis* isolates in human infections (83). This indicates that although antibiotic resistance is an important mechanism that contributes to successful

colonization and infection by enterococci, other mechanisms must be important in promoting infection, particularly by *E. faecalis*. One of these mechanisms is biofilm formation (66).

Biofilm Formation

Genetic Factors

To date, numerous genes have been identified that play a role in *E. faecalis* biofilm formation, which primarily include genes that encode adhesins, sortases, autolysins, and proteases (52; 119). One of the earliest of these genetic factors to be discovered is *esp*, the gene encoding enterococcal surface protein (Esp) (146). This gene was first identified in *E. faecalis* in 1999 and determined to encode a surface protein that was enriched in disease-causing isolates (146). *esp* was later determined to be located within a pathogenicity island (PAI), although the PAI is not present in all *E. faecalis* strains, and *esp* is not present in all strains containing the PAI (144). Ensuing studies indicated a strong correlation between the presence of *esp* and the biofilm-forming ability of *E. faecalis* strains (159; 167). Toledo-Arana et al. showed that *esp* was important for *E. faecalis* biofilm formation by demonstrating biofilm formation impairment in *esp* insertion mutants (167). Tendolkar et al. demonstrated an increase in biofilm formation of strains transformed with an *esp*-expressing plasmid (159). Another study established the importance of Esp in a murine urinary tract infection model, specifically to colonization and persistence of *E. faecalis* at the site of infection (145). Despite this breadth of research, it is crucial to note that other studies determined that Esp is not necessary for *E. faecalis* biofilm formation (94) – OG1RF is one example of an *esp*- negative strain that forms biofilms fine – or found no correlation between Esp and biofilm formation of 83 *E.*

faecalis isolates from varying sources, most being from infections (44). Despite this, *esp* is still commonly looked for in *E. faecalis* biofilm studies as a marker for virulence and biofilm formation, and biofilm formation is often more frequently detected in *esp*+ strains than *esp*- strains (6; 103; 179; 180).

Another pair of well-known factors are the genes *prgB* and *asa1*, which encode surface proteins Asc10 and Asa1, respectively, and together are known as aggregation substance (AS) (119). AS is an adhesin that mediates essential cell-to-cell contact during mating of two *E. faecalis* cells, a donor and a recipient, in response to a pheromone signal (32). As with *esp*, the genes that encode the AS proteins are not located in the core genome of *E. faecalis*, but are found on mobile genetic elements (MGEs) that include assorted naturally occurring plasmids and the aforementioned PAI (63; 76; 144). Various studies have demonstrated a role for AS in biofilm formation, indicating how AS is an important factor for biofilm formation in *E. faecalis* (2; 17; 28; 29).

Other notable *E. faecalis* biofilm-associated genes include the protease-encoding genes, *gelE* (7) and *sprE*, and the global biofilm regulator-encoding genes, *fsrABDC* (5; 119). *gelE* and *sprE* encode a metalloprotease and serine protease, respectively, that are cotranscribed and positively regulated by the Fsr quorum-sensing system (127; 130; 148). *gelE* specifically encodes gelatinase, which has been shown to be important in a rabbit model of enterococcal endocarditis and also activates the autolysin (AtlA) that plays a critical role in *E. faecalis* biofilm formation (104; 162; 165). The serine protease encoded by *sprE* has been specifically characterized as a glutamyl endopeptidase I and can be considered a counterpart to gelatinase in that it interacts with AtlA to inhibit autolysis (89; 162; 163). *sprE* and *gelE* have been shown to be important in a mouse model of

enterococcal peritonitis, in a zebrafish larvae model, in killing of the nematode *Caenorhabditis elegans*, in a rabbit model of enterococcal endophthalmitis, and in a rat model of enterococcal endocarditis (53; 128; 129; 148-150). The *fsr* locus is comprised of four genes, *fsrA, fsrB, fsrC, and fsrD* that encode a quorum-sensing two-component regulatory system (113; 114). This system is known to regulate virulence factors that are involved in biofilm formation, such as *gelE* and *sprE* , and a number of less well-defined factors that may be involved in biofilm formation and other processes; it is the only known quorum-sensing system that regulates biofilm development in *E. faecalis* (20; 119; 129; 130; 158). All of these genes are found within the core genome of *E. faecalis* (119). Numerous other genetic factors have also been identified and documented to date (52; 76; 119).

Biofilm Matrix

The biofilm extracellular matrix is crucial for providing many of the properties for which biofilms are known. This is likely also true for *E. faecalis* biofilms, although much is still unknown about the biochemical composition of enterococcal biofilm matrices. Enhancing our understanding of this would provide much needed context for elucidating how enterococcal biofilms as a whole contribute to disease. Our current knowledge sits with knowing that extracellular DNA (eDNA) is a component of the *E. faecalis* biofilm matrix, at least in strains OG1RF and V583 (15; 70; 162; 163). Interestingly, these studies provide conflicting evidence as to where biofilm matrix eDNA originates. Thomas et al. first showed that matrix eDNA in 24-hour biofilms came from a fratricidal mechanism mediated by the secreted protease GelE, in coordination with the autolysin AtlA (162). In contrast, Barnes et al. demonstrated that eDNA is present in as early as 4-

hour biofilms, that this eDNA did not appear to originate from a lytic means, and that eDNA was present in both long intercellular structures and bulkier structures that more broadly encompassed cells in the biofilm (15). Both mechanistic origins of *E. faecalis* biofilm matrix eDNA likely occur, and it would be further illuminating to determine if both of these mechanisms occur in strains beyond the commonly studied strains OG1RF and V583. Also around the same time as these studies, Iyer et al. showed the *E. faecalis* strain V583 to actually form denser biofilms when mutated to lack the ability of autolysis, by deletion of the alternate sigma factor σ^{54}, which impaired the release of eDNA to be used in biofilm formation (85). This was an important finding in being one of the first pieces of evidence, if not the first, to indicate that other components beyond eDNA may contribute to biofilm matrix composition of *E. faecalis*. This study even demonstrated that Proteinase K disrupted 6, 12, and 24-hour biofilms of the mutant strain, and 24-hour biofilms of the wild-type and complemented strains (85), providing support for proteins as an additional component of importance in *E. faecalis* biofilm matrix composition.

Despite this relative dearth of knowledge about the composition of *E. faecalis* biofilm matrices, recent studies have tackled this gap of insight and have begun to provide some additional answers. Schlafer et al. demonstrated that DNase I treatment decreased adherence of *E. faecalis* strain DSM 20478 (ATCC 19433) to 96-well plates, but was not sufficient to prevent both adhesion to teeth and biofilm dispersal on 96-well plates and teeth (140). These data support the notion that eDNA is an important component of the early *E. faecalis* biofilm matrix, yet also suggest that the matrix composition of older *E. faecalis* biofilms consists of other or additional components that

are crucial to biofilm integrity. Torelli et al. determined that alginate lyase (AlgL), which is known for cleaving the glycosidic bonds of alginate, enhanced vancomycin killing of biofilm cells of 48-hour biofilms of three UTI-derived *E. faecalis* isolates, suggesting a role for polysaccharides, particularly alginate, in *E. faecalis* biofilm matrix composition and recalcitrance to antibiotic treatment (169). They provided similar findings with DNase I, in addition to showing that treatment with either AlgL or DNase I disrupted adhesion of the biofilms compared to lack of treatment (169). Altogether, these data are in concordance with Schlafer et al. (140) that the E. faecalis biofilm matrix structure is complex and consists of at least eDNA and polysaccharides. Another recent study showed that the biofilm matrix composition of *E. faecalis* strain ATCC 29212 was influenced by the conditioning of the substrate the biofilm was formed on (e.g. conditioned with serum, saliva, or collagen) and the age of the biofilm (4). Through this, the study measured polysaccharide and protein content of the biofilm matrix, providing evidence that the ATCC 29212 strain contained both components in its biofilm matrix (4). Furthermore, this study raised the important methodological concern of the appropriate conditions under which biofilms should be grown in biofilm studies. The authors of the study were concerned with the environment of a human oral cavity, provoking the question of what may be the optimal conditions that mimic other sites of *E. faecalis* biofilm infections, such as a human heart valve. At the very least, this concern provides good reason to test multiple growth conditions in *E. faecalis* biofilm studies. In conjunction with this study, a separate study conducted around the same time provided evidence that recombinant human matrix metalloprotease-1 (MMP1), an enzyme that broadly cleaves protein substrates, significantly inhibited biofilm formation and

significantly dispersed formed biofilms of *E. faecalis* strains FA2-2 and V583, indicating the importance of proteins in the biofilm matrix composition of both strains (96). It may still be noted in this study that the biofilms, which varied from one to seven days old, were never completely inhibited or disrupted (96), in agreement with the notion that *E. faecalis* biofilm matrix composition is complex, and the well-defined evidence that eDNA is an important component of the *E. faecalis* biofilm matrix. These various studies provide a number of pieces that contribute to understanding the enigma that is the composition of the *E. faecalis* biofilm matrix; yet it is clear that more research is needed to fully elucidate this gap of knowledge. Furthermore, most of these studies only used one strain in their experimental designs, leaving one to wonder how their findings might fare in different strains.

Interactions with Host Immunity

As previously described, one of the most significant characteristics of biofilms is increased resistance to the host immune system. Similarly to that of *E. faecalis* biofilm matrices, there is much that is still unknown about how *E. faecalis* biofilms interact with the immune systems of the hosts they have colonized. For example, what is currently known of *E. faecalis* and host immune system interactions has primarily come from studies only looking at planktonic cells (67; 131; 154; 170; 181). A set of these studies focused on the role of AS during *E. faecalis* interactions with host immunity. They demonstrated that AS promotes bacterial adhesion to neutrophils and macrophages in OG1RF, but that these genetically engineered AS-bearing OG1RF cells are more resistant to killing by the neutrophils and macrophages upon phagocytosis than their AS- counterparts (131; 154; 170). These data indicate that strains natively lacking AS, which

includes strain OG1RF, interact with the immune system differently than strains that express AS. It would be further illuminating to observe how strains that naturally contain these genetic determinants fare in experiments such as these, in addition to how biofilms may fare in these experiments. Several other studies with a focus of *E. faecalis* and host immune system interactions have considered strain diversity and utilized multiple *E. faecalis* strains in their work, including V583, MMH594, and DS16C2, a derivative of DS16 (67; 166; 181); yet these studies still highlight a need for similar experiments to be conducted with *E. faecalis* biofilms, particularly since biofilm cells display a different transcription profile and metabolic state than their planktonic counterparts.

With this said, some studies in the last decade have been conducted with specific intent to look at *E. faecalis* biofilm cells or models of *E. faecalis* biofilm infections and host immune system interactions (27; 105; 182). One such study provides a link of the biofilm-associated protein Esp to a greater immune response, providing evidence for this in a mouse peritonitis model (182). This study was conducted with only *E. faecalis* strain MMH594 (182), but does provide evidence that the genetic make-up of a strain affects its interaction with a host's immune system. Another study observed immunomodulation of the host immune response by OG1RF in a wound infection model in mice, and specifically determined that the multiple peptide resistance factor (Mprf) was more important to resistance of immune response clearance in the wound infections than traditional biofilm-associated factors (27). Mathew et al. looked at immunogenic properties of biofilms of *E. faecalis* strains OG1RF, ATCC 29212, and FA2-2 under nutrient-rich and nutrient-deprived conditions, and also compared these properties to that of planktonic cells of these strains (105). They observed some similarities among the

strains' elicitation of various host immune responses; yet they also recorded a number of differences among the strains in biofilms and in the two growth conditions in their immunogenic properties, in addition to observing quite notable distinctions of various immune response outcomes between biofilm and planktonic cells (105). A paper by Daw et al., which looked at biofilms of strains other than OG1RF, noted that two distinct *E. faecalis* strains' biofilms elicited two different sets of various host immune responses (41). The results of these four studies provide evidence in support of the importance of studying *E. faecalis* biofilm properties in the context of multiple strains, particularly with distinct genetic backgrounds, and under multiple growth conditions.

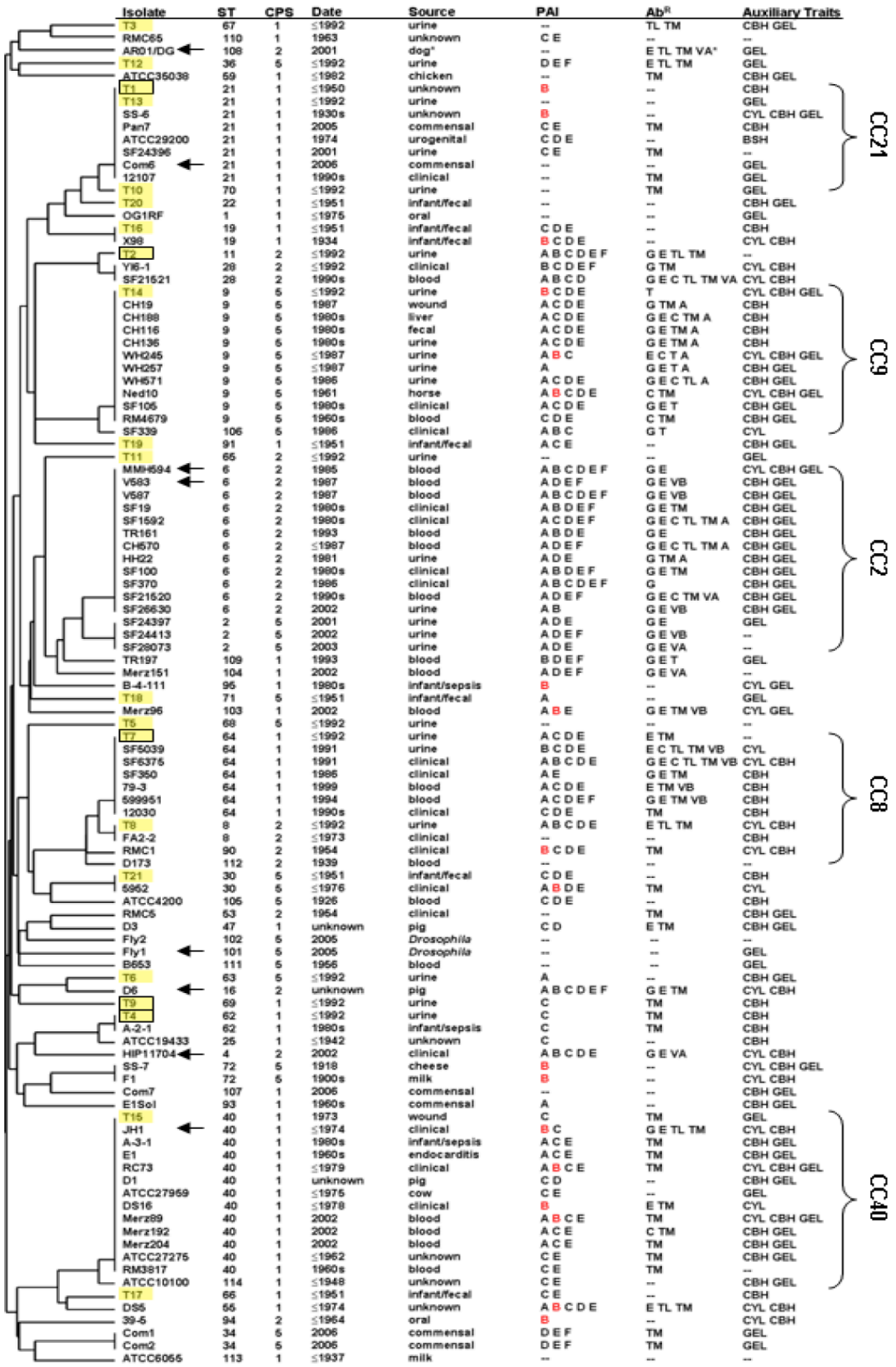
GOALS AND AIMS

McBride et al. used multi-locus sequence typing (MLST) schemes to better understand the genetic diversity that may exist among *E. faecalis* at a subspecies level (107). To do this, they analyzed 106 *E. faecalis* strains (107). A quick glance at Figure 2 in McBride et al. (Fig. 1) reveals the extent of genetic diversity among distinct *E. faecalis* strains (107). A number of the genes that are not ubiquitous among the many noted strains in the figure are biofilm-associated genes, such as *esp* and *gelE* (107; 144). This raises the point that the genetic background of a strain being researched in a biofilm study needs to be highly considered. Observing how genetic heterogeneity actually affects the variability seen in *E. faecalis* biofilm formation properties among strains is illuminating. Wild-type OG1RF is a fine biofilm former, while wild-type FA2-2 is a poor biofilm former (74; 94); both of these strains natively lack the *esp* gene. However, when transformed with plasmids that expressed *esp*, both strains had increased amounts of biofilm formation (159). A different study showed that *E. faecalis* strains V583 and

This figure and figure legend are taken from McBride et al. (107) and are available under the Creative Commons Attribution license.

Figure 1. "Dendrogram of Isolates Aligned with Capsule Type, Pathogenicity Island Segments, and Antibiotic Resistance Traits"

"MLST-based dendrogram showing genetic relationship of all *E. faecalis* isolates in this study. Small yellow highlights indicate a serotyping type strain, while black boxes designate the five most common serotypes. Arrows designate isolates used for comparative genomic microarray analysis. Abbreviations are defined as follows: ST = sequence type; CPS = capsule type; PAI = pathogenicity island fragment (A = *nuc1*; B = *cylB*; C = *esp*; D = hydrolase homolog similar to *xylS*; E = *psaA* homolog; F = *gls-24* like); a red letter B indicates strains that readily transfer cytolysin via mating; Ab^R = antibiotic resistance; T = tetracycline resistance; TM = *tetM*+; TL = *tetL*+; E = *ermB*+; VA = *vanA*+; VB = *vanB*+; G = gentamicin resistant; C = *cat*+; A = *blaZ*+; CBH = bile salt hydrolase; GEL = gelatinase; CYL = cytolysin".


Isolate ST CPS Date Source PAI Ab^R Auxiliary Traits
T3 67 1 ≤1992 urine -- TL TM CBH GEL
RMC65 110 1 1963 unknown C E -- --
AR01/DG 108 2 2001 dog* -- E TL TM VA* GEL
T12 36 5 ≤1992 urine D E F E TL TM GEL
ATCC35038 59 1 ≤1982 chicken -- TM CBH GEL
T1 21 1 ≤1950 unknown B -- CBH
T13 21 1 ≤1992 urine -- -- GEL
SS-6 21 1 1930s unknown B -- CYL CBH GEL
Pan7 21 1 2005 commensal C E TM CBH
ATCC29200 21 1 1974 urogenital C D E -- BSH
SF24396 21 1 2001 urine C E TM --
Com6 21 1 2006 commensal -- -- GEL
12107 21 1 1990s clinical -- TM GEL
T10 70 1 ≤1992 urine -- TM GEL
T20 22 1 ≤1951 infant/fecal -- -- CBH GEL
OG1RF 1 1 ≤1975 oral -- -- GEL
T16 19 1 ≤1951 infant/fecal C D E -- CBH
X98 19 1 1934 infant/fecal B C D E -- CYL CBH
T2 11 2 ≤1992 urine A B C D E F G E TL TM --
YI6-1 28 2 ≤1992 clinical B C D E F G TM CYL CBH
SF21521 28 2 1990s blood A B C D G E C TL TM VA CYL CBH
T14 9 5 ≤1992 urine B C D E T CYL CBH GEL
CH19 9 5 1987 wound A C D E G TM A CBH
CH188 9 5 1980s liver A C D E G E C TM A CBH
CH116 9 5 1980s fecal A C D E G E TM A CBH
CH136 9 5 1980s urine A C D E G E TM A CBH
WH245 9 5 ≤1987 urine A B C E C T A CYL CBH GEL
WH257 9 5 ≤1987 urine A G E T A CBH GEL
WH571 9 5 1986 urine A C D E G E C TL A CBH GEL
Ned10 9 5 1961 horse A B C D E C TM CYL CBH GEL
SF105 9 5 1980s clinical A C D E G E T CBH GEL
RM4679 9 5 1960s blood C D E C TM CBH GEL
SF339 106 5 1986 clinical A B C G T CYL
T19 91 1 ≤1951 infant/fecal A C E -- CBH GEL
T11 65 2 ≤1992 urine -- -- GEL
MMH594 6 2 1985 blood A B C D E F G E CYL CBH GEL
V583 6 2 1987 blood A D E F G E VB CBH GEL
V587 6 2 1987 blood A B C D E F G E VB CBH GEL
SF19 6 2 1980s clinical A B D E F G E TM CBH GEL
SF1592 6 2 1980s clinical A C D E F G E C TL TM A CBH GEL
TR161 6 2 1993 blood A B D E G E CBH GEL
CH570 6 2 ≤1987 blood A D E F G E C TL TM A CBH GEL
HH22 6 2 1981 urine A D E G TM A CBH GEL
SF100 6 2 1980s clinical A B D E F G E TM CBH GEL
SF370 6 2 1986 clinical A B C D E F G CBH GEL
SF21520 6 2 1990s blood A D E F G E C TM VA CBH GEL
SF26630 6 2 2002 urine A B G E VB CBH GEL
SF24397 2 5 2001 urine A D E G E GEL
SF24413 2 5 2002 urine A D E F G E VB --
SF28073 2 5 2003 urine A D E G E VA --
TR197 109 1 1993 blood B D E F G E T GEL
Merz151 104 1 2002 blood A D E F G E VA --
B-4-111 95 1 1980s infant/sepsis B -- CYL GEL
T18 71 5 ≤1951 infant/fecal A -- GEL
Merz96 103 1 2002 blood A B E G E TM VB CYL GEL
T5 68 5 ≤1992 urine -- -- --
T7 64 1 ≤1992 urine A C D E E TM --
SF5039 64 1 1991 urine B C D E E C TL TM VB CYL
SF6375 64 1 1991 clinical A B C D E G E C TL TM VB CYL CBH
SF350 64 1 1986 clinical A E G E TM CBH
79-3 64 1 1999 blood A C D E E TM VB CBH
599951 64 1 1994 blood A C D E F G E TM VB CBH
12030 64 1 1990s clinical C D E TM CBH
T8 8 2 ≤1992 urine A B C D E E TL TM CYL CBH
FA2-2 8 2 ≤1973 clinical -- -- CBH
RMC1 90 2 1954 clinical B C D E TM CYL CBH
D173 112 2 1939 blood -- -- --
T21 30 5 ≤1951 infant/fecal C D E -- CBH
5962 30 5 ≤1976 clinical A B D E TM CYL
ATCC4200 105 5 1926 blood C D E -- CBH
RMC5 53 2 1954 clinical -- TM CBH GEL
D3 47 1 unknown pig C D E TM CBH GEL
Fly2 102 5 2005 Drosophila -- -- --
Fly1 101 5 2005 Drosophila -- -- GEL
B653 111 5 1956 blood -- -- GEL
T6 63 5 ≤1992 urine A -- CBH GEL
D6 16 2 unknown pig A B C D E F G E TM CYL CBH
T9 69 1 ≤1992 urine C TM CBH
T4 62 1 ≤1992 urine C TM CBH
A-2-1 62 1 1980s infant/sepsis C TM CBH
ATCC19433 25 1 ≤1942 unknown C -- CBH
HIP11704 4 2 2002 clinical A B C D E G E VA CYL CBH
SS-7 72 5 1918 cheese B -- CYL CBH GEL
F1 72 5 1900s milk B -- CYL CBH
Com7 107 1 2006 commensal -- -- CBH GEL
E1Sol 93 1 1960s commensal A -- CBH GEL
T15 40 1 1973 wound C TM GEL
JH1 40 1 ≤1974 clinical B C G E TL TM CYL CBH
A-3-1 40 1 1980s infant/sepsis A C E TM CBH GEL
E1 40 1 1960s endocarditis A C E TM CBH GEL
RC73 40 1 ≤1979 clinical A B C E TM CYL CBH GEL
D1 40 1 unknown pig C D -- CBH GEL
ATCC27959 40 1 ≤1975 cow C E -- GEL
DS16 40 1 ≤1978 clinical B E TM CYL
Merz89 40 1 2002 blood A B C E TM CYL CBH GEL
Merz192 40 1 2002 blood A C E C TM CBH GEL
Merz204 40 1 2002 blood A C E TM CBH GEL
ATCC27275 40 1 ≤1962 unknown C E TM CBH GEL
RM3817 40 1 1960s blood C E TM --
ATCC10100 114 1 ≤1948 unknown C E -- CBH GEL
T17 66 1 ≤1951 infant/fecal C E -- CBH
DS5 55 1 ≤1974 unknown A B C D E E TL TM CYL CBH
39-5 94 2 ≤1964 oral B -- CYL CBH
Com1 34 5 2006 commensal D E F TM GEL
Com2 34 5 2006 commensal D E F TM GEL
ATCC6055 113 1 ≤1937 milk -- -- --
CC21
CC9
CC2
CC8
CC40

MMH594 exhibited differing amounts of biofilm formation relative to that of OG1RF, particularly when grown in a medium commonly used for *E. faecalis* biofilm growth *in vitro*, tryptic (trypticase) soy broth without dextrose (TSB^{-D}) (94). It should be noted that all three of these strains exhibit distinct genetic backgrounds.

The examples discussed in the preceding paragraph represent only a minuscule proportion of the studies conducted that demonstrate the importance of considering the varying biofilm formation traits among distinct *E. faecalis* strains. Yet, these studies are representative of the majority of work describing *E. faecalis* biofilms in the context of pathogenesis: specifically, the primary measurement in such studies is the relative amount of biofilm formation. In spite of this, there are bodies of research that have implemented means of measuring the importance of biofilms to *E. faecalis* pathogenesis beyond only looking at relative biofilm formation. One of these is the 2012 study by Daw et al., which showed that biofilms of two *E. faecalis* strains with different genetic backgrounds elicited discrepant responses from innate immune system components (41). Another example is a set of studies by Frank et al. that looked at biofilm formation in a rabbit model of endocarditis (59; 60) and in mouse catheter-associated urinary tract infections (60). Although the Frank et al. studies did not compare biofilm formation properties among genetically different wild-type strains, they did explore biofilm formation within different *in vivo* models of infection, which can more accurately determine how a strain's biofilms behave during actual infection of a human host, and in different forms of infection, than *in vitro* characterization may be able to establish. In the context of experimental limitations where *in vivo* work is not feasible, this supports the argument that broadening the scope of biofilm properties studied and how these

properties are studied, such as how biofilms form in environmental conditions that more closely mimic what may be found within a host, is a necessity. We aim to expand this literature with our investigations detailed herein, which explore how a genetically diverse panel of *E. faecalis* strains form biofilms.

We first measured biofilm formation of five strains from varying genetic backgrounds in different media and on different substrates over a course of 48 hours to generate a whole picture of biofilm formation kinetics under multiple growth conditions. The conditions we chose included growth medium typically used for *E. faecalis* biofilm formation and this same medium supplemented with heat-inactivated fetal bovine serum (FBS), which facilitated the inclusion of components that may more closely mimic an environment at a site of enterococcal infection in a host. We also included tissue culture medium to observe how the strains may form biofilms in an environment conducive to immune cells. The substrate types we chose included polystyrene, which is commonly used in 96-well biofilm experiments, and Aclar, a type of fluoropolymer that supports *E. faecalis* biofilm formation and is optimal for use in fluorescent microscopy experiments (15; 38). Our experimental design takes numerous growth conditions into account in order to broadly address how the physical and chemical environment a biofilm forms in can impact biofilm development.

One of the most unknown aspects of *E. faecalis* biofilms is the discrete biochemical composition of their EPS matrix (52). Therefore, in the second part of our biofilm strain panel characterization, we began to investigate the biofilm matrix composition of each strain. The importance of understanding this in greater depth is only compounded by how important the EPS matrix is to the functions and properties of

biofilms. We employed a three-pronged approach to gather as much information as we could about the biofilm matrix compositions: 1) conduct chemical and enzymatic treatment assays in order to determine which type(s) of macromolecule(s) - eDNA, polysaccharides, and proteins - may be important to the integrity of each strain's biofilm matrix; 2) conduct confocal fluorescence microscopy, targeting individual components to visualize what may compose each matrix; and, 3) conduct biochemical analysis, specifically measuring the concentrations of potential macromolecules that may compose each matrix. For the first experimental approach, all biofilms were grown for 24 hours in typical medium used for *E. faecalis* biofilm studies. For the other two approaches, measurements were taken from biofilms, also grown in typical biofilm medium, over a 48 hour time course to get an idea of the dynamics of biofilm matrix composition during biofilm formation. We also measured biofilm thickness, area, and volume to get as complete a picture of the strains' biofilm matrices as possible, and more so, to characterize the biofilms as a whole.

The results described in this thesis demonstrate the biofilm properties of different *Enterococcus faecalis* strains and establish that distinct strains form biofilms with unique properties. The knowledge gained from this work may serve to inform of particular pathogenic characteristics of specific strains and may be useful as a guide for specific avenues to consider for treatment of individual *E. faecalis* strains.

CHAPTER 2: Diverse *Enterococcus faecalis* Strains Show Heterogeneity in Biofilm Properties

To be submitted for publication as: **Scott D. Schaffer, Nontokozo V. Mdluli, Arielle Weinstein, Candace N. Rouchon, Kristi L. Frank.** "Diverse *Enterococcus faecalis* Strains Show Heterogeneity in Biofilm Properties."

The work presented here is the sole work of S.D. Schaffer with the following exceptions: N.V. Mdluli characterized the biofilm accumulation of the *E. faecalis* strains in Figure 2; A. Weinstein tested the effect of DNase, sodium (meta)periodate, and Proteinase K on biofilm formation of the *E. faecalis* strains in Figure 7; K.L. Frank conducted the MLST.

ABSTRACT

Enterococcus faecalis is an opportunistic pathogen that causes a range of healthcare-associated infections. Biofilm formation is important for successful *E. faecalis* colonization and pathogenesis. Prior studies have reported variability in biofilm formation potential among *E. faecalis* strains and isolates. However, it is unclear how the biofilms of those strains that do form biofilms vary in parameters such as development and composition. We hypothesized that differences in biofilms exist among distinct *E. faecalis* strains. In this work, we evaluated *in vitro* biofilm formation and matrix characteristics of a panel of five genetically diverse *E. faecalis* lab-adapted strains and clinical isolates (OG1RF, V583, DS16, MMH594, and VA1128). We compared biofilm formation in different growth conditions and investigated the composition of the biofilm

matrix of these strains. All strains formed biofilms similarly when grown in TSB^{-D}, 90% TSB^{-D}, or 90% TSB^{-D} + 10% FBS. However, DMEM with 10% FBS restricted the accumulation of an appreciable amount of biofilm biomass for all strains except VA1128. Treatment of pre-formed biofilms with sodium (meta)periodate, DNase, or Proteinase K indicated that the composition of the *E. faecalis* biofilm matrix is complex. None of the biofilms were dispersed by single treatments alone, but the biofilm biomass of both OG1RF and DS16 was significantly decreased by a sequential treatment of sodium (meta)periodate and DNase. Reversing the treatment order was not effective, suggesting that the extracellular DNA targeted by DNase was obscured by polysaccharides that are susceptible to sodium (meta)periodate degradation. Fluorescence microscopy further demonstrated differences between OG1RF and VA1128 in biofilm development and matrix macromolecular composition over a 48 hour time course. This study highlights the existence of heterogeneity in biofilm properties among diverse *E. faecalis* strains, which may have implications for the design of novel anti-biofilm treatment strategies.

INTRODUCTION

Enterococci are commensal bacteria of the human GI tract that can also flourish as opportunistic pathogens. Enterococcal infections have increased over the last several decades, which has led to the emergence of more antibiotic-resistant enterococci and a rise in hospital-acquired infections (9; 10; 21). Depending on the region of the world, *Enterococcus faecalis* causes between 63-74% of human enterococcal infections (126), which include infective endocarditis and urinary tract infections. The formation of biofilms, which are communities of cells attached to an interface or each other, embedded

in a self-produced matrix of extracellular polymeric substances, is an important mechanism for successful *E. faecalis* colonization and infection. Biofilms contribute to bacterial growth and survival by providing properties such as resource accessibility, social interactions, and resistance to antibiotics, which in turn can promote infection (57; 58). Significant research efforts have established the importance of biofilms for *E. faecalis* pathogenesis (24; 52).

Enterococci are known for harboring a plethora of MGEs, which include plasmids, insertion sequences, transposons, integrons, and PAIs (32). Many of these elements express genes that contribute to virulence or antimicrobial resistance, and these elements are not conserved between all enterococci or even among strains of the same species. Indeed, *E. faecalis* strains display a large amount of genetic heterogeneity, much of which derives from variability among the MGEs found in the strains (107; 122). A number of genes that are specifically associated with biofilm formation in *E. faecalis* are also found on MGEs or are only part of the accessory genome (29; 33; 144; 160; 161), and in consequence, are not conserved among all *E. faecalis* strains. One example is the *esp* gene, which encodes the enterococcal surface protein Esp that is localized to the PAI found in some *E. faecalis* strains (144). Moreover, the *esp* gene is not present in all strains containing the PAI, as seen with the commonly studied strain V583: the PAI in this strain contains a deletion of the *esp*-encoding region (144).

Numerous reports have shown that there is significant variability in *E. faecalis* biofilm formation among isolates or strains, as defined by the relative accumulation of biofilm biomass or the presence or absence of known biofilm formation-associated genes (6; 94; 100; 103; 159; 179; 180). However, data on biofilm formation phenotypes among

strains that extend beyond the simple measurement of relative biofilm biomass accumulation in a single type of growth medium are sparse, and what limited work has been published does not give a clear picture of the extent to which genetic heterogeneity influences the full scope of enterococcal biofilm formation properties. On one hand, the genetically divergent *E. faecalis* strains OG1RF, V583, and MMH594 displayed consistent medium-dependent biofilm formation and maintenance patterns across three types of growth media (94). On the other hand, biofilms of two *E. faecalis* strains of different genetic backgrounds elicited discrepant responses from components of the innate immune system (41). Thus, in this study, we tested the hypothesis that differences exist in biofilms among genetically diverse strains of *E. faecalis*. To this end, we characterized a panel of five *E. faecalis* strains that represent a mix of lab-adapted strains and clinical isolates in several biofilm growth conditions and sought to gain insight on the composition of the biofilm matrix of each strain.

RESULTS

E. faecalis strain panel.

We characterized biofilm formation properties of five wild type *E. faecalis* strains: OG1RF, DS16, MMH594, V583, and VA1128 (Table 1). These strains were selected based on use in previous studies, clinical relevance, genetic heterogeneity, and biofilm formation ability. Four of the strains were previously analyzed by MLST and determined to belong to three distinct sequence types (STs) (107); V583 and MMH594 belong to the same ST (Table 1). The ST of VA1128 is 414. OG1RF, V583, and MMH594 are the three most commonly studied strains in the panel (107). OG1RF is a

Table 1. *Enterococcus faecalis* Strains Used in this Study

Strain name	Description	Source or reference	MLST
OG1RF	Rifampicin and fusidic acid resistant derivative of OG1. Isolated from human oral cavity in 1970s.	**(19; 51; 69)**	1
DS16	Resistant to erythromycin, kanamycin, streptomycin, and tetracycline. Isolated from a patient at St. Joseph's Mercy Hospital, Ann Arbor, MI, prior to 1979. Harbors plasmids pAD1 and pAD2.	**(107; 168)**	40
MMH594	Resistant to erythromycin and gentamicin. Isolated from the bloodstream of a patient in Wisconsin in 1985.	**(84; 106; 107; 144)**	6
V583	Resistant to vancomycin, erythromycin, and gentamicin. Isolated from the bloodstream of a patient at Barnes Hospital, St. Louis, MO, in 1987. Harbors the plasmids pTEF1, pTEF2, and pTEF3.	**(123; 136)**	6
VA1128	Isolated from an indwelling Foley catheter in a patient at Minneapolis VA hospital in 1990s.	**(77; 78)**	414

rifampicin- and fusidic acid-resistant derivative (51) of the strain OG1, a human oral isolate (69). V583 and MMH594 are human bloodstream isolates (84; 136; 144). V583 was the first vancomycin-resistant enterococcal isolate identified in the United States (123; 136). OG1RF and V583 have fully annotated genome sequences (19; 123), while only a scaffold genome sequence assembly is available for MMH594 (39 contigs, GenBank accession AJDZ00000000.1). The two other strains of the panel, DS16 and VA1128, are not as well characterized. DS16 is a human clinical isolate (168) for which only a scaffold genome assembly is available (24 contigs; JGI IMG taxon ID 2558860757). VA1128 is an unsequenced human clinical isolate that was isolated from an indwelling Foley catheter (12).

E. faecalis has a propensity to acquire foreign DNA (32), and the strains in our panel represent a range of genomic complexities. OG1RF is a plasmid-free strain with very few MGEs (19), likely due to its functional genome defense systems (82; 121). In contrast, the V583 genome is comprised of more than 25% MGEs, many of which confer virulence and antibiotic-resistance traits and include three plasmids (123). MMH594 was reported to contain the first identified and sequenced PAI within the genus (144). DS16 is the host of two large plasmids, the 60 kb pheromone-responsive conjugative plasmid pAD1 and the ~26 kb non-conjugative plasmid pAD2, which together carry multiple antibiotic resistance determinants (31; 168). The MGE composition of strain VA1128 is not known.

OG1RF (13; 60; 90; 94; 95; 176) and V583 (74; 85; 96; 125; 162) are commonly used in *E. faecalis* biofilm studies. In contrast, biofilm formation by MMH594 (23; 39; 94) and VA1128 (12; 77; 78) has been studied less extensively. Strain DS16 has not been

documented to form biofilms prior to this study. Fig. 2 confirms that the five *E. faecalis* strains in our panel form *in vitro* biofilms in a microtiter plate biofilm assay.

Characterization of biofilm formation in different media.

We first characterized biofilm biomass accumulation and biofilm formation kinetics of the five strains in microtiter plate biofilms over 48 hours. To do this, we measured total bacterial cell growth (OD_{600}) and biofilm biomass (OD_{450}) at 2, 4, 8, 24, and 48 hours in three types of media. All strains reached similar cell densities at 48 hours in TSB^{-D}, our standard biofilm growth media (Fig. 3A). However, two distinct cell growth patterns emerged among the five strains (Fig. 3A): the OD_{600} values of DS16 and VA1128 increased sharply until four hours then showed only a minimal increase out to 48 hours, while the OD_{600} values for OG1RF, MMH594, and V583 increased until eight hours then reproducibly demonstrated a >50% decrease at 24 hours before recovering to reach the highest average values at 48 hours. The five strains had similar biofilm formation kinetics, but accumulated different amounts of biofilm biomass over the 48-hour time course (Fig. 3B). OG1RF and DS16 formed biofilms with the greatest biomass, while the biofilm biomass of MMH594 and V583 was approximately 50% lower across all time points. The average biomass values for VA1128 biofilms were intermediate to both groups. Curiously, OG1RF had the highest biofilm biomass of all the strains at 24 hours (Fig. 3B) despite having a low cell density at the same time point (Fig. 3A).

We next tested how the five strains formed biofilms in a growth condition that is more similar to a host environment by supplementing 90% TSB^{-D} with 10% FBS. Cell

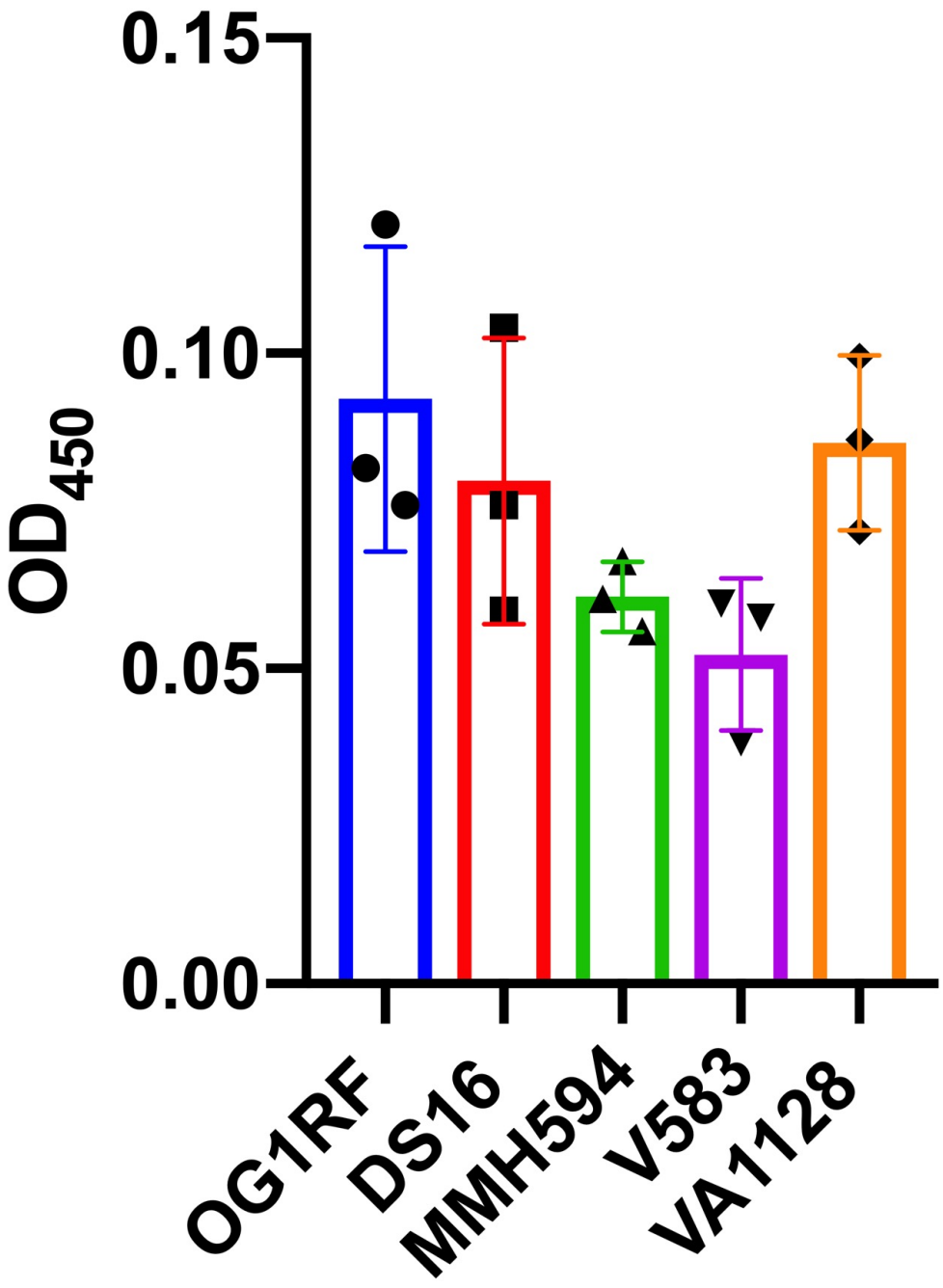


Figure 2. Biofilm Biomass Accumulation of *E. faecalis* Strains in a Microtiter Plate Assay

Biofilms were grown in TSB^{-D} for 20 hr. Each bar indicates the mean OD_{450} of safranin-stained biofilm biomass for three biological replicates shown as individual data points. Error bars display standard deviation.

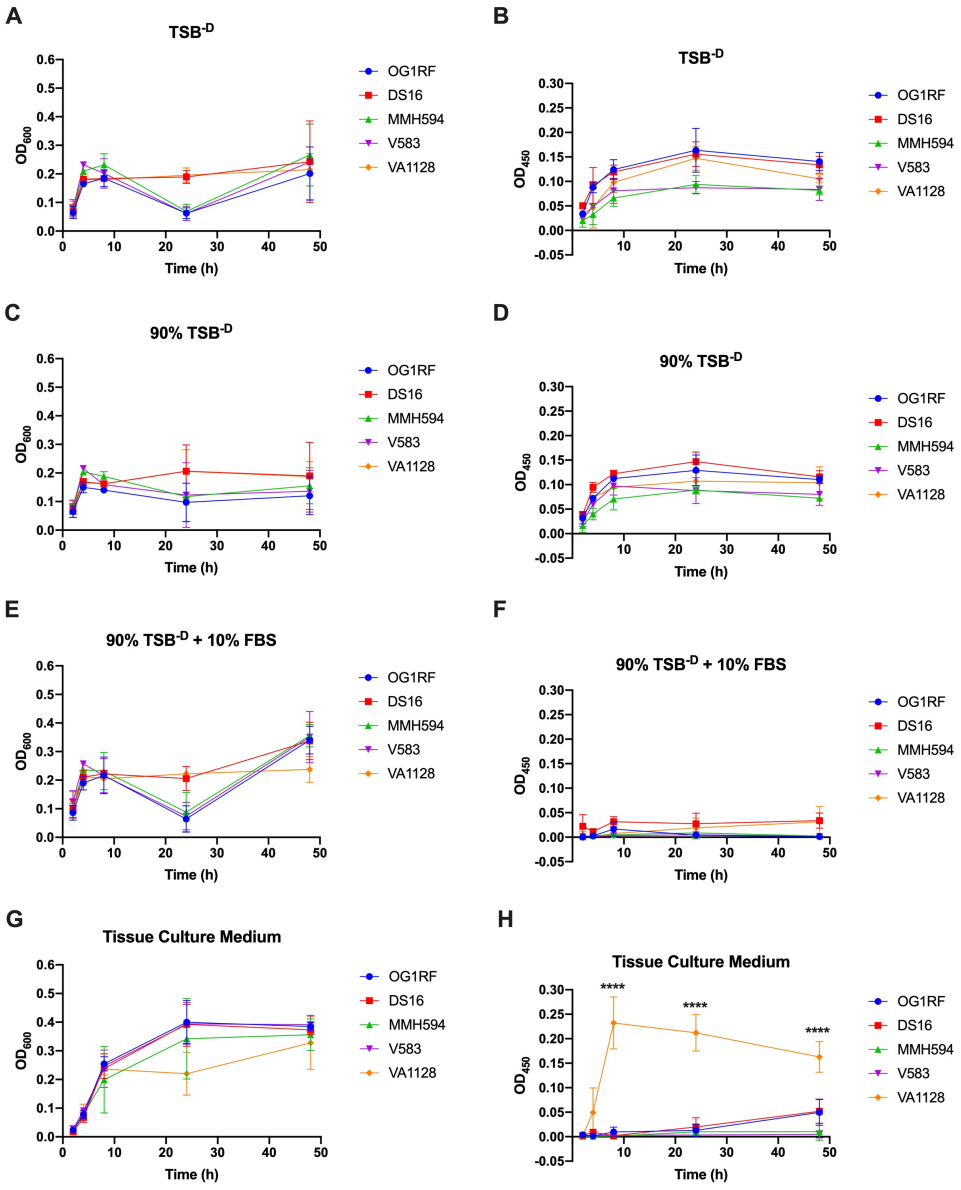


Figure 3. Biofilm Formation Kinetics of *E. faecalis* Strains in Different Media
Biofilms were grown over a period of 48 hr in either TSB^{-D} (A and B), 90% TSB^{-D} (C and D), 90% TSB^{-D} + 10% FBS (E and F), or tissue culture medium containing 10% FBS (G and H) in a microtiter plate assay. Planktonic cell growth (A, C, E, and G), indicated by the OD_{600} of biofilm cultures prior to decanting and washing, and biofilm development (B, D, F, and H), indicated by the OD_{450} of safranin-stained biofilm biomass, were measured at 2, 4, 8, 24, and 48 hr. Each point indicates the mean for three biological replicates; error bars display standard deviation. *P* values were determined by 2-way ANOVA followed by Tukey's multiple comparison test: ****, $P < 0.0001$. *P* values indicate levels of significance for comparisons between the means of VA1128 and all other strains.

growth and biofilm formation for all strains in 90% TSB^{-D} alone (Figs. 3C, 3D), which was used as a control, were similar to the undiluted TSB^{-D} results (Figs. 3A, 3B). Upon addition of 10% FBS (Fig. 3E), cell growth patterns among the strains were similar to those seen in TSB^{-D} (Fig. 3A). A notable exception to this was an increase in the density measurement for DS16 between 24 and 48 hours that was not observed for VA1128 (Fig. 3E). Overall, the optical densities for all strains except VA1128 were ~0.1 unit greater in 90% TSB^{-D} with 10% FBS (Fig. 3E) compared to TSB^{-D} (Fig. 3A) at 48 hours. Interestingly, none of the strains accumulated appreciable amounts of biofilm biomass accumulation (Fig. 3F). These results suggest that the presence of 10% FBS in a medium that is standardly used to cultivate *E. faecalis* biofilms (13; 94) disrupts normal biofilm biomass accumulation. It is unclear if this repression is due to the inhibition of biofilm production or the formation of less adherent biofilms that are more readily removed during the washing steps of the assay.

We also evaluated cell growth and biofilm formation in tissue culture medium. Growth measurements of all strains except VA1128 increased at each time point up to 24 hours then plateaued (Fig. 3G). VA1128 growth measurements remained stationary between 8 and 24 hours before increasing at 48 hours to an optical density that was similar to the other strains (Fig. 3G). Biofilm biomass accumulation was negligible for MMH594 and V583 at all time points, and a minimal amount of stained biomass (OD_{450} ~0.05) was measured in OG1RF and DS16 biofilm wells only at 48 hours (Fig. 3H). In contrast, VA1128 accumulated a strikingly high amount of biofilm biomass by 8 hours, which persisted through the remainder of the 48-hour assay (Fig. 3H). Taken together, the results shown in Figs. 3F and 3G indicate that biofilm biomass accumulation is delayed

or disrupted for numerous *E. faecalis* strains when 10% FBS is present in the biofilm growth media, yet a component of the utilized tissue culture medium uniquely induces VA1128 biofilm formation relative to the four other strains.

Direct comparison of the cell growth and biofilm biomass accumulation phenotypes for VA1128 from Fig. 3 further confirmed the inducing effect that tissue culture medium had on this strain (Fig. 4). VA1128 cell growth was significantly greater at 2, 4, and 48 hours in tissue culture medium than in one or more of the three media types (Fig. 4A). Biofilm biomass accumulation was also significantly higher at 4, 8, 24, and 48 hours in tissue culture medium than in one or more of the three media types (Fig. 4B). Thus, VA1128 displayed different biofilm formation kinetics in tissue culture medium compared to the other strains (Fig. 3), and to itself (Fig. 4) when grown in the other types of media.

Characterization of biofilm matrix composition by enzymatic and chemical treatments.

We next performed enzymatic and chemical treatments of 24 hour-old microtiter plate biofilms of the five strains in an attempt to characterize biofilm matrix composition. We hypothesized that the biofilm matrices of the five *E. faecalis* strains may be differentially dispersed following treatment with DNase, sodium (meta)periodate, and Proteinase K, to disrupt eDNA, polysaccharides, and proteins, respectively. However, when treated with either a single concentration of DNase (Fig. 5A) or three concentrations of sodium (meta)periodate (Fig. 5B), biofilm biomass remained the same

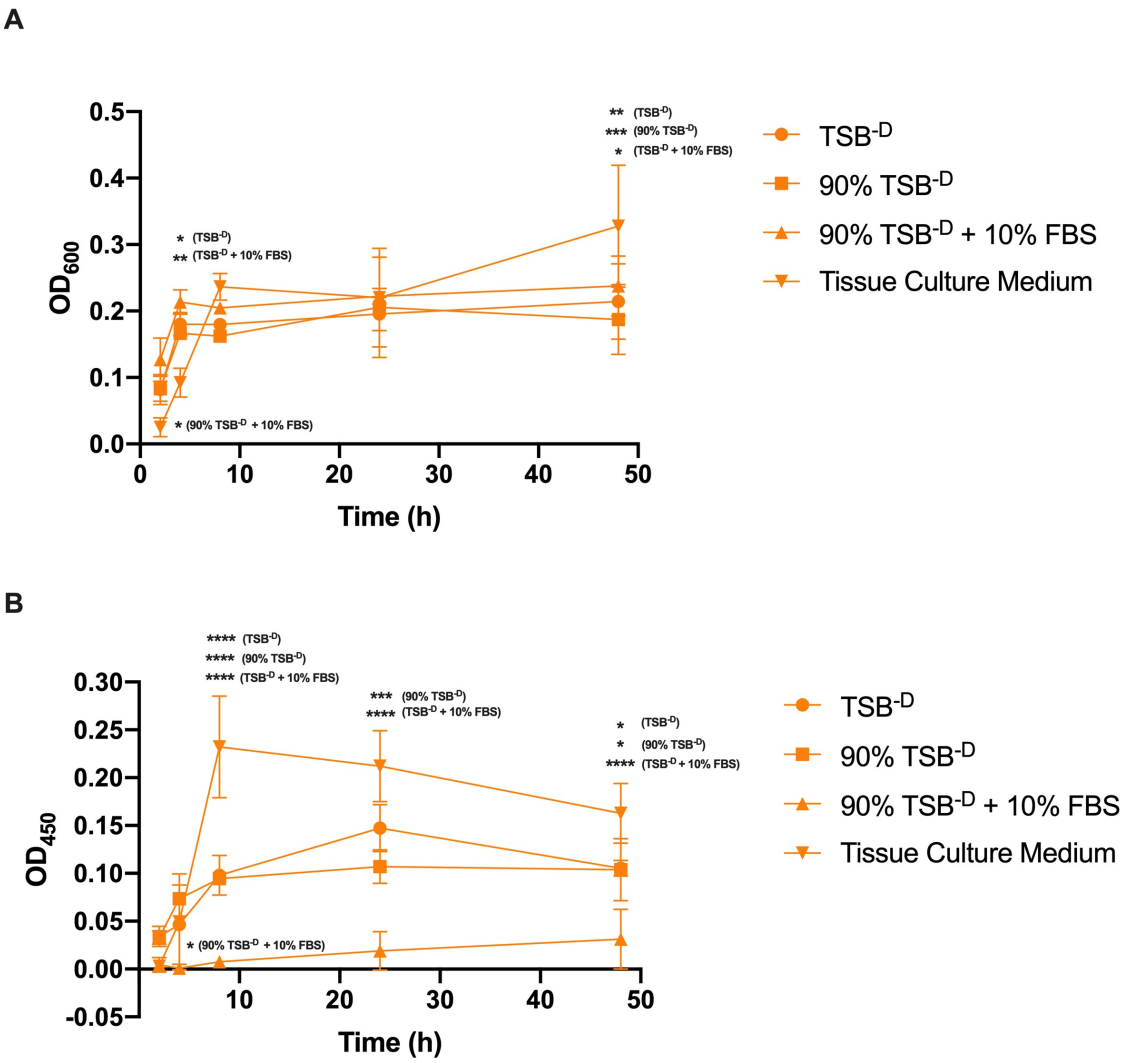


Figure 4. Direct Comparison of the Biofilm Formation Kinetics of *E. faecalis* VA1128 in Different Media

The planktonic cell growth (A) and biofilm development (B) data shown for *E. faecalis* VA1128 in Figure 3 are directly compared here. Each point indicates the mean for three biological replicates; error bars display standard deviation. *P* values were determined by 2-way ANOVA followed by Tukey's multiple comparison test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. *P* values indicate levels of significance for comparisons between the means of the medium indicated in parentheses and tissue culture medium.

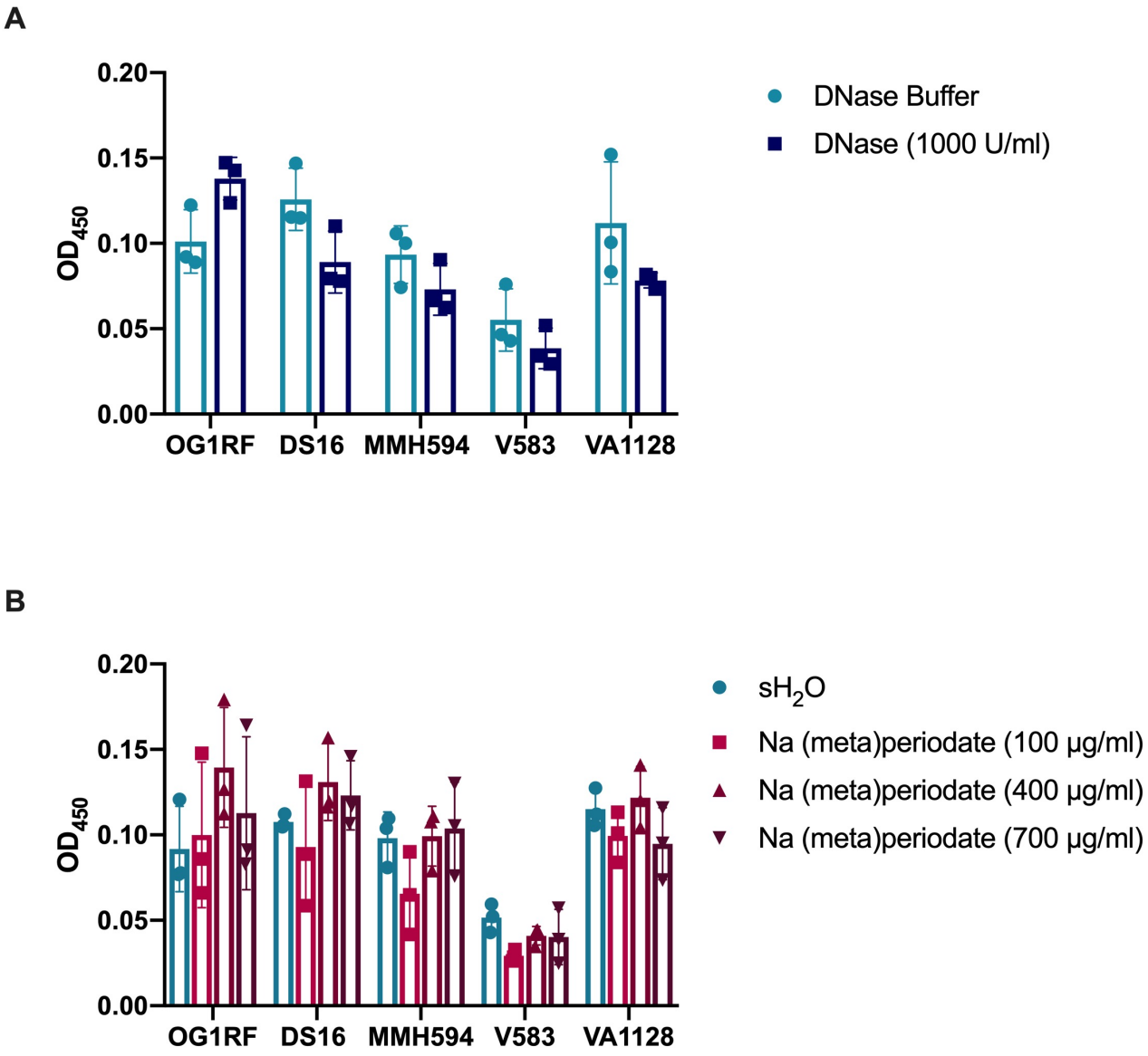


Figure 5. Effects of DNase and Sodium (meta)periodate on Biofilms of *E. faecalis* Strains
Biofilms grown in a microtiter plate assay in TSB^{-D} for 24 hr were treated with 1000 U/ml DNase (A) or 100 µg/ml, 400 µg/ml, or 700 µg/ml sodium (meta)periodate (B) for 2 hr at 37°C. Each bar indicates the mean for three biological replicates shown as individual data points. Error bars display standard deviation.

for all strains compared to the biofilm biomass of untreated biofilms. These results suggest that the stability of the biofilm matrix formed by these five *E. faecalis* strains is not dependent on eDNA or polysaccharides alone.

The stained biofilm biomass of OG1RF, DS16, and V583 did not change compared to untreated controls when treated with three concentrations (400, 700, and 1000 μg/ml) of Proteinase K for two, six, and 20 hours (Fig. 6A, B, and D). The amount of stained biomass in MMH594 biofilm wells was significantly higher after treatment with the highest concentration of Proteinase K (1000 μg/ml) and the longest duration of treatment (20 hours) compared to the untreated control at the same time point (Fig. 6C); no change in biofilm biomass was observed across the other treatment conditions. This unexpected increase in biomass staining led us to determine the CFU/ml of MMH594 biofilms treated for 20 hours with all three concentrations of Proteinase K. The CFU/ml of MMH594 biofilms was higher for all Proteinase K-treated conditions compared to the untreated control; specifically, 700 and 1000 μg/ml Proteinase K-treated biofilms were higher by ~1 $\log_{10}$ CFU/ml, and 400 μg/ml Proteinase K-treated biofilms were higher by ~0.5 $\log_{10}$ CFU/ml (data not shown). Thus, the CFU/ml results do not provide a definitive explanation for the unexpected significant increase in MMH594 stained biomass after treatment with a high concentration of Proteinase K for 20 hours. In contrast to the Proteinase K results observed for the other four strains (Fig. 6A-D), VA1128 biofilm biomass was significantly reduced after treatment with 1000 μg/ml Proteinase K for six hours and after treatment with 400 μg/ml and 1000 μg/ml Proteinase K for 20 hours (Fig. 6E). Together, the results of Fig. 6 indicate that only the biofilm matrix of strain VA1128

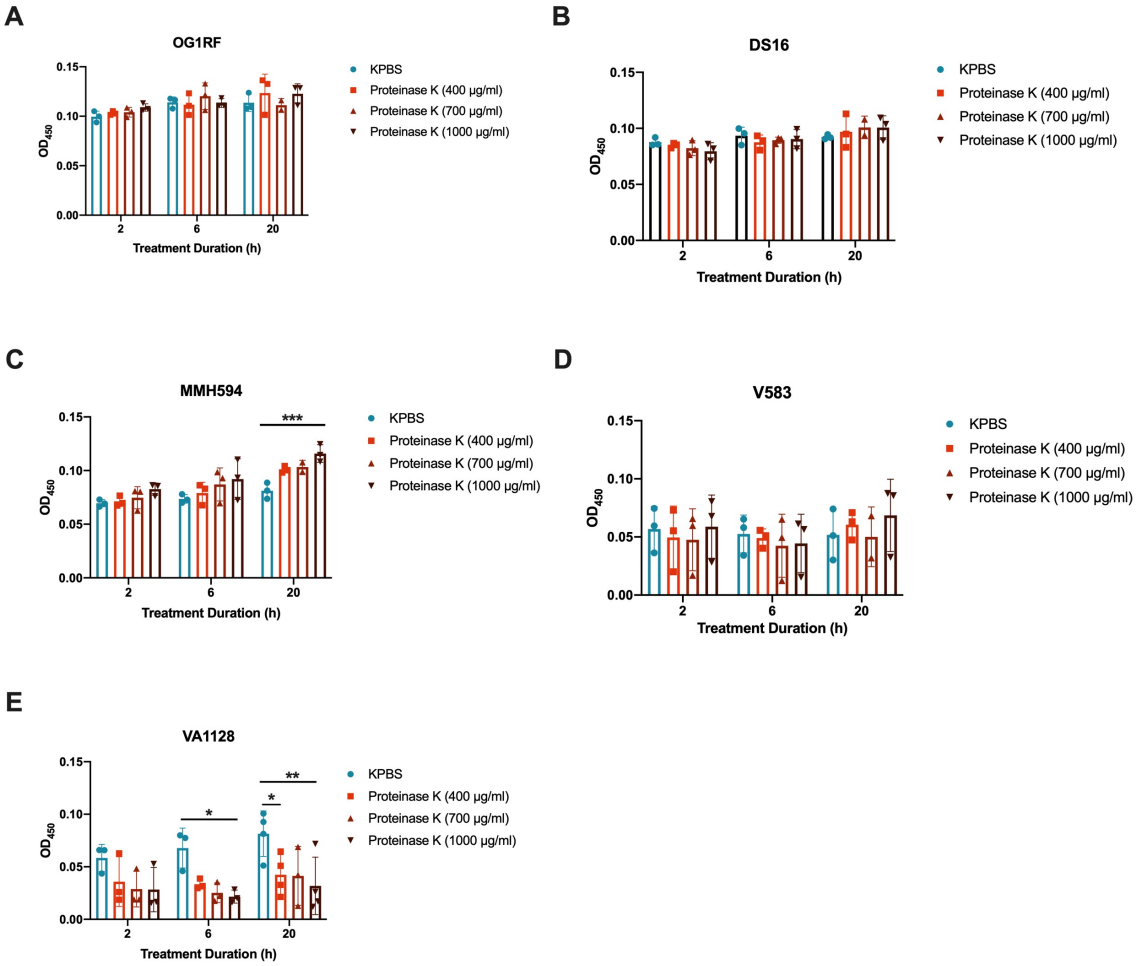


Figure 6. Effects of Proteinase K on Biofilms of *E. faecalis* Strains

Biofilms grown in a microtiter plate assay in TSB^{-D} for 24 hr were treated with 400 µg/ml, 700 µg/ml, or 1000 µg/ml Proteinase K for 2, 6, or 20 hr at 37°C. Each bar indicates the mean for three biological replicates for all strains and conditions, except for OG1RF, DS16, MMH594, and V583 for T=20, 700 µg/ml (n=2); and, VA1128 for T=20, KPBS, 400 µg/ml, and 1000 µg/ml (n=4). Means are shown as individual data points. Error bars display standard deviation. *P* values were determined by 2-way ANOVA followed by Tukey's multiple comparison test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for the indicated comparisons.

is partially susceptible to cleavage by Proteinase K. The biofilm matrices of the other four strains either do not contain proteins or contain proteins that are resistant to, or not accessible to, digestion by Proteinase K.

We also evaluated biofilm biomass accumulation of the five strains in the presence of 1000 U/ml DNase, 400 μg/ml sodium (meta)periodate, or 400 μg/ml Proteinase K. There were no differences in biomass accumulation between the buffer only or enzyme/chemical-exposure conditions after 24 hours (Fig. 7). Therefore, targeted disruption of eDNA, polysaccharides that are targeted by sodium (meta)periodate, or proteins that are susceptible to digestion by Proteinase K had no effect on the biofilm formation process of any of the strains studied.

Based on the results of the single treatment experiments (Figs. 5-7), our follow-up hypothesis was that the biofilm matrices of these five *E. faecalis* strains may be differentially comprised of complex amalgamations of macromolecules that render the action of any one chemical or enzymatic disruption agents ineffective. To test this hypothesis, we used Proteinase K, DNase, and sodium (meta)periodate in a series of consecutive treatment experiments. Biofilms were treated with varying permutations of separate, sequential two-hour treatments with either two or three of the treatment agents. Treatment with DNase followed by sodium (meta)periodate had no effect (Fig. 8). Conversely, treatment with sodium (meta)periodate followed by DNase yielded a significant decrease of biofilm biomass for OG1RF and DS16 compared to the untreated control biofilms, but did not cause statistically significant decreases in MMH594, V583, or VA1128 biofilm biomass (Fig. 9A). When Proteinase K was added to this treatment,

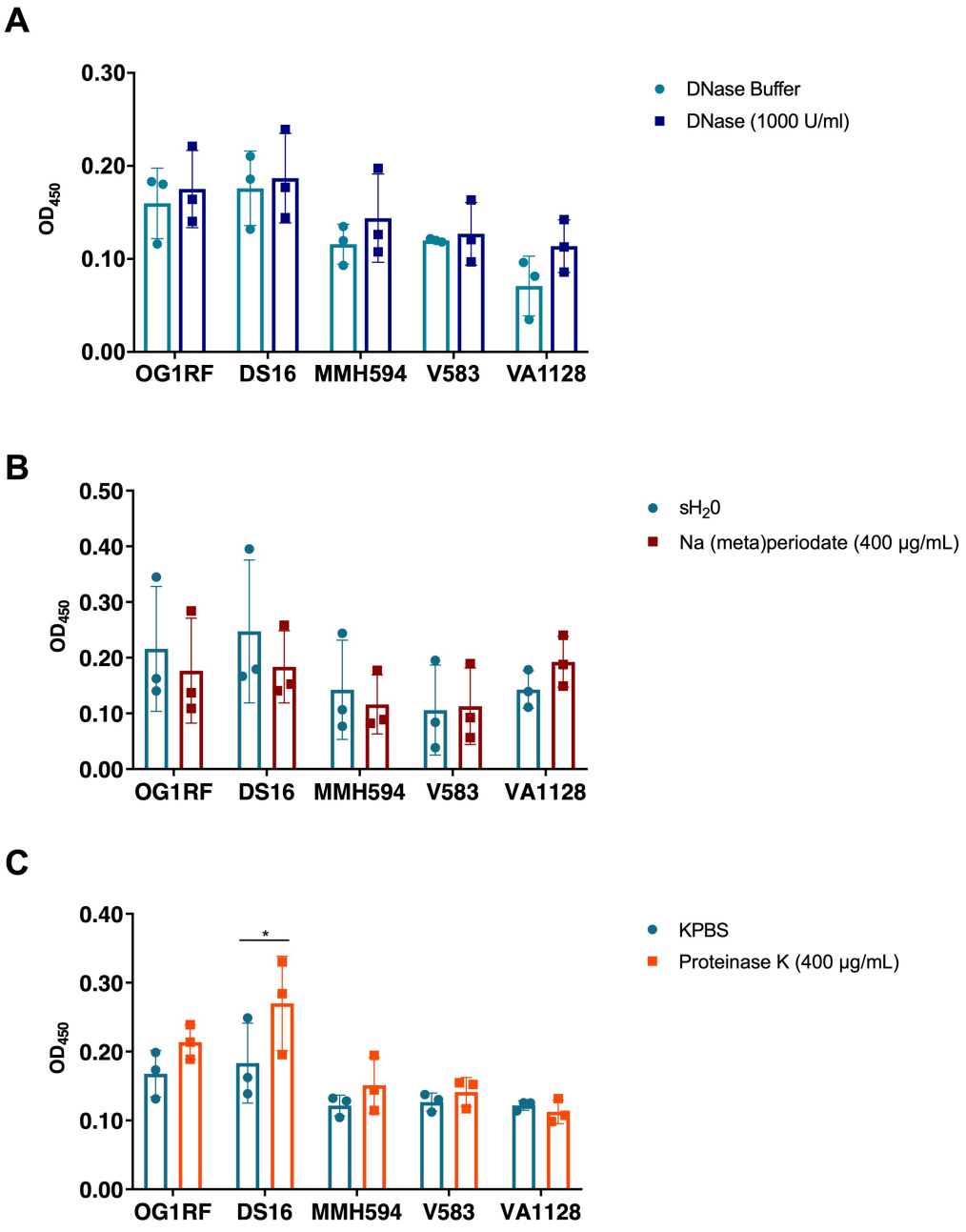


Figure 7. Effect of DNase, Sodium (meta)periodate, and Proteinase K on Biofilm Formation by Five *E. faecalis* Strains

Biofilms were grown for 24 hr in a microtiter plate assay in TSB^{-D} containing 1000 U/ml DNase (A), 400 µg/ml sodium (meta)periodate (B), or 400 µg/ml Proteinase K (C). Each bar indicates the mean for three biological replicates shown as individual data points. Error bars display standard deviation. *P* values were determined by 2-way ANOVA followed by Sidak's multiple comparison test: *, $P < 0.05$ for the indicated comparison.

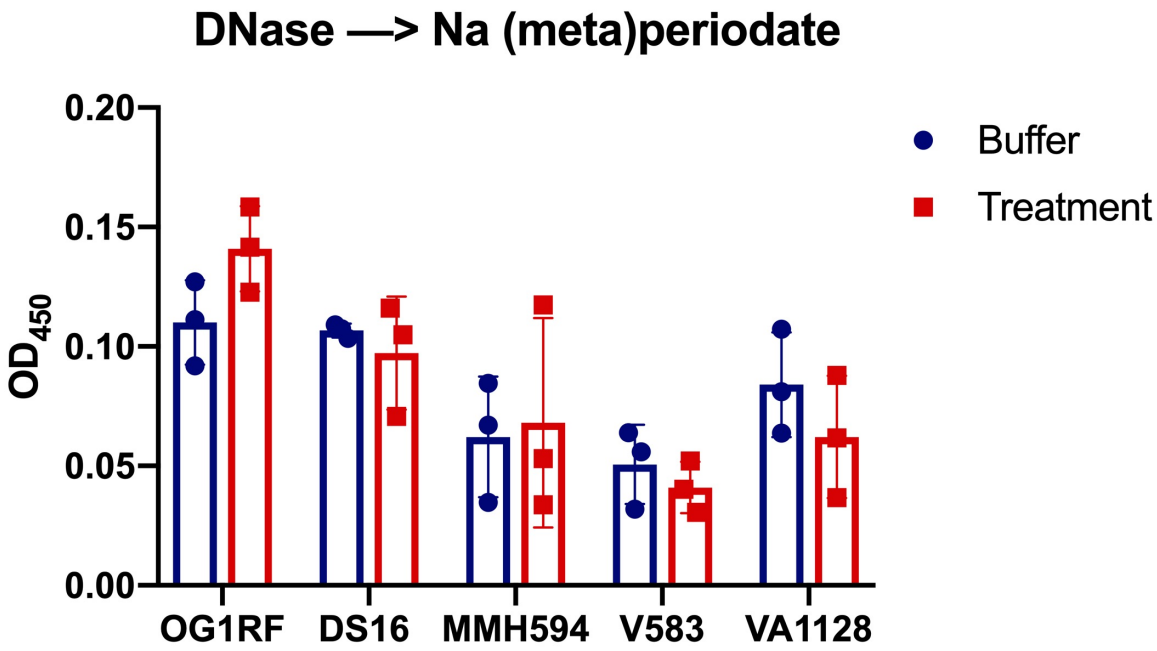


Figure 8. Effects of Consecutive Treatments of DNase Followed by Sodium (meta)periodate on *E. faecalis* Biofilms
Biofilms grown in a microtiter plate assay in TSB^{-D} for 24 hr were treated with 1000 U/ml DNase for 2 hr, washed, and subsequently treated with 400 µg/ml sodium (meta)periodate for 2 hr. Each bar indicates the mean for three biological replicates shown as individual data points. Error bars display standard deviation.

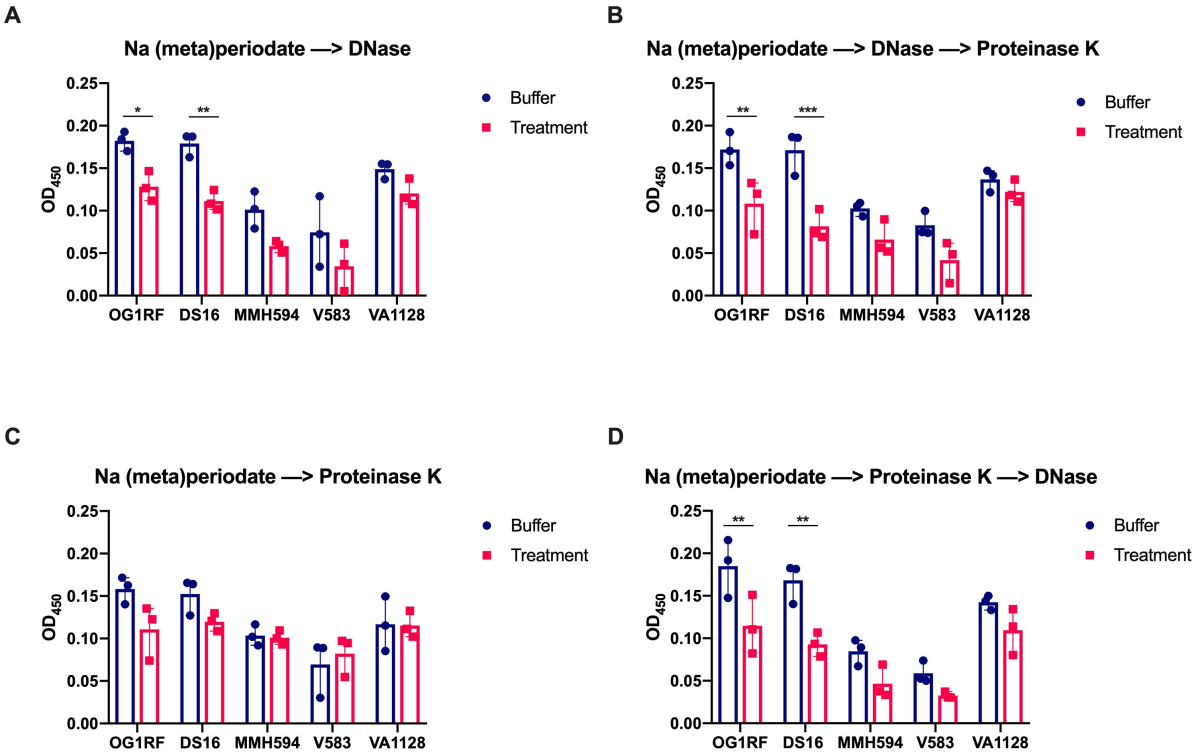


Figure 9. Effects of Consecutive Treatments of DNase, Sodium (meta)periodate, and Proteinase K on Biofilms of *E. faecalis* Strains

Biofilms grown in a microtiter plate assay in TSB^{-D} for 24 hr were treated with 400 µg/ml sodium (meta)periodate followed by 1000 U/ml DNase (A), 1000 U/ml DNase followed by 1000 µg/ml Proteinase K (B), 1000 µg/ml Proteinase K (C), or 1000 µg/ml Proteinase K followed by 1000 U/ml DNase (D). All treatments were conducted for 2 hr at 37°C, and biofilms were washed after each treatment. Each bar indicates the mean for three biological replicates shown as individual data points. Error bars display standard deviation. *P* values were determined by 2-way ANOVA followed by Sidak's multiple comparison test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for the indicated comparisons.

either immediately after treatment with sodium (meta)periodate or immediately following treatment with DNase, biofilm biomass was again significantly lower for OG1RF and DS16 compared to the untreated biofilms (Fig. 9B and D). Treatment with sodium (meta)periodate followed by Proteinase K had no effect on any of the strains (Fig 9C). Together, the results in Figs. 8-9 support a model in which initial degradation of polysaccharides in the biofilm matrix by treatment with sodium (meta)periodate exposed underlying eDNA that was subsequently susceptible to digestion by DNase for two of the five *E. faecalis* strains. There were no changes in biofilm biomass observed for any of the strains after testing treatment permutations of Proteinase K followed by DNase, Proteinase K followed by DNase and then Na(meta)periodate, Proteinase K followed by sodium (meta)periodate, and Proteinase K followed by sodium (meta)periodate and then DNase (data not shown). It is unknown why treatment with Proteinase K prior to subsequent treatments of sodium (meta)periodate and then DNase did not yield similar results to what is shown in Figs. 9A, B, and D.

Characterization of *E. faecalis* OG1RF and VA1128 biofilm biomass accumulation in the presence of shear force.

The remainder of this study focused on a comparison of the biofilm properties of the best-studied strain, OG1RF, and the least-studied clinical isolate, VA1128, of the *E. faecalis* strain panel (Table 1) when grown in the presence of shear force over time. To do this, we measured biofilm biomass accumulation and biofilm formation kinetics of the two strains when grown on Aclar fluoropolymer in TSB^{-D} under shaking conditions at 4, 8, 24, 32, and 48 hours. OG1RF biofilm biomass increased gradually for 24 hours, rose sharply at 32 hours and then plateaued (Fig. 10A). In contrast, VA1128 biofilm biomass

steadily increased until 24 hours, remained stable through 32 hours, then increased again by 48 hours, ultimately reaching an OD_{595} value approximately double what was observed for OG1RF at the same time point (Fig. 10B). While OG1RF and VA1128 displayed near-identical values of biofilm biomass accumulation and patterns of biofilm formation kinetics over time on polystyrene (Fig. 3B), the results in Fig. 10A and B demonstrate that a different surface and shear forces are associated with distinct biofilm formation patterns for these two strains.

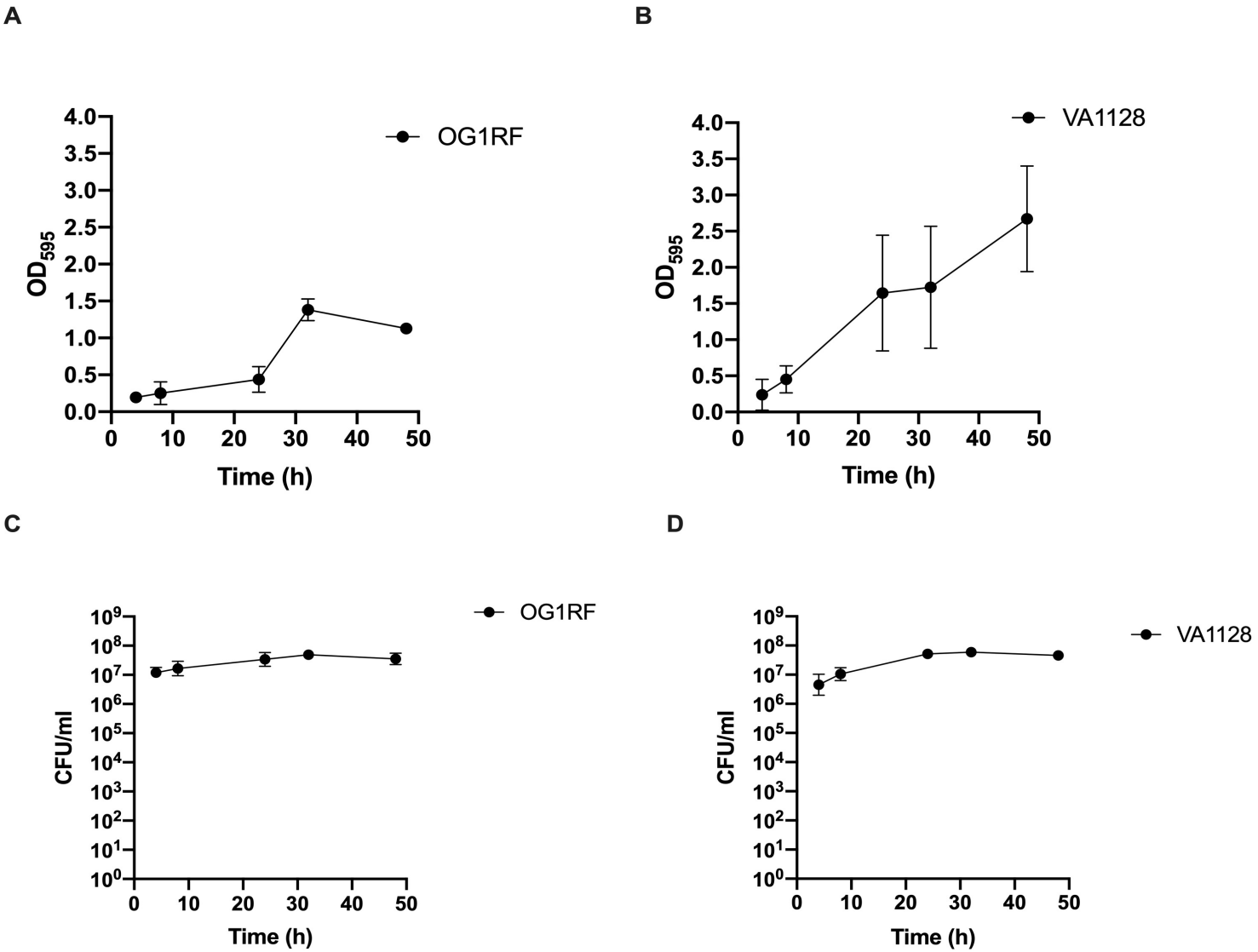
OG1RF and VA1128 Aclar biofilms were vortexed to detach biofilm cells for the determination of viable cell densities at each time point over 48 hours (Fig. 10C and D). The number of viable cells recovered from biofilms was nearly identical for both strains and did not correlate with the differences in biofilm biomass accumulation observed for the two strains (Fig. 10A and B). Subsequent crystal violet staining of Aclar membranes after vortexing revealed that considerable levels of stained biomass remained on the membranes for both OG1RF and VA1128, especially for biofilms grown for 24, 32, and 48 hours (Fig. 11). Thus, the viable cell counts of biofilm cells detached from Aclar membranes by vortexing are likely under representative of the total viable cells present in the biofilms.

Characterization of *E. faecalis* OG1RF and VA1128 biofilm development by fluorescence microscopy.

OG1RF and VA1128 biofilms grown on Aclar membranes for 4, 8, 24, 32, and 48 hours were stained with the Filmtracer LIVE/DEAD Biofilm Viability Kit and imaged to characterize cellular organization during the process of biofilm development. At the early

Figure 10. Biofilm Formation Kinetics and Viable Biofilm Cell Counts of *E. faecalis* OG1RF and VA1128 on Aclar Membranes

Biofilms were grown over a period of 48 hr in TSB^{-D} on 11-mm-diameter Aclar membranes in shaking 6-well plates. Biofilm development (A and B) and viable detached biofilm cells recovered by vortexing (C and D) were measured at 4, 8, 24, 32, and 48 hr. Each point indicates the mean for three biological replicates; error bars display standard deviation.


A
B
C
D
OG1RF
VA1128
OD595
CFU/ml
Time (h)
0
10
20
30
40
50
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
10^0
10^1
10^2
10^3
10^4
10^5
10^6
10^7
10^8
10^9

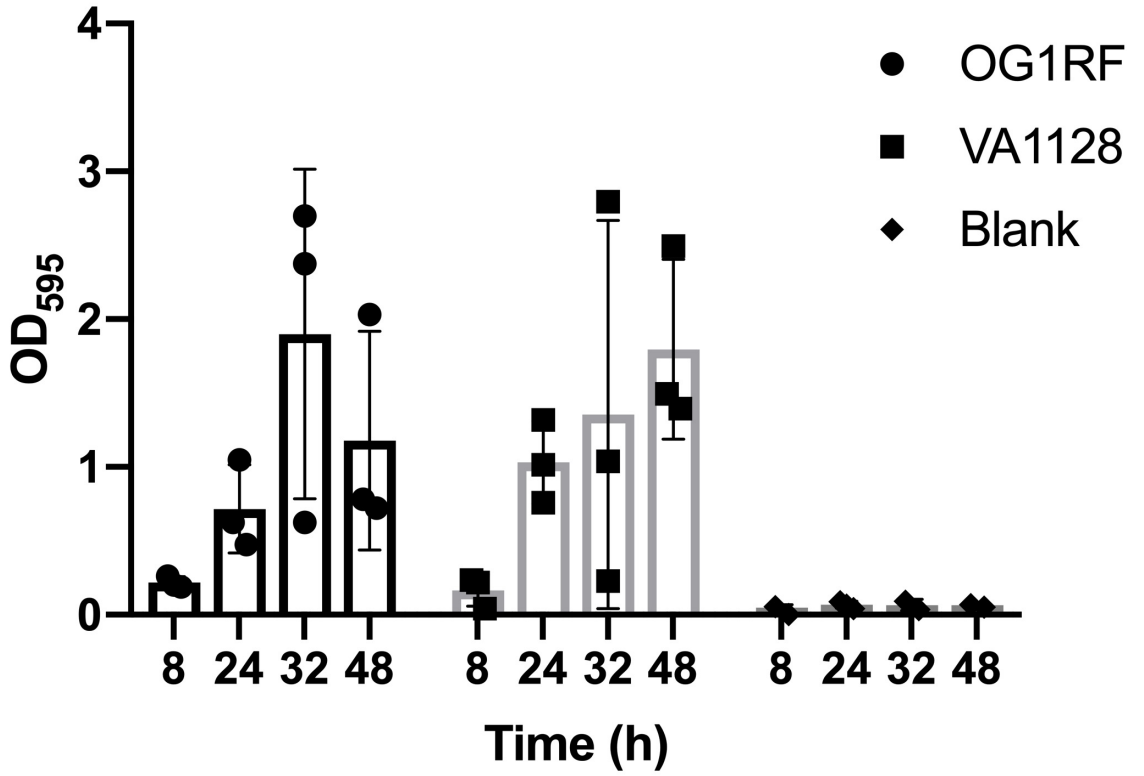


Figure 11. *E. faecalis* OG1RF and VA1128 Biofilm Biomass Remaining on Aclar Membranes After Vortexing

OG1RF and VA1128 biofilms were grown in TSB^{-D} on 11-mm-diameter Aclar membranes for 8, 24, 32, and 48 hr. At each time point, membranes were placed into 2-ml Eppendorf tubes and vortexed on a multi-tube vortexer for 6 min. Post-vortexing, residual biomass remaining on the membranes was stained with 0.1% crystal violet and quantitated. Uninoculated, blank control membranes were prepared in parallel. Each bar indicates the mean for three biological replicates shown as individual data points. Error bars display standard deviation.

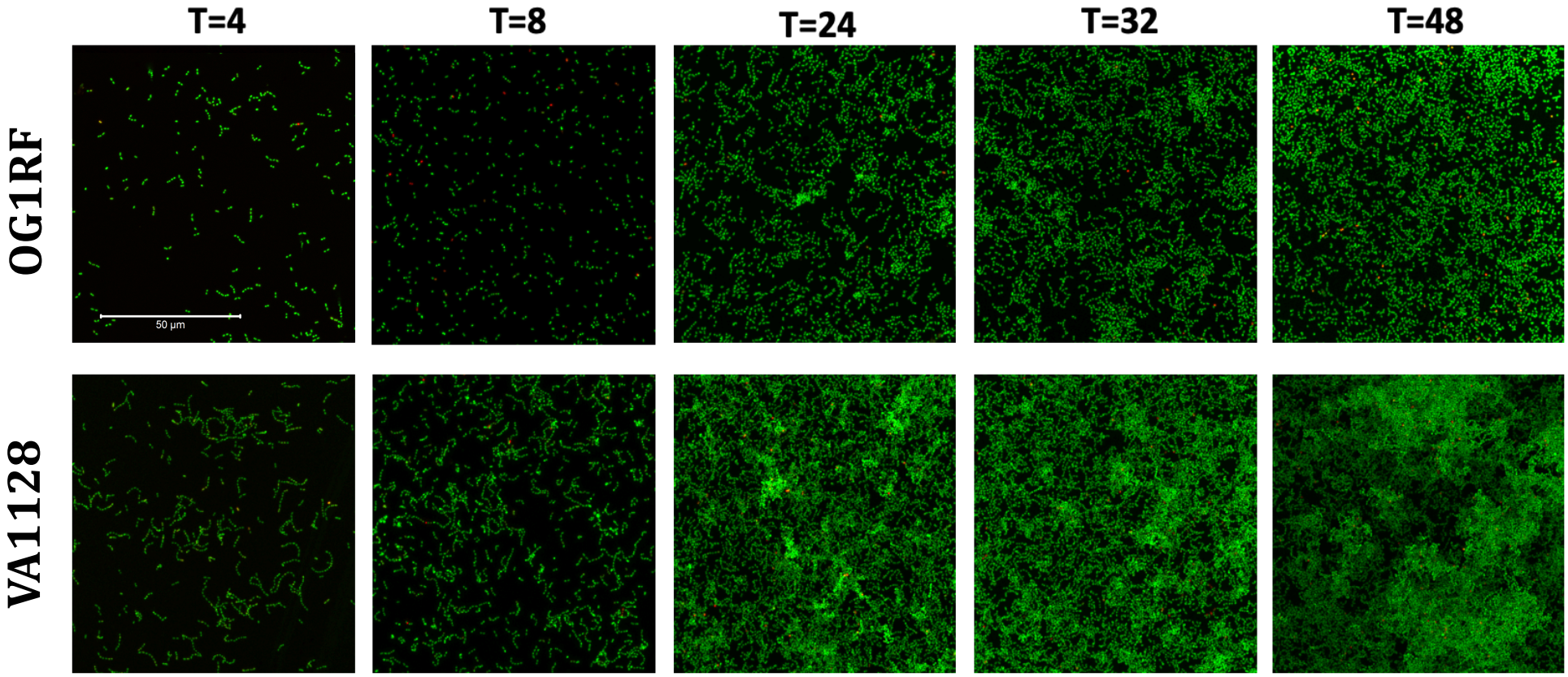
time points, OG1RF colonized the Aclar membranes as individual diplococci and short chains while VA1128 predominantly existed as long chains (Fig. 12). VA1128 biofilms had greater cell density and architectural diversity compared to OG1RF biofilms by 24 hours, and this persisted through the 32 and 48 hour time points (Fig. 12). Biofilm thickness, area, and volume based on the STYO-9 staining were determined for all biofilms imaged. Consistent with the visual analysis (Fig. 12), all three parameters increased over the 48-hour time course (Fig. 13). All parameters were significantly greater for VA1128 than for OG1RF at 32 and 48 hours (Fig. 13).

Characterization of *E. faecalis* OG1RF and VA1128 biofilm matrix macromolecular content by biochemical quantification.

OG1RF and VA1128 biofilms grown on Aclar membranes for 8, 24, 32, and 48 hours were vortexed in KPBS to dislodge biofilm matrix components for biochemical quantification of protein, carbohydrate, and eDNA. The concentrations of protein, carbohydrate, and eDNA content recovered from OG1RF and VA1128 biofilm matrices remained relatively static over 48 hours of growth on Aclar (Fig. 14). Furthermore, the protein, carbohydrate, and eDNA concentrations did not differ greatly between the strains at any time point (Fig. 14). These results confirm the presence of protein, carbohydrates, and eDNA in the biofilm matrices of OG1RF and VA1128. However, the concentrations of each macromolecule determined in these assays may be under representative of the actual concentrations in the biofilm matrix given the high amount of stained biofilm biomass that persisted on Aclar membranes in Fig. 11.

Figure 12. Visualization of biofilm development of *E. faecalis* strains OG1RF and VA1128 on Aclar membranes

OG1RF and VA1128 biofilms were grown in TSB^{-D} on 11-mm-diameter Aclar membranes and stained with the Filmtracer LIVE/DEAD Biofilm Viability Kit. Biofilm formation was visualized by fluorescence confocal microscopy after 4, 8, 24, 32, and 48 hr of incubation. Each dye was captured on a single channel, and a representative image of a single biofilm per time point is shown at a magnification of X63. Images shown were merged and are of compressed z-stacks. Bar, 50 μm


T=4
T=8
T=24
T=32
T=48
OG1RF
VA1128
50 µm

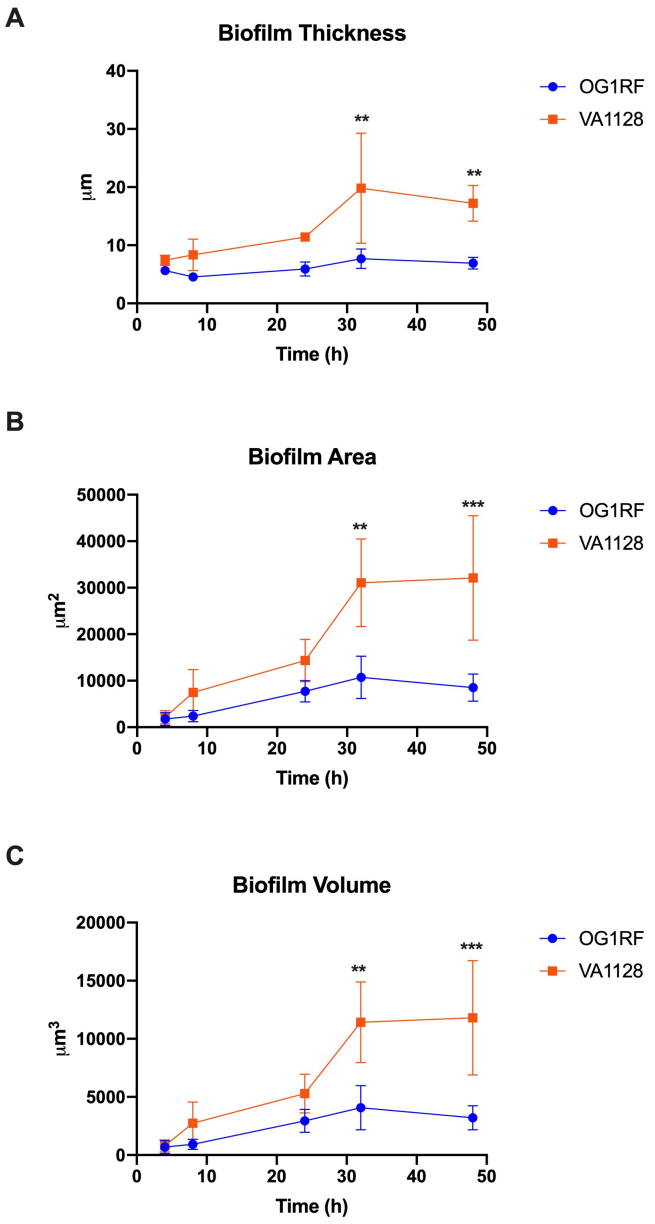
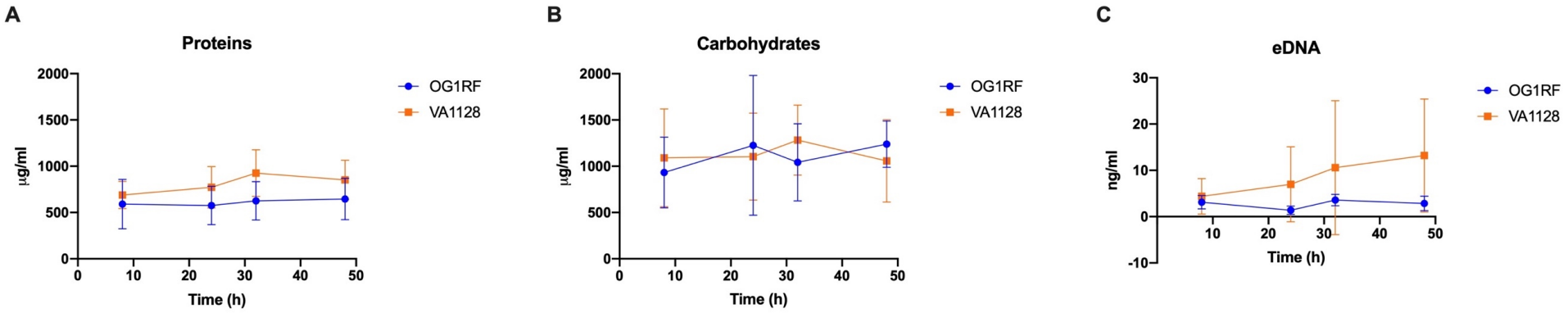


Figure 13. Image analysis of biofilm development of *E. faecalis* strains OG1RF and VA1128

OG1RF and VA1128 biofilms were grown in TSB^{-D} on 11-mm-diameter Aclar membranes and stained with the Filmtracer LIVE/DEAD Biofilm Viability Kit. Biofilm formation was visualized by fluorescence confocal microscopy after 4, 8, 24, 32, and 48 hr of incubation. Biofilm thickness, area, and volume based on the Syto-9 fluorescence were calculated using ImageJ. Each point indicates the mean for three biological replicates, in which each biological replicate indicates the mean for three individual images of a single biofilm. Error bars display standard deviation. *P* values were determined by 2-way ANOVA followed by Tukey's multiple comparison test: **, $P < 0.01$; ***, $P < 0.001$. *P* values indicate levels of significance for comparisons between the means of the two strains at each time point.

Figure 14. Concentrations of protein, carbohydrate, and eDNA dislodged from OG1RF and VA1128 biofilm matrices

OG1RF and VA1128 biofilms grown in TSB^{-D} on 11-mm-diameter Aclar membranes for 8, 24, 32, and 48 hr were vortexed in KPBS to dislodge biofilm matrix macromolecules. Protein, carbohydrate, and eDNA concentrations in vortexed samples were determined. Each point indicates the mean for three biological replicates; error bars display standard deviation.


A
Proteins
µg/ml
2000
1500
1000
500
0
0
10
20
30
40
50
Time (h)
OG1RF
VA1128
B
Carbohydrates
µg/ml
2000
1500
1000
500
0
0
10
20
30
40
50
Time (h)
OG1RF
VA1128
C
eDNA
ng/ml
30
20
10
0
-10
10
20
30
40
50
Time (h)
OG1RF
VA1128

Characterization of biofilm matrix composition by fluorescence confocal microscopy.

OG1RF and VA1128 biofilms grown on Aclar membranes for 4, 8, 24, 32, and 48-hours were stained to visualize cellular DNA, eDNA, proteins, and carbohydrates by fluorescence confocal microscopy. Fig. 15 shows the progression of biofilm matrix development and organization during the process of biofilm formation. Hoechst dye (blue), a cell-permeable DNA stain, was used as a counterstain to visualize all *E. faecalis* cells in the biofilms. At the early time points (4 and 8 hours, Fig. 16A, B, F, and G), the majority of cells were co-localized with one or more of the other fluorescent stains for both *E. faecalis* strains. The proportion of cells labeled solely with Hoechst increased as cell density in the biofilms increased. There was broad staining with wheat germ agglutinin (WGA; red), a lectin that binds to N-acetylglucosamine and sialic acid, across the fields imaged for both strains (Figs. 15 and 16). In contrast, the SYPRO (pink) and TOTO-1 (green) dyes, corresponding to proteins and eDNA respectively, frequently co-localized with one another in punctate forms (Figs. 15 and 16). This was also observed for both strains across all time points and was specifically prominent at 4 and 8 hours.

Figure 17 shows the corresponding biofilm images of Fig. 15 in an X-Z orientation. This perspective further confirmed variation between OG1RF and VA1128 biofilm development, particularly with respect to cellular and matrix macromolecule distribution over time. WGA was the prominent stain observed in biofilms of both strains at 4 and 8 hours. However, differences in the staining patterns suggest that WGA-labeled carbohydrates were diffuse throughout the height of OG1RF biofilms but more concentrated at the base of VA1128 biofilms. By the 32 hour time point, biofilms of both

Figure 15. Visualization of biofilm matrix development of *E. faecalis* strains OG1RF and VA1128

OG1RF and VA1128 biofilms were grown in TSB^{-D} on 11-mm-diameter Aclar membranes, stained with Hoechst 33342 Solution (blue; *E. faecalis* cellular DNA), FilmTracer SYPRO Ruby Biofilm Matrix Stain (pink; proteins), Wheat Germ Agglutinin - Alexa Fluor 647 Conjugate (red; carbohydrates), and TOTO-1 Iodide (green; eDNA), and visualized by fluorescence confocal microscopy after 4, 8, 24, 32, and 48 hr of incubation. Each dye was captured on a single channel, and one representative image of a single biofilm per time point is shown at a magnification of X63. Images shown were merged and are of compressed z-stacks. Bar, 50 μm

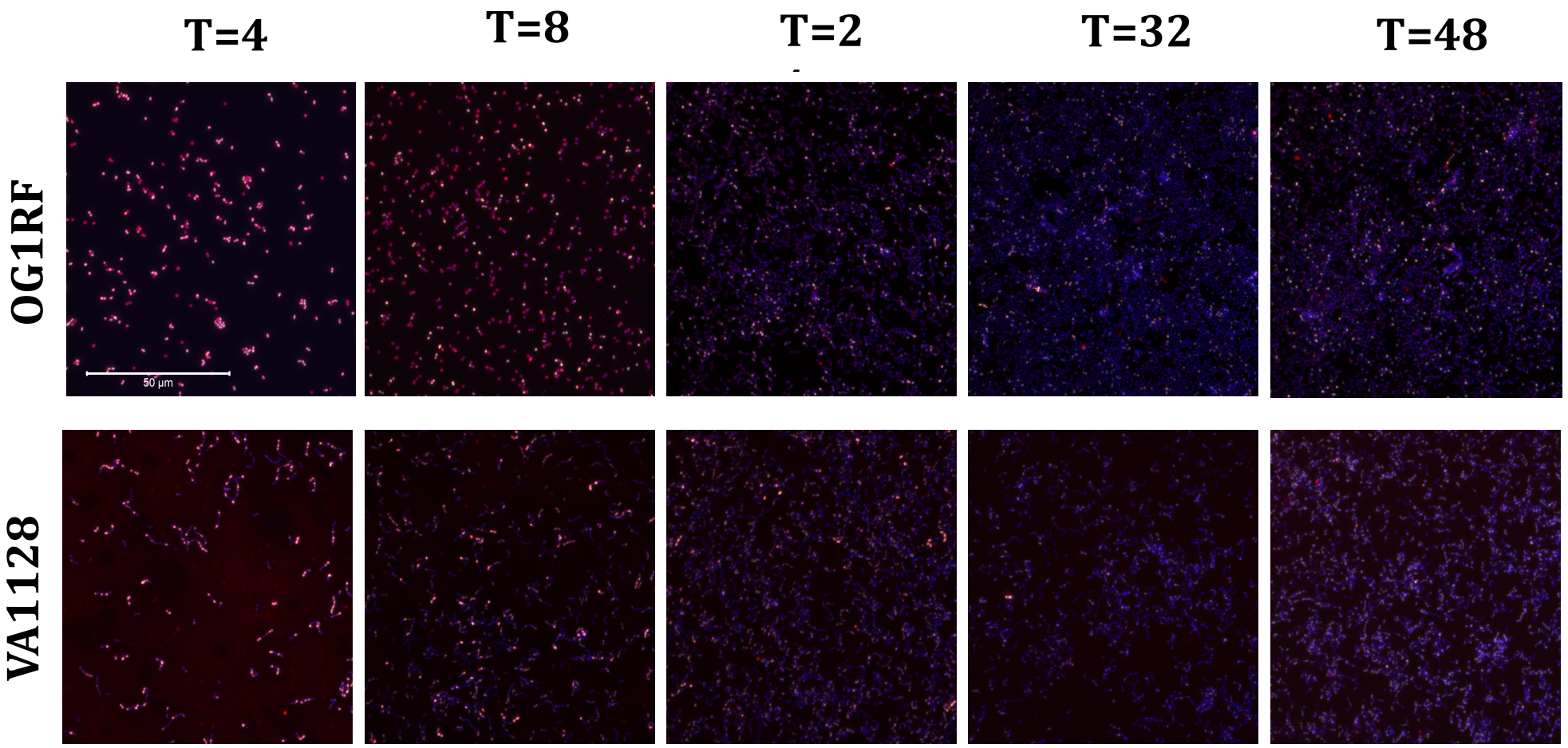
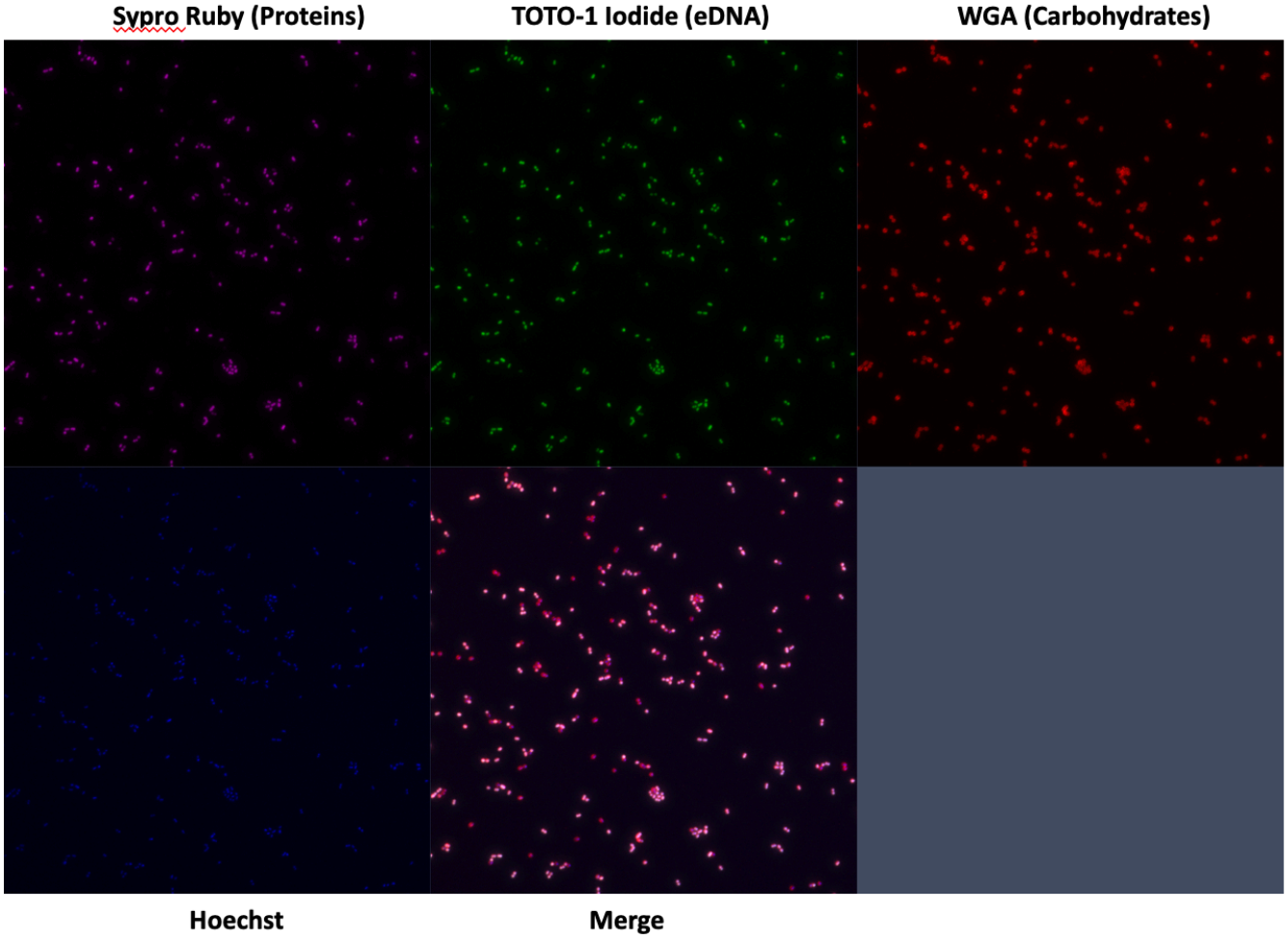

T=4
T=8
T=2
T=32
T=48
OG1RF
VA1128
50 µm

Figure 16. Single channel images of each panel shown in Figure 15

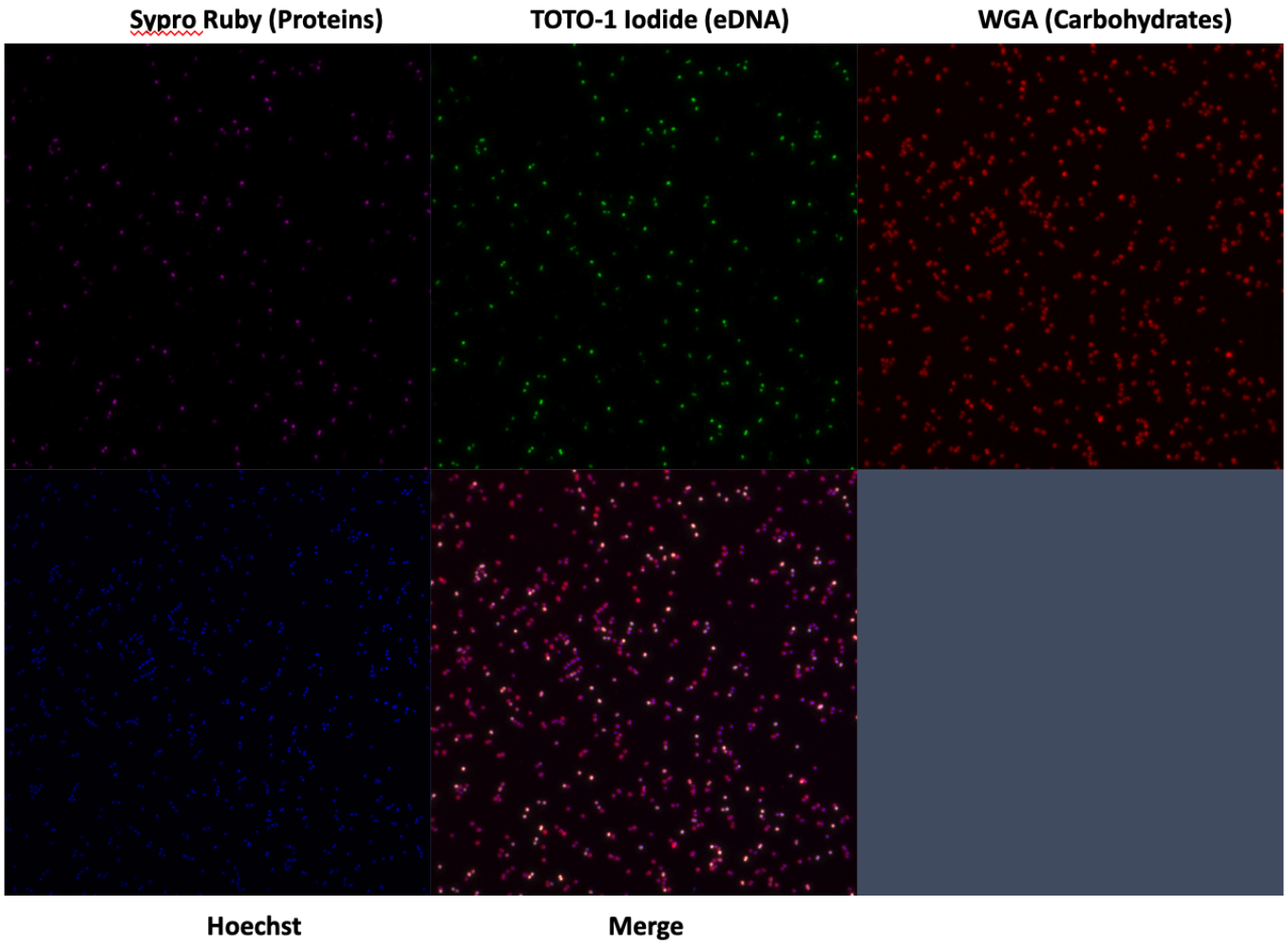
T=4

OG1RF


Sypro Ruby (Proteins)
TOTO-1 Iodide (eDNA)
WGA (Carbohydrates)
Hoechst
Merge

T=8

OG1RF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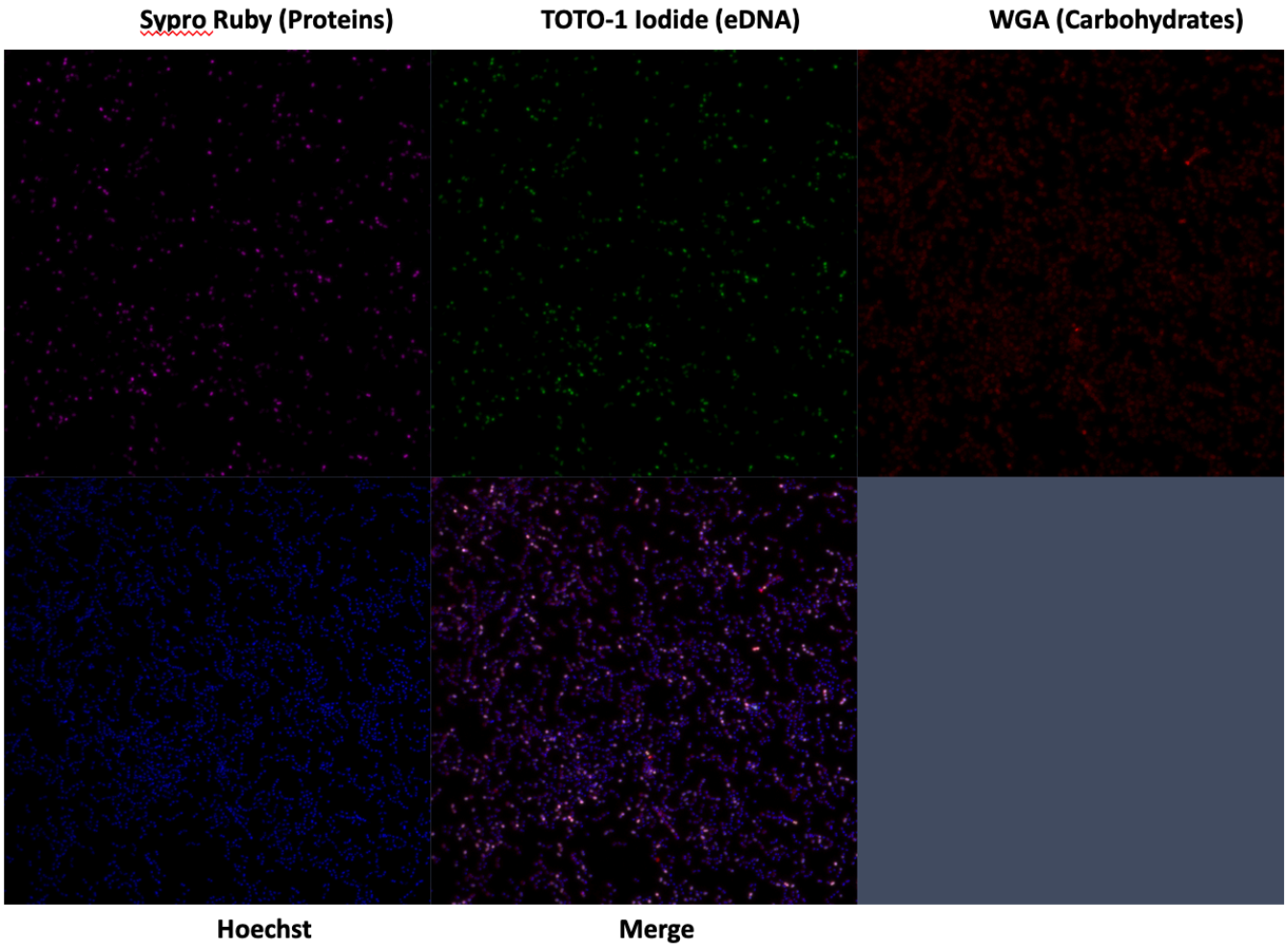
TOTO-1 Iodide (eDNA)
WGA (Carbohydrates)
Hoechst
Merge

T=24

OG1RF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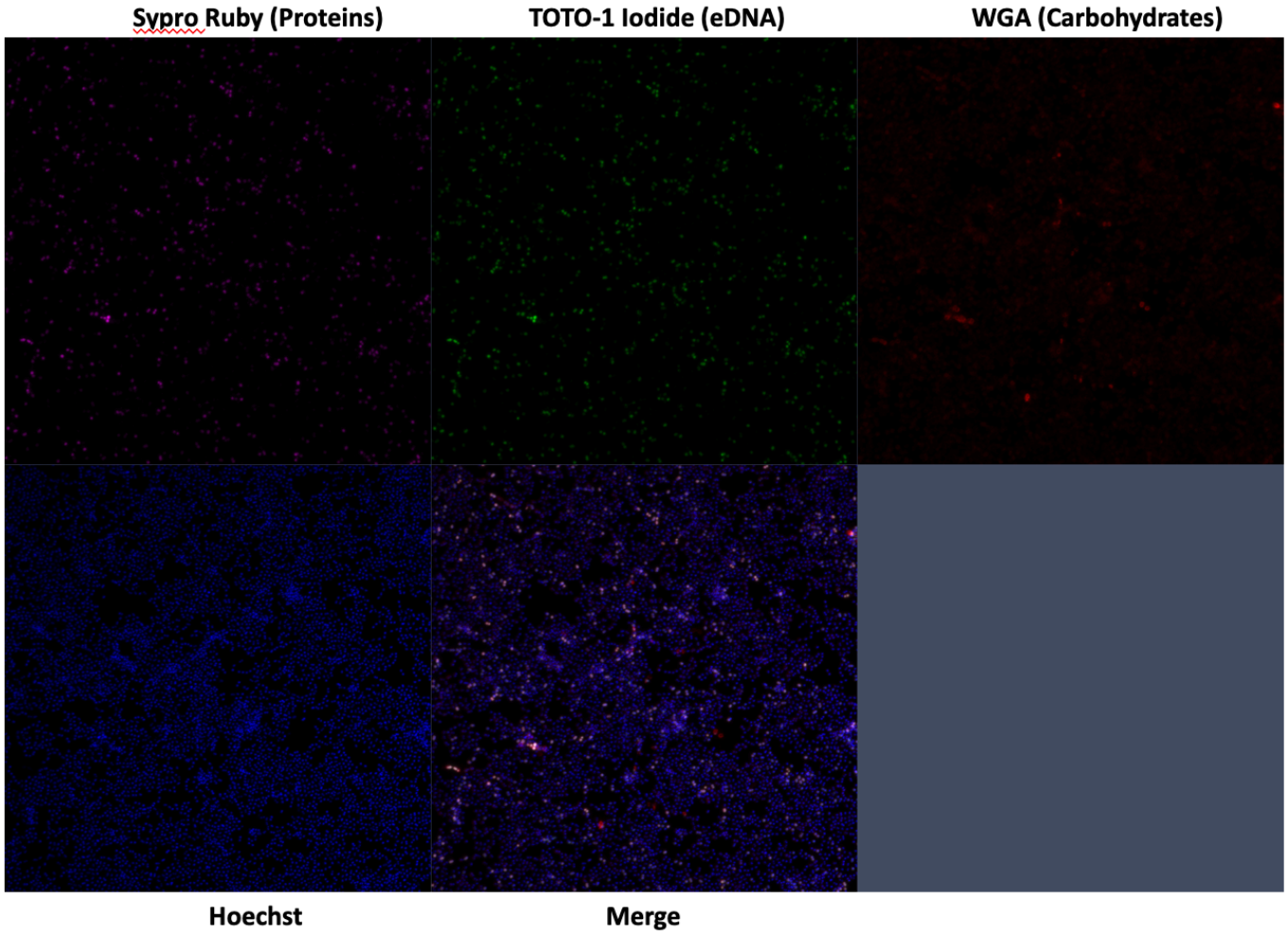
TOTO-1 Iodide (eDNA)
WGA (Carbohydrates)
Hoechst
Merge

T=32

OG1RF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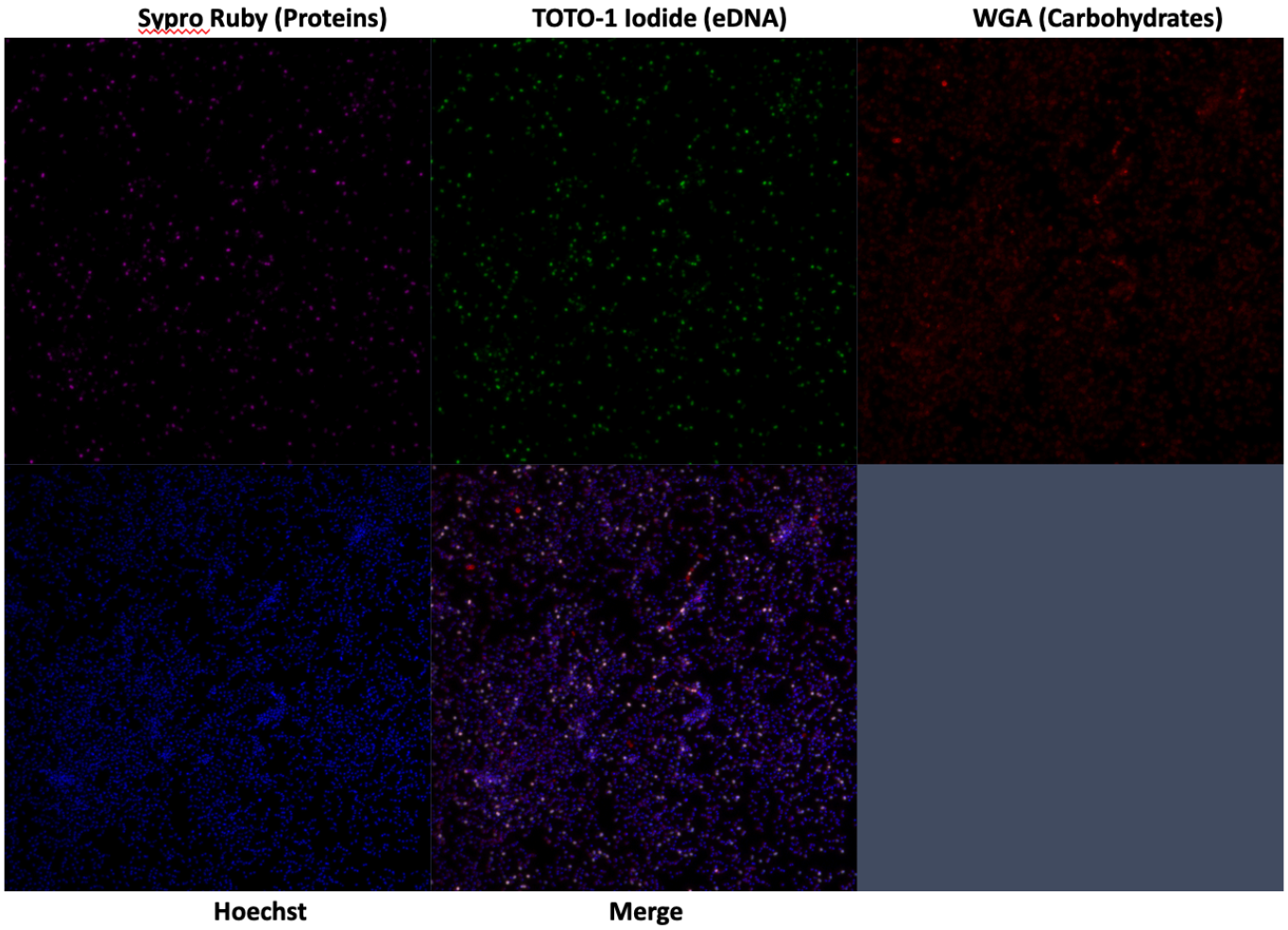
TOTO-1 Iodide (eDNA)
WGA (Carbohydrates)
Hoechst
Merge

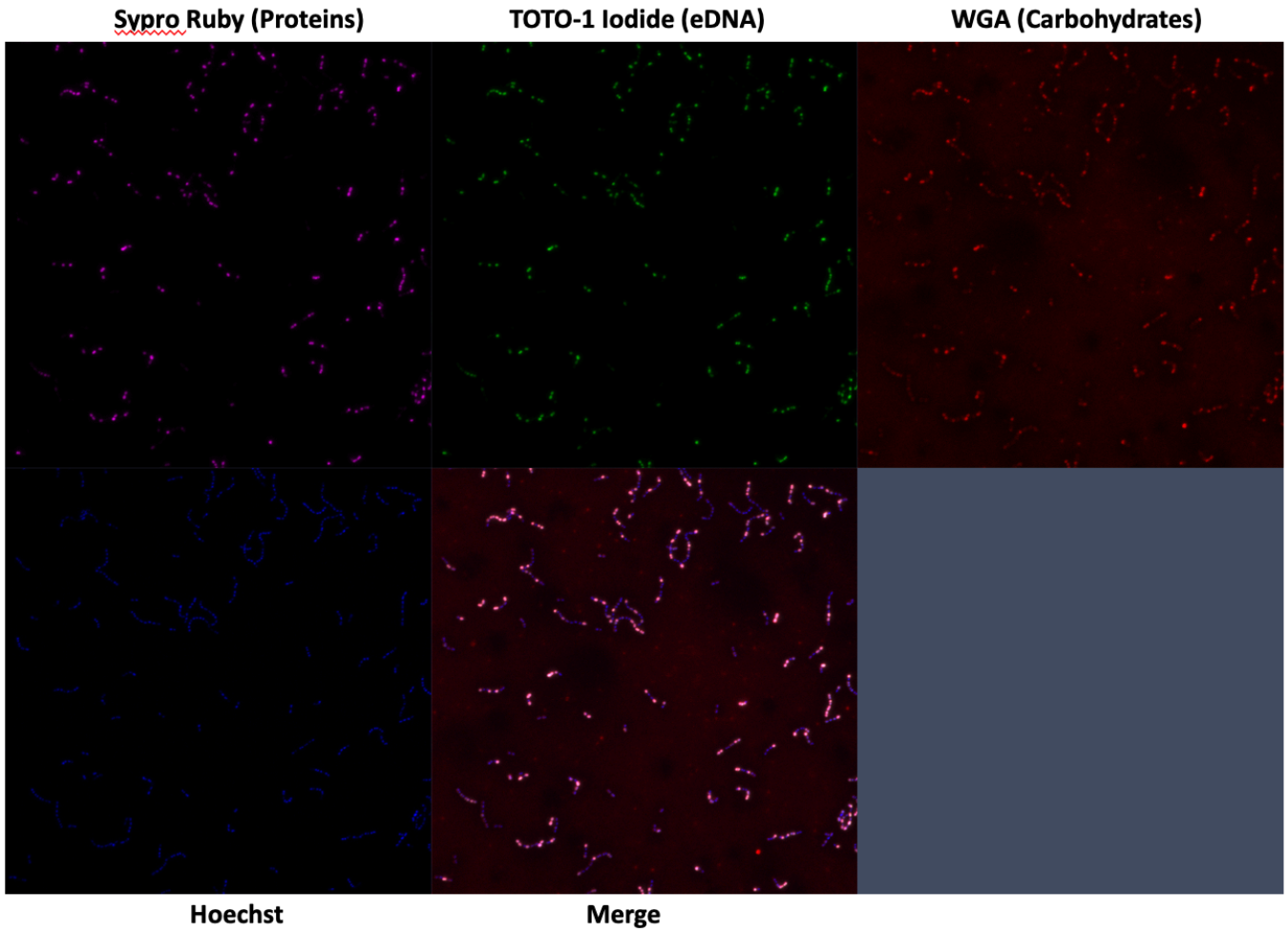
T=48

OG1RF


Sypro Ruby (Proteins)
TOTO-1 Iodide (eDNA)
WGA (Carbohydrates)
Hoechst
Merge

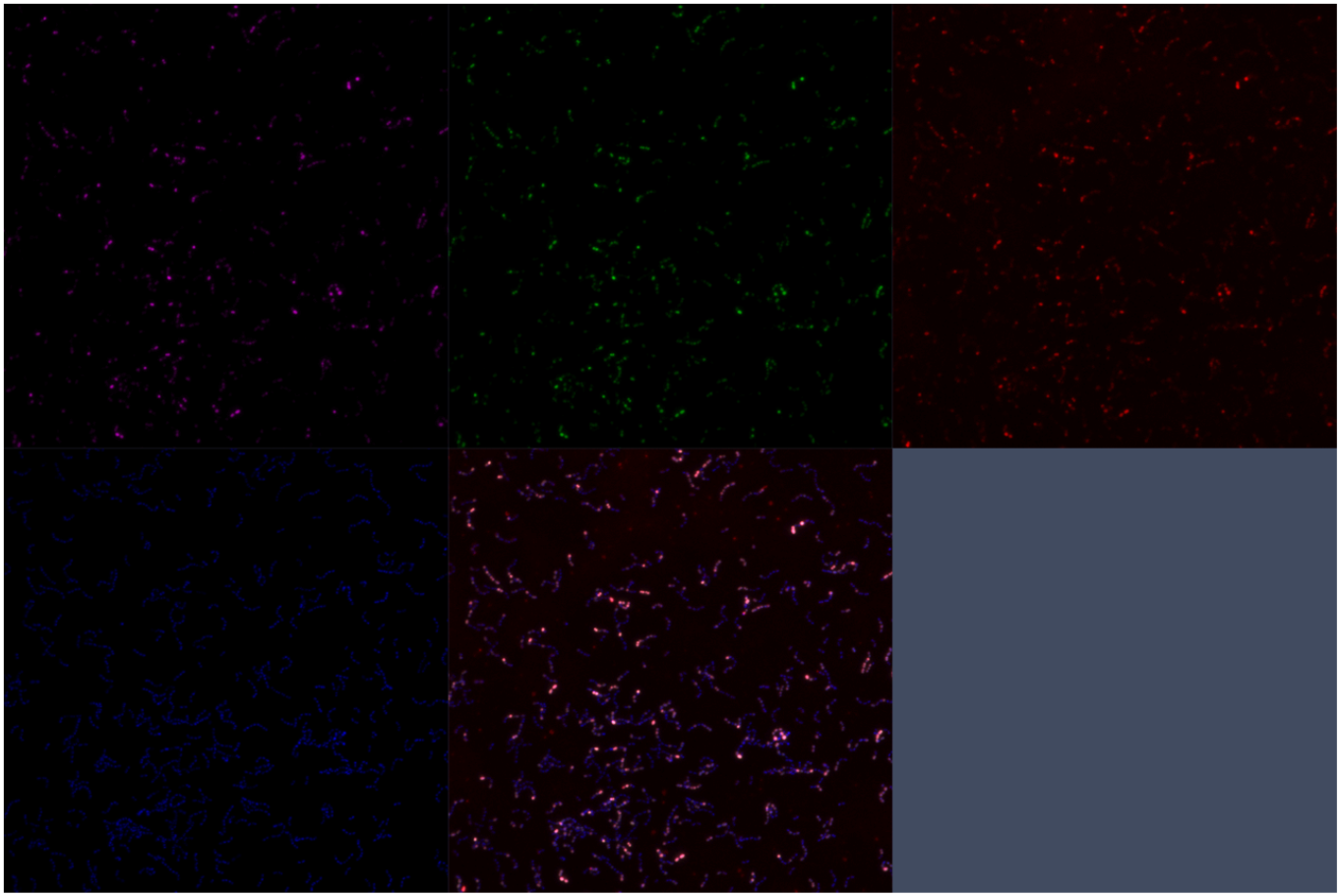
T=4

VA1128


Sypro Ruby (Proteins)
TOTO-1 Iodide (eDNA)
WGA (Carbohydrates)
Hoechst
Merge

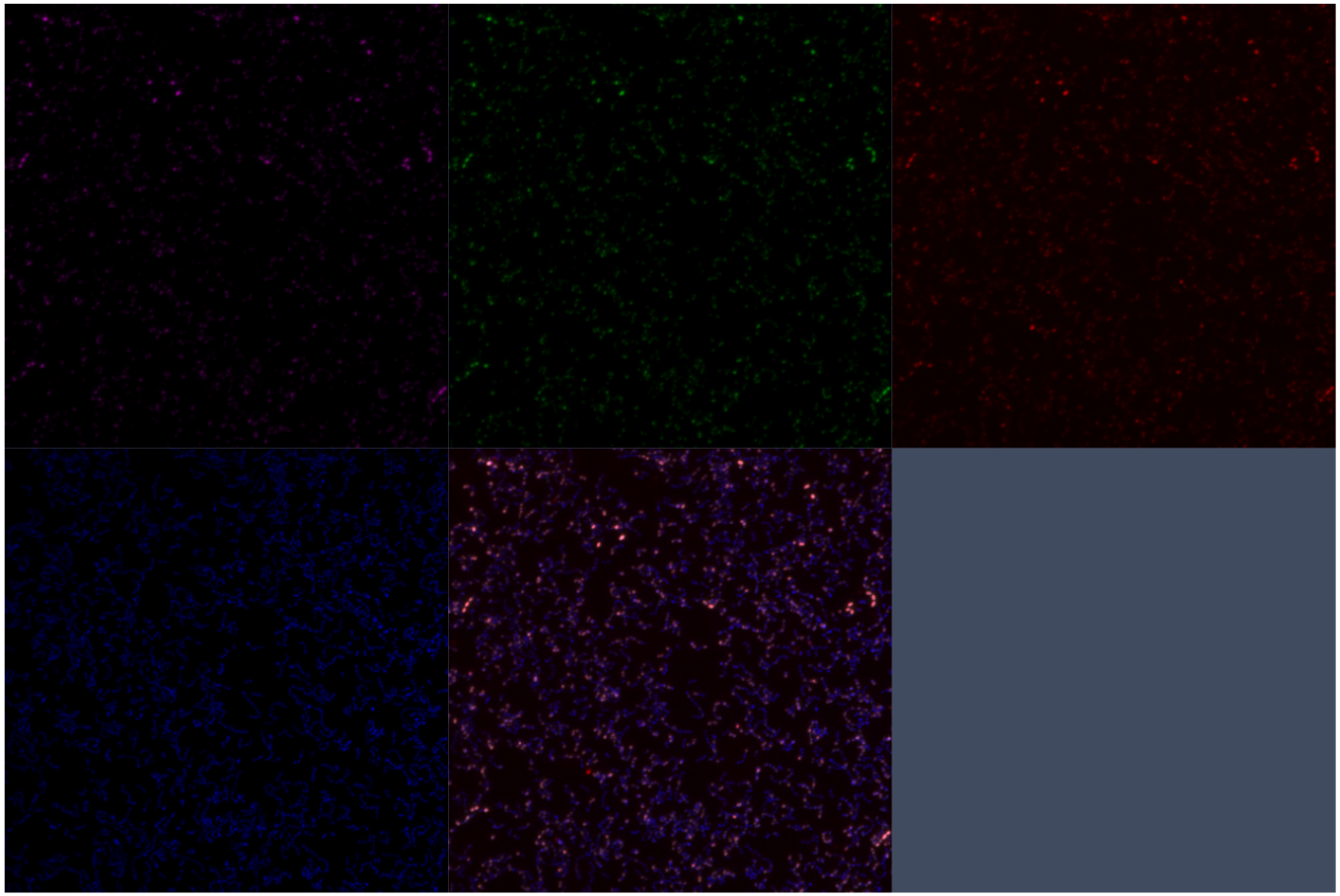
T=8

VA1128


Sypro Ruby (Proteins)
TOTO-1 Iodide (eDNA)
WGA (Carbohydrates)
Hoechst
Merge

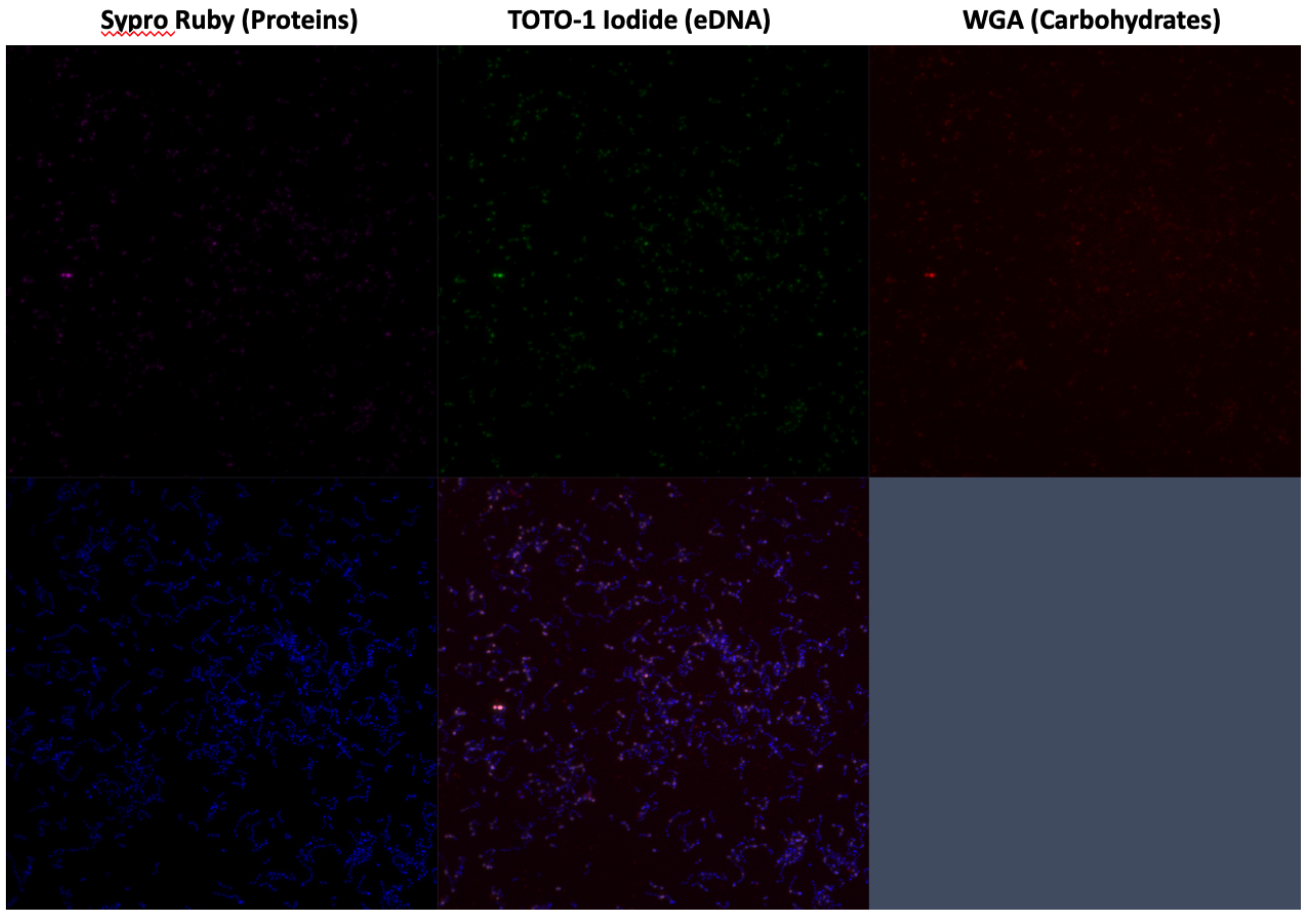
T=24

VA1128


Sypro Ruby (Proteins)
TOTO-1 Iodide (eDNA)
WGA (Carbohydrates)
Hoechst
Merge

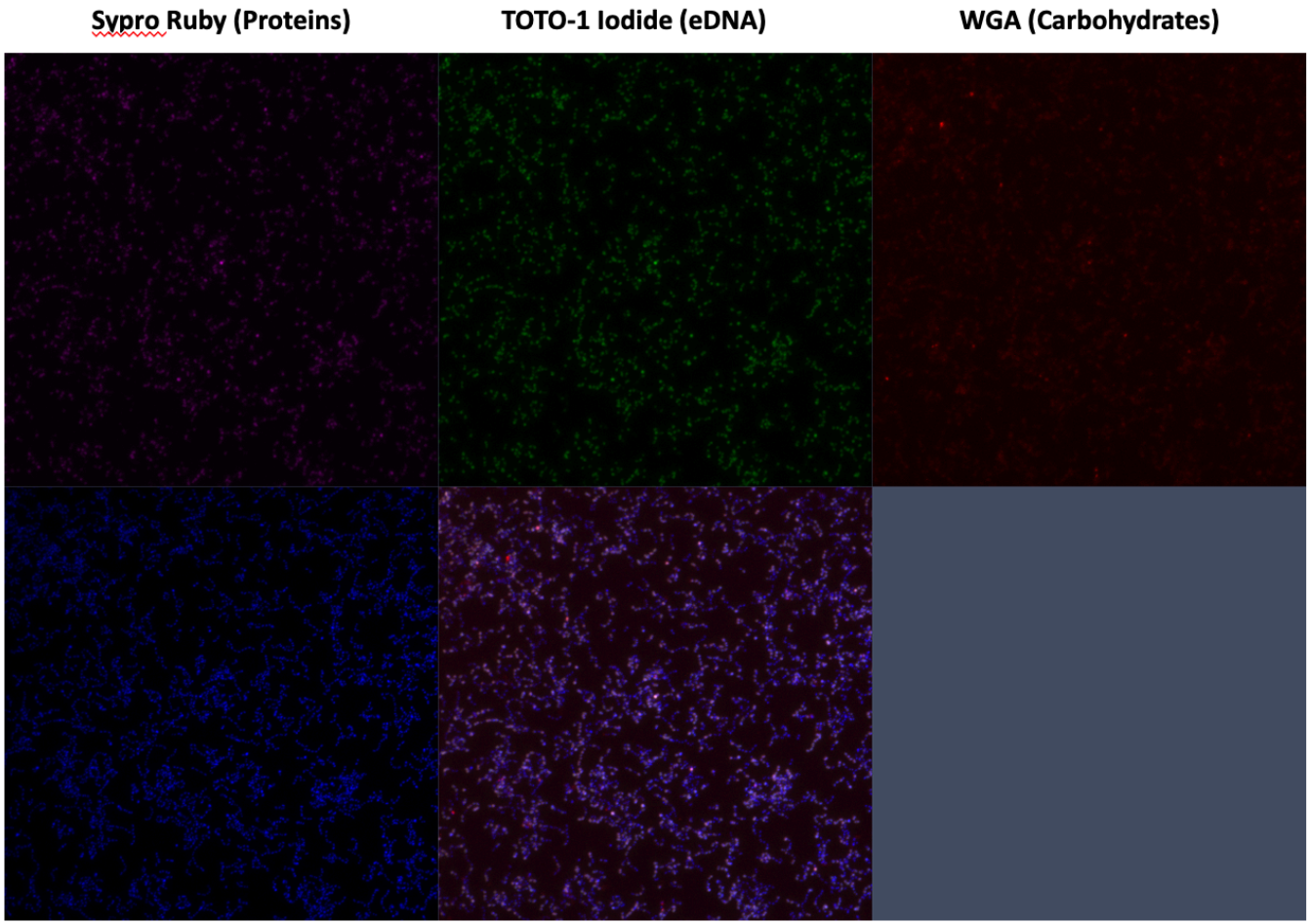
T=32

VA1128


Sypro Ruby (Proteins)
TOTO-1 Iodide (eDNA)
WGA (Carbohydrates)
Hoechst
Merge

T=48

VA1128


Sypro Ruby (Proteins)
TOTO-1 Iodide (eDNA)
WGA (Carbohydrates)
Hoechst
Merge

strains had co-localized matrix molecules at the base and were covered by several layers of cells; the presence of macromolecules was minimal in the upper layers. Matrix macromolecules were more homogenously spread throughout the biofilms of both strains by 48 hours. Lastly, in agreement with the images in Fig. 15, these images indicate that VA1128 formed biofilms more rapidly than OG1RF: VA1128 biofilms had towers of cells and macromolecular components by 8 hours, while similar structures did not begin to appear in OG1RF biofilms until 24 hours (Fig. 17). VA1128 biofilms also appeared denser than OG1RF biofilms at the last time point.

DISCUSSION

Biofilm formation is well established as an important aspect of many enterococcal diseases, including endocarditis (108), urinary tract infection (143), and endodontic failure (50; 52). Strains that cause these diseases are genetically diverse (107); however, it is unknown if biofilms produced by such strains are biologically equivalent, or if they display variation in characteristics such as biomass accumulation kinetics or matrix macromolecular composition. Given the high resistance of enterococcal biofilms to traditional antibiotics (137), novel therapies are needed for the treatment of biofilm-associated infections caused by *E. faecalis*. If each biofilm has unique properties, then efforts to develop new therapies may need to take such variations into account. In this study, we characterized how five *E. faecalis* strains from four distinct MLST groups form biofilms in diverse growth conditions. We also studied the biofilm matrix composition of each strain through enzymatic and chemical assays, and of two of the strains through

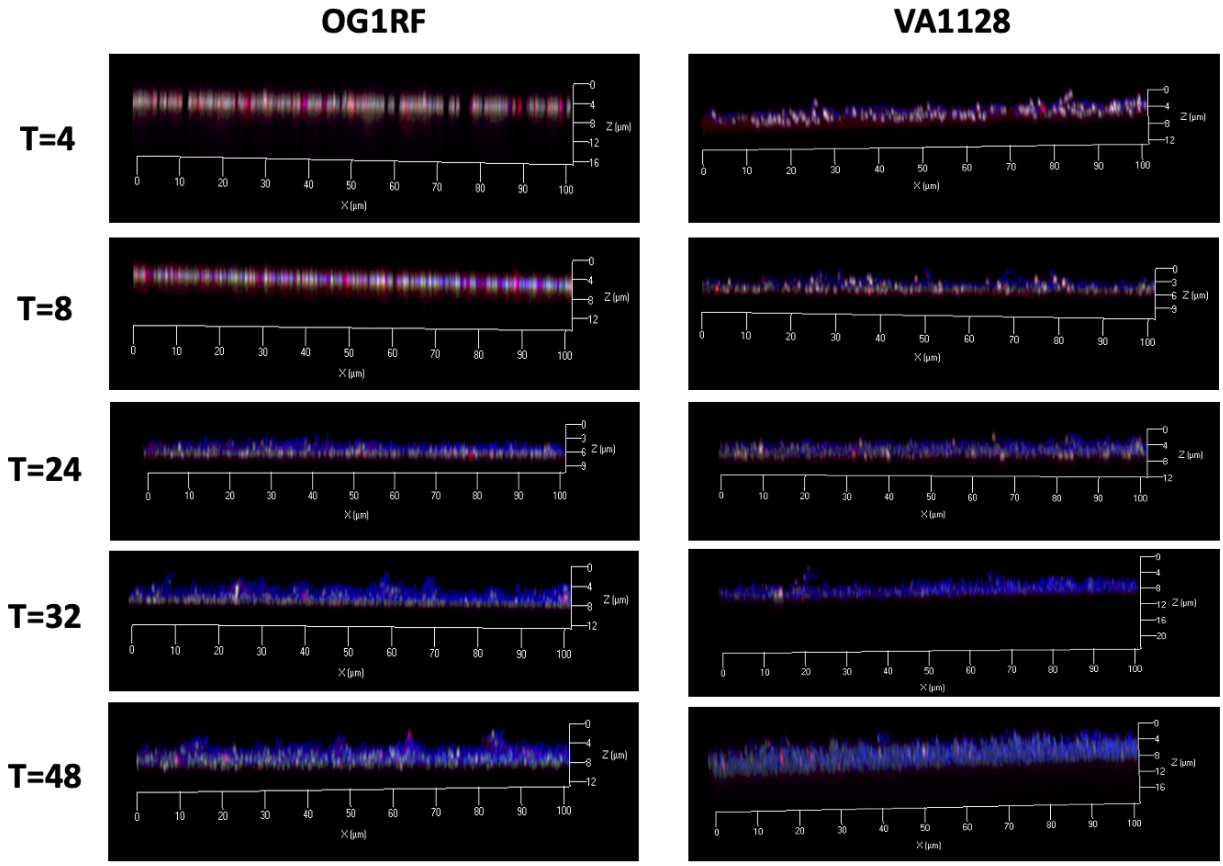


Figure 17. Time-course XZ-plane visualization of biofilm matrix development of *E. faecalis* strains OG1RF and VA1128

XZ-plane profiles corresponding to the individual biofilm images in Figure 15 are shown for each strain.

biochemical analysis and fluorescence microscopy. Our results demonstrate that differences exist in biofilm properties among *E. faecalis* strains, that these properties are contingent on environmental growth conditions, and that variation can exist in biofilm properties of a single strain of *E. faecalis* across diverse growth conditions. In addition, this work suggests that *E. faecalis* biofilm matrices are composed of multiple types of macromolecules.

Numerous studies have explored how environmental conditions affect *E. faecalis* biofilm formation (23; 94; 102; 142; 159). A previous report on OG1RF, MMH594, and V583 biofilms formed in TSB^{-D} medium showed that, while all three strains followed similar biofilm formation kinetics, OG1RF produced substantially more biomass than the other two strains as early as four hours (94). In this work, we replicated these previously reported results and show that the biofilm formation kinetics of strains DS16 and VA1128 are similar to the other strains (Fig. 3B). In addition, DS16 and VA1128 accumulate more biofilm biomass than MMH594 and V583 at later time points (Fig. 3B). Unexpectedly, the addition of 10% heat-inactivated FBS to TSB^{-D} completely suppressed biofilm biomass accumulation, despite supporting cell growth for all five strains (Figs. 3E and F). Further, only VA1128 formed biofilms when grown in tissue culture medium (DMEM containing 10% heat-inactivated FBS; Fig. 3H). In fact, growth in tissue culture medium resulted in the greatest amount of biofilm biomass accumulation observed for VA1128 across the three medium types assayed (Fig. 4). A low molecular weight, non-proteinaceous component of both human serum and FBS was reported to inhibit biofilm formation of two *Staphylococcus aureus* strains (1). Similarly, serum has also been shown to inhibit biofilms of *Pseudomonas aeruginosa* and *Candida albicans* (45; 73).

This is the first description of serum negatively affecting *E. faecalis* biofilm formation, as previous work established that horse serum induces enterococcal cell wall adhesins that bind to host extracellular matrix molecules (115; 116).

The mechanism of the observed biofilm inhibition by serum (Fig. 3F), and why tissue culture medium strongly induced strain VA1128 biofilm formation (Fig. 3H), is presently unclear. FBS is a complex mix containing undefined amounts of bovine serum albumin, growth factors, other proteins, and nutrients such as sugars, amino acids, and lipids. The DMEM used for the experiments in Fig. 3 contained defined amounts of inorganic salts, amino acids, phenol red, sodium pyruvate, vitamins, and D-glucose (4.5 g/L). The presence of glucose contributed by the DMEM in the tissue culture medium biofilm condition provides one plausible explanation for the enhanced biofilm formation phenotype of VA1128 that was absent from the other strains (Fig. 3H). It has been shown that *E. faecalis* forms biofilms with greater biomass in the presence of increased glucose levels (102; 142); however, this property is dependent on strain genetic background (102). Additional experiments will be necessary to identify the nature of the *E. faecalis* biofilm-suppressing component(s) in FBS and to test the hypothesis that serum-specific biofilm inhibition can be overcome in VA1128 by exposure to glucose.

Biofilm matrices are known to consist of diverse biochemical macromolecules, and matrix composition can differ between bacterial genera and even across species (57; 58). Treatment of pre-formed biofilms with a single degradative agent (i.e., Proteinase K, sodium (meta)periodate, or DNase, depending on species or strain) has been shown to have moderate to profound dispersal effects on strains of *Staphylococcus epidermidis* and *Staphylococcus lugdunensis* (25; 61), *Staphylococcus haemolyticus* (62), *Helicobacter*

pylori (177), [*Pasteurella*] *pneumotropica* biotypes Jawetz and Heyl (135), and *Actinobacillus pleuropneumoniae* (88). In comparison, for the five *E. faecalis* strains tested in this work, the only effect we observed from single treatment with any of the disruption compounds was a partial reduction of VA1128 biofilm biomass with Proteinase K (Figs. 5 and 6). These observations suggest that the biofilm matrices of the five *E. faecalis* strains in this study are (1) heterogeneous and (2) may be more complex than the biofilm matrices of many other bacterial genera. Both of these hypotheses are supported by the data in Figs. 8 and 9, which demonstrated partial disruption of OG1RF and DS16 by sequential treatment of sodium metaperiodate followed by DNase but not the reverse combination. Figs. 14, 15, 16, and 17 support the conclusion that the *E. faecalis* strains we have studied contain more than one major macromolecular biofilm matrix component, and the macromolecules may be interwoven in a manner that prevented any one of the degradative enzymes from showing any dramatic effects. Enterococcal biofilm matrices may also contain macromolecules that are not targeted by Proteinase K, sodium (meta)periodate, or DNase.

Previous *in vitro* and *in vivo* microscopy studies determined that OG1RF forms biofilms on a substratum as discrete microcolonies of diplococci, rather than as chains, clumps, or confluent monolayers of cells (16; 38; 39; 54), and our results are consistent with this (Fig. 12). In contrast, VA1128 biofilms formed by the attachment of long chains of cells to the growth surface (Fig. 12). Compared to OG1RF, VA1128 biofilms were thicker, covered more area, and occupied a greater volume (Fig. 13). Fluorescent labeling of various matrix macromolecules revealed that distribution patterns of matrix molecules between OG1RF and VA1128 were significantly divergent at early time points but

became more similar as the biofilms matured (Fig. 17). eDNA in the extracellular matrix of OG1RF is surrounded by another matrix component, likely a protein or lipoprotein (15). Our images revealed fluorescently-labeled eDNA almost exclusively co-localized with Sypro Ruby-labeled proteins in both OG1RF and VA1128 (Figs. 15 and 16), which provides additional evidence that the previously unknown biofilm matrix component found with the eDNA is proteinaceous.

In conclusion, our findings demonstrate that genetically distinct *E. faecalis* strains can display varied biofilm phenotypes under the same environmental conditions, and the same *E. faecalis* strain can display distinct biofilm phenotypes under diverse environmental conditions. The mechanisms driving the observed heterogeneity of biofilm formation phenotypes among *E. faecalis* strains from different genetic backgrounds remains to be determined. Overall, these data suggest that successful treatment for enterococcal diseases complicated by biofilm formation may need to be tailored to the biofilm strain being treated. Finally, this work emphasizes the importance of measuring biofilm formation through more rigorous means when studying the contribution of biofilms in *E. faecalis* disease.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions.

The *E. faecalis* strains used in this study are listed in Table 1. Strains were streaked from stocks stored at -80°C in 40% (v/v) glycerol prior to each replicate experiment. Strains were cultivated in brain heart infusion broth (BHI; BD Bacto, Becton, Dickinson and Company, Sparks, MD) or on BHI agar under static conditions at

37°C in ambient air. For biofilm assays, unless otherwise noted, bacteria grown overnight in BHI were diluted in tryptic soy broth without added dextrose (TSB^{-D}; BD Bacto); biofilms were grown at 37°C in ambient air. In specific biofilm experiments, bacteria grown overnight in BHI were diluted in TSB^{-D} diluted to 90% in sterile water [90% TSB^{-D}], TSB^{-D} containing 10% heat-inactivated fetal bovine serum (FBS; VWR International, LLC, Radnor, PA) [90% TSB^{-D} + 10% FBS], or DMEM without L-glutamine (Quality Biological, Gaithersburg, MD) containing 10% heat-inactivated FBS and 1% 0.2 M L-glutamine (Quality Biological), referred to hereafter as tissue culture medium. FBS was heat inactivated by incubating 50 ml frozen aliquots at 55°C for 1 h.

Multi-locus sequence typing.

The DNeasy blood and tissue kit (Qiagen, Germantown, MD) was used to extract genomic DNA from overnight broth cultures of strains DS16 and VA1128. Regions of genes *aroE*, *gdh*, *gki*, *gyd*, *pstS*, *xpt*, and *yqiL* were PCR amplified with PfuUltra II Fusion HotStart DNA Polymerase (Agilent, Santa Clara, CA) using oligonucleotides (Thermo Fisher Scientific, Waltham, MA) previously described for *E. faecalis* MLST (107). Bidirectional Sanger sequences of the PCR products were obtained from Eurofins Genomics (Louisville, KY). Sequence files were assembled and analyzed with Geneious Prime software (v. 2020.2.4; Biomatters, Inc., San Diego, CA). Allelic profiles and sequence types were assigned by searching the *Enterococcus faecalis* typing database maintained by PubMLST (https://pubmlst.org/organisms/enterococcus-faecalis).

Microtiter plate biofilm assay.

Quantitative microtiter plate biofilm assays were performed as described previously, with minor changes (60). Briefly, each strain was streaked for isolated

colonies onto BHI agar and incubated at 37°C overnight. 2 ml BHI broth were inoculated with three colonies of each strain and incubated at 37°C overnight. These cultures were diluted 1:100 in TSB-D, and 0.1 ml of each diluted strain was pipetted into eight replicate wells of a 96-well tissue culture treated flat-bottom polystyrene plate (Costar, Corning Inc., Corning, NY). Wells with sterile medium were used as blanks. Plates were placed in a sealed plastic container lined with damp paper towels and statically incubated at 37°C for the time indicated in the figure legends. Culture turbidity was measured following incubation by reading the optical density at 600 nm (OD600) on a Synergy HTX multi-mode reader (BioTek, Winooski, VT). The medium was removed, wells were washed 3 times with 0.1 ml 10 mM potassium phosphate-buffered saline (KPBS), and plates were dried for at least two hr at room temperature. Wells were stained with 0.1 ml of 0.1% safranin for 10 min. The stain was removed, wells were washed five times with 0.1 ml KPBS, and plates were dried for at least two hr at room temperature. The safranin stain was quantified by reading the optical density of the dried wells at 450 nm (OD450). Technical replicates in each experiment were averaged, and the mean OD450 of three biological replicates is reported.

Enzymatic and chemical biofilm dispersal assays.

Pre-formed microtiter plate biofilms were treated as described below with varying concentrations of Proteinase K (Qiagen), Deoxyribonuclease I from bovine pancreas (DNase, Sigma-Aldrich, St. Louis, MO), or sodium (meta)periodate (Sigma-Aldrich) for varying durations. Treatment concentrations are indicated in the figure legends. For DNase, 10 mg/ml stock solutions of DNase were prepared in 0.15 M NaCl; aliquots were stored frozen at -20°C and used for up to one week. DNase treatment solutions were

prepared with DNase buffer (10 mM Tris HCl pH 7.5, 2.5 mM MgCl2, 0.5 mM CaCl2). Proteinase K treatment solutions were prepared in KPBS. Sodium (meta)periodate treatment solutions were prepared with sterile water. Biofilms were grown for 24 hr and washed as described above. A 0.1 ml aliquot of treatment solution or corresponding buffer was pipetted into each well containing a washed biofilm. Empty wells filled with 0.1 ml of treatment solution or corresponding buffer served as blanks. For single treatments, plates were incubated at 37°C in ambient air for the time indicated in the figure legends. For consecutive treatments, plates containing the first treatment were incubated at 37°C in ambient air for 2 hr then washed 3 times with 0.1 ml KPBS prior to the application of the subsequent treatment, which was also incubated at 37°C for 2 hr. Following treatment, the treatment solutions and buffers were removed, and the wells were washed, dried, stained, and assayed to quantify the remaining biofilm biomass as described above. Technical replicates in each experiment were averaged, and the mean OD450 of three biological replicates is reported.

Biofilm formation in the presence of Proteinase K, DNase, or sodium (meta)periodate.

Biofilms were prepared as described above in the microtiter plate biofilm quantification section with the following changes: (1) Three colonies of each strain were grown in 3 ml BHI broth overnight. (2) Cultures were diluted 1:100 in TSB^{-D} containing 1000 U/ml DNase, 400 μg/ml sodium (meta)periodate, or 400 μg/ml Proteinase K in control TSB^{-D} containing the equivalent amount of the appropriate corresponding buffer. Blanks lacking bacteria were prepared in TSB^{-D} with the equivalent amounts of enzyme or chemical or appropriate corresponding buffers. (3) Plates were incubated for 24 hours.

Aclar membrane biofilms.

Biofilms were grown on Aclar fluoropolymer membranes (Aclar embedding film, 7.8-mil thickness; Electron Microscopy Sciences, Hatfield, PA) as described previously, with minor changes (59). For each strain, 2 ml of BHI broth was inoculated with 3 colonies and incubated at 37°C overnight. These cultures were diluted 1:100 in TSB^{-D}, and 3 ml of each diluted strain was pipetted into a well of a 6-well plate (Costar, tissue culture treated, Corning Inc.). Sterile 11-mm-diameter Aclar membranes were placed at the bottom of each well. Plates were placed on an orbital shaker at 120 rpm and incubated at 37°C for the time indicated in the figure legends. The medium was replaced at 8, 24, and 32 h. Following incubation, membranes were removed from each well and washed by dipping consecutively into 3 wells of a 24-well flat-bottom polystyrene plate (Nunclon Delta Surface; Thermo Fisher Scientific) filled with 2 ml KPBS. No more than 3 membranes were washed in one set of 3 wells. Biofilm membranes were processed for the following, as described further below: (1) biofilm biomass quantification by crystal violet staining, (2) CFU enumeration, (3) biochemical analysis of detached biofilm matrix macromolecules, and (4) confocal microscopy.

Aclar membrane biofilm biomass quantification by crystal violet staining.

Aclar membrane biofilms were stained with crystal violet, and biofilm biomass was measured as described previously, with minor changes (59). Briefly, washed membranes were placed, biofilm-side up, into individual wells of a new 24-well flat-bottom polystyrene plate to dry for at least 30 min in a biosafety cabinet. Membranes were stained with 0.5 ml 0.1% crystal violet for 30 min, washed in KPBS as described previously (no more than 1 membrane was washed in 1 set of 3 wells), and placed into

separate unused wells of the 24-well plate and dried for at least 30 min in a biosafety cabinet. Crystal violet stain was solubilized by soaking membranes in 0.5 ml 95% ethanol for 20 min while gently agitating on a Nutating Mixer (VWR International). Duplicate 0.2 ml aliquots of the crystal violet-ethanol solution were removed from each well, placed into individual wells of a 96-well flat-bottom polystyrene plate (Costar, tissue culture treated, Corning Inc.), and quantified at 595 nm (OD595) on a plate reader. The duplicate aliquots for each biological replicate were averaged, and the mean OD595 of three biological replicates is reported.

CFU enumeration of Aclar membrane biofilms.

Cell viability of Aclar membrane biofilms was measured as described previously, with minor changes (59). Briefly, washed membranes were placed into 2-ml tubes filled with 0.5 ml KPBS. Tubes were placed into a rack that was clamped securely on a multi-tube vortexer (DVX-2500, VWR International) and vortexed for 6 minutes at speed 1000 at room temperature. Following vortexing, 0.1 ml aliquots were removed from each tube, and 10-fold serial dilutions were prepared and plated on BHI agar plates. Colony forming units (CFU) were counted after overnight incubation at 37°C, and CFU/ml was calculated.

Biochemical quantification of protein, carbohydrates, and eDNA dislodged from biofilm matrices.

The concentrations of protein, carbohydrate, and eDNA dislodged from biofilm matrices were measured as described previously, with minor changes (4; 156). Biofilms grown on Aclar membranes were washed and placed into 2 ml tubes filled with 0.75 ml KPBS for protein and carbohydrate analysis, and into 2 ml tubes filled with 1 ml KPBS

for eDNA analysis. Tubes were placed into a rack that was clamped securely on a multi-tube vortexer and vortexed for 6 min at speed 1000 at room temperature to detach biofilms from the membranes into KPBS. Following vortexing, the biofilm matrix samples for protein and carbohydrate analysis were fixed at a concentration of 2% paraformaldehyde overnight at room temperature and kept at 4°C until analyzed, and the biofilm matrix samples for eDNA analysis were kept frozen until analyzed. Prior to analysis, samples were filtered through 0.2 μm syringe filters (polyethersulfone membrane; Whatman Uniflo, Cytiva Life Sciences, Marlborough, MA). Aliquots of the filtered samples were analyzed with the Total Carbohydrate Assay Kit (Sigma-Aldrich), Micro BCA Protein Assay Kit (Thermo Fisher Scientific), and Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturers' instructions.

Preparation of glass slides for confocal microscopy.

12.8-mm-diameter holes were punched from double-sided SecureSeal adhesive (Electron Microscopy Sciences; 7.25"x10", 0.24 mm thick; Hatfield, PA), forming wells for placement of the 11-mm-diameter Aclar membranes. Squares of the punched adhesive were affixed to the center of each glass slide.

Confocal microscopy of Aclar membrane biofilms.

Washed Aclar membrane biofilms were placed, biofilm-side up, into individual wells of a 24-well flat-bottom polystyrene plate and stained with Filmtracer LIVE/DEAD Biofilm Viability Kit (Invitrogen, Thermo Fisher Scientific) or a combination of four dyes to stain individual cells and components of the biofilm matrices. All staining incubations were performed in the dark. For LIVE/DEAD staining, membranes were

stained according to the manufacturer's directions with 0.25 ml of staining solution for 15 min. For individual cell and biofilm matrix staining, membranes were stained first with FilmTracer SYPRO Ruby Biofilm Matrix Stain (Invitrogen, Thermo Fisher Scientific) for 20 min, then with TOTO-1 Iodide (Invitrogen, Thermo Fisher Scientific) for three min, then with Wheat Germ Agglutinin - Alexa Fluor 647 Conjugate (Invitrogen, Thermo Fisher Scientific) for 15 min, and then with Hoechst 33342 Solution (Invitrogen, Thermo Fisher Scientific) for five min. Membranes were washed with sterile water between each dye. Stained, washed membranes were placed into separate unused wells of the 24-well plate and fixed with 1 ml of 2% paraformaldehyde overnight in the dark at room temperature. Membranes were removed from each well, washed as described above, and placed onto prepared glass slides (see above). A single drop of ProLong Diamond Antifade Mountant (Invitrogen, Thermo Fisher Scientific) was placed into the center of each membrane, and 22x22mm glass coverslips (Erie Scientific LLC, Portsmouth, NH) were affixed to the SecureSeal adhesive surrounding the membranes. Slides were placed at room temperature in the dark overnight. Each side of the coverslips was sealed with clear nail polish and placed at 4°C for at least 1 hour before viewing on a Zeiss 700 confocal laser microscope with a 63X 1.4-numerical-aperture objective housed in the Biomedical Instrumentation Center at Uniformed Services University (Carl Zeiss AG, Oberkochen, Germany). Three representative images from separate, randomly chosen fields of each biofilm were obtained. Images were merged and compressed z-stacks were obtained (Zeiss Zen 3.2 black edition imaging software).

Statistical analysis.

Statistical analyses were conducted using GraphPad Prism (version 8.0.0; GraphPad Software, Inc., La Jolla, CA). A 2-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons was used to identify differences in biofilm formation kinetics between strains at each time point, in growth conditions at each time point for VA1128, and in biofilm biomass between different treatment conditions in the enzymatic and chemical biofilm dispersal assays with single treatments. A 2-way ANOVA with Sidak's correction for multiple comparisons was used to identify differences in biofilm biomass between different treatment conditions in enzymatic and chemical biofilm dispersal assays with consecutive treatments.

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CHAPTER 3: Discussion

The studies described in this thesis established that heterogeneity in biofilm properties exists among *E. faecalis* strains from distinct genetic backgrounds. This was observed among the five strains tested in biofilm biomass accumulation differences when grown in various media conditions and in biofilm dispersion differences after treatment with several agents. Differences in biofilm formation patterns for OG1RF and VA1128 in the presence of shear force, and differences in biofilm development and matrix composition of OG1RF and VA1128 when characterized by fluorescence microscopy, were also observed. Biofilm biomass accumulation was delayed or completely impeded for all strains except VA1128 in tissue culture medium (Fig. 3H). Biofilm dispersal assays indicated that eDNA and polysaccharides are components of the OG1RF and DS16 biofilm matrices (Fig. 9). When grown under shear forces on Aclar membranes, OG1RF and VA1128 displayed differences in biofilm formation kinetics and biomass accumulation (Fig. 10). Finally, fluorescence confocal microscopy revealed how OG1RF and VA1128 form biofilms with distinct kinetics, architecture, and staining patterns of specific macromolecules over a 48 hour time course (Figs. 12, 13, 15, 16, and 17).

Furthermore, these studies contribute a number of observations that broadly encompass the biofilm formation properties of *E. faecalis*. Our data show biofilm formation heterogeneity for single strains in different growth conditions (Figs. 3 and 4). Specifically, the presence of 10% FBS in TSB^{-D} resulted in nearly no biofilm development for all five strains, which contrasted with all five strains developing biofilms in TSB^{-D} alone (Fig. 3F *vs*. 3B). Notably, VA1128 formed biofilms with

different kinetics and biofilm biomass accumulation in three of the four media conditions tested (Fig. 4B). Additionally, OG1RF and VA1128 displayed different biofilm formation properties when grown on polystyrene under static conditions in TSB^{-D} (Fig. 3B) compared to when grown on Aclar under shear forces in TSB^{-D} (Fig. 10A and B), which further demonstrated how a single *E. faecalis* strain can have differential biofilm formation properties under different growth conditions. The biofilm dispersal assays broadly demonstrated that all five strains' matrices are likely differentially comprised of a complex composite of macromolecules (Figs. 5, 6, 8, and 9), and biochemical quantification and fluorescence microscopy confirmed the presence of protein, carbohydrates, and eDNA in the biofilm matrices of two of the five strains (Figs. 14, 15, 16, and 17). In the biofilm dispersal assays, treatment with one dispersal agent alone did not completely disrupt any of the strains' biofilms (Fig. 5 and 6), which contrasts with other bacterial genera that lose complete or near-complete biofilm integrity after treatment with a single dispersal agent (25; 61; 62; 88; 135; 177). Fluorescence microscopy showed that a dye corresponding to carbohydrates diffusely stained both OG1RF and VA1128 biofilms, while dyes corresponding to proteins and eDNA frequently co-localized and were observed in punctate forms in the strains' biofilms (Fig. 15 and 16), supporting the results of the biofilm dispersal assays and the conclusion that the biofilm matrix composition of *E. faecalis* is complex.

A recent study looking at clinical and laboratory strains of *E. faecalis* demonstrated that strains accumulate varying amounts of biofilm biomass in media with increasing concentrations of glucose or sucrose (91). Specifically, OG1RF accumulated more biofilm biomass than the type strain ATCC 29212 in media supplemented with 1%

glucose or 0.5% and 1% sucrose, and three clinical *E. faecalis* isolates accumulated more biofilm biomass than OG1RF and ATCC 29212 in both 0.5 and 1% sucrose-supplemented media (91). An older study similarly demonstrated that strains accumulate differing amounts of biofilm biomass in disparate media conditions: strains MMH594 and V583 accumulated less biofilm biomass than OG1RF over 48 hours in M17 and THYE media, and also, more notably so, in TSB (94). In line with these studies, we demonstrated that distinct *E. faecalis* strains can accumulate varying amounts of biomass in TSB^{-D} and tissue culture medium, as we observed MMH594 and V583 to accumulate less biofilm biomass than OG1RF and DS16 in TSB^{-D} and VA1128 to accumulate vastly more biofilm biomass than the other four strains in tissue culture medium (Fig. 3B and H). Together, these data suggest that different *E. faecalis* strains cannot be assumed to share similar biofilm formation properties.

If one is to assume the biofilm matrix of enterococcal species is similar in composition to that of other bacterial species' biofilms, for which this information has been much more well-defined, then enterococcal biofilm matrix composition likely consists of mostly polysaccharides, protein, and eDNA (40; 57; 99; 175). In 2004, a study suggested an important role for polysaccharides in enterococcal biofilm formation and biofilm matrix structure (54). Later work defined a role for eDNA in enterococcal biofilm function and structure (15; 70; 163). That eDNA contributes to enterococcal biofilm composition is one of the few pieces that has been more so fleshed out about enterococcal biofilm matrix composition, and has been cited as so (24; 52). These data from the first decade of the present century have provided a starting point for understanding the composition of enterococcal biofilm matrices, yet also highlight how much is still to be

understood about this important subject. According to Dunny et al. in 2014, "a detailed biochemical characterization of the extracellular matrix of an enterococcal biofilm has not been presented" (52). Since then, some studies have made advances toward this: a 2019 study looked at the influence of substrate surface conditioning and biofilm age on the composition of *E. faecalis* biofilms, a separate 2019 study isolated components of *E. faecalis* biofilm matrices to test their inflammatory potential, and a 2017 study tested the effects of alginate lyase and DNase I in conjunction with vancomycin on biofilms of clinical *E. faecalis* isolates (4; 132; 169). Although these studies provide more information than the relative dearth of knowledge that existed in 2014 of the biochemical composition of enterococcal species, the overall picture of what enterococcal biofilm matrices consist of still remains mostly unknown.

To address this lack of understanding, we began by treating biofilms of the five *E. faecalis* strains with reagents that broadly target polysaccharides (sodium (meta)periodate), protein (Proteinase K), and eDNA (DNase I) to observe which of these treatments may disperse a strain's biofilm, thereby indicating a likely important component of that strain's biofilm matrix. For all conditions, treatment with one of these reagents alone either did not disperse any of the strains' biofilms, or did so incompletely (Fig. 5 and 6). Additionally, biofilm formation in the presence of any one of these reagents alone had no effect on the strains (Fig. 7). One possible explanation for the Proteinase K treatment results is that the biofilm matrix proteins made by these strains could be resistant to this specific protease. This would be pertinent to test in future studies by conducting the same experiment with alternate proteases. Another, and arguably more likely possibility, is that these strains' biofilms are more complex and thus

more than one major group of macromolecules is crucial to the function and structure of the biofilm matrix. Previous studies demonstrated that biofilms of both Gram-positive and Gram-negative bacteria could be dispersed entirely by a single dispersal agent (25; 61; 62; 88; 135; 177), which provided a suggestion that the same may be the case for the *E. faecalis* strains in these studies; yet general biofilm knowledge indicates how bacterial biofilms are often more complex than this (56-58). In fact, this possibility would be congruent with the little information that is known about the composition of enterococcal biofilm matrices, in which eDNA (15; 70; 163) and possibly polysaccharides (4; 54; 169) and proteins (4; 85; 96) are crucial components.

Upon performing varying sequential treatments with permutations of the three reagents, we observed that sodium (meta)periodate and DNase significantly dispersed OG1RF and DS16 biofilms (Fig. 9). This may indicate that eDNA and carbohydrates provide the structural stability of these strains' biofilms, and that without these components, the biofilms, at least partially, fall apart. Furthermore, since significant dispersal was only observed upon sequence-specific treatment, in which dispersal was only observed upon the specific treatment order of sodium (meta)periodate followed by DNase, this may specifically indicate that eDNA serves as the primary component for the structural integrity of these strains' biofilms and that carbohydrates serve more of an auxiliary role, such as a protective function to protect against potential digestion of the eDNA. In fact, a recent study by Jennings et al. established evidence in *P. aeruginosa* of Pel, one of the three major exopolysaccharides found in the biofilm matrices of these bacteria, and eDNA interactions within *P. aeruginosa* biofilms found in the sputum of CF patients (86). In addition to this, they demonstrated that the interactions between the

cationic Pel and anionic eDNA provided protection for the eDNA against nuclease digestion (86), which is consistent with our hypothesis that carbohydrates protect eDNA in *E. faecalis* biofilm matrices. Although the authors of this work show that *P. aeruginosa* biofilm formation and integrity is dependent on exopolysaccharides, they suggest that other components, such as eDNA, may also be vital to the stability of these biofilms (86). Additionally, this study is not alone in providing evidence for the role of polysaccharide-eDNA complexes within biofilm matrices for giving structure and stability to bacterial biofilms. A 2015 study showed that interactions between Psl, one of the other major exopolysaccharides found within *P. aeruginosa* biofilm matrices, and eDNA created what they termed "a biofilm skeleton," providing structural backing for *P. aeruginosa* biofilms (173); and a 2020 study showed that exopolysaccharide-eDNA interactions defined the three-dimensional architecture of *Bacillus subtilis* biofilms, particularly in the early stages of biofilm formation (124).

In light of this, it is important to remember that these bacterial species are not closely related to *E. faecalis* and that the interactions between these biomolecular components may be completely different in *E. faecalis* than these other bacterial species. Despite this, these initial observations from our study certainly elicit further experimentation to potentially understand what may be an important relationship between carbohydrates and eDNA within *E. faecalis* biofilm matrices. It is also important to note that the biofilms of OG1RF and DS16 still did not completely disperse after this dual treatment or after sequential treatment with all three reagents. This displays the complexity of these strains' biofilms and raises the question, what really is integral to the composition and integrity of these strains' biofilms? Furthermore, the other three strains'

biofilms remained intact after these sequential treatment experiments, further adding to the conclusion of the complexity of *E. faecalis* biofilm composition. Overall, these data additionally demonstrate how it cannot be assumed that distinct *E. faecalis* strains share similar biofilm formation properties.

It is clear from the breadth of enterococcal biofilm research over the past couple decades that the conclusions made from the experiments encapsulating this work can vary greatly on how biofilm formation is quantified (52; 100). As such, it is best practice to measure biofilm formation by more than one method. Therefore, in this work, we measured biofilm composition with multiple methods. In addition to enzymatic and chemical treatment assays to determine what major class of macromolecules is most important to the integrity of the strains' biofilms, we also used biochemical assays to directly quantify protein, carbohydrate, and eDNA concentrations in the matrices of OG1RF and VA1128 biofilms. As mentioned, prior studies have demonstrated a role for eDNA in biofilm development for OG1RF (15; 17; 70; 162) and V583 (163). Our findings here are consistent with the conclusions of these prior studies, as we quantified detectable concentrations of eDNA in the biofilms of OG1RF and VA1128 grown on Aclar membranes (Fig.14C). This is also corroborated by results we observed from the enzymatic treatment assays for OG1RF and DS16 (Fig. 9). In addition to eDNA, we also quantified detectable levels of proteins and carbohydrates within the biofilms of OG1RF and VA1128 grown on Aclar membranes (Fig. 14A and B). Barnes et al. observed complex eDNA structures within OG1RF biofilms, specifically noting these structures to be enveloped by what they termed "mats of extracellular matrix (ECM)" (15). Although Barnes et al. did not elaborate to determine the biochemical make-up of these mats (15),

our findings may very well indicate the content of them to be a mix of proteins and polysaccharides.

It should be noted that upon testing the effectiveness of the method we used to recover biofilm from the Aclar membranes (see Chapter 2, Page 72), in which we measured the absorbance of crystal violet left on the membranes after implementing this method, we observed appreciable amounts of biofilm remaining on the membranes for both OG1RF and VA1128 (Fig. 11). This presented a limitation in our work's experimental design as this method was inadequate for removal of biofilm from the membranes. This limitation indicates that the concentration values in Figure 14 and the CFU/ml values in Figure 10 may be inaccurate, likely under representative of the actual values. Regardless, it is impossible to draw definitive conclusions from these data about the specific concentration and CFU/ml values; for example, we cannot know if the lack of increase of proteins, carbohydrates, or eDNA over 48 hours in Figure 14 is real or only an artifact due to the inadequate removal of biofilm from the Aclar membranes. To overcome this limitation, several methods could be utilized in future work. To measure bacterial cell viability within biofilms, an ATP-detection assay could be used such as the BacTiter-Glo™ Microbial Cell Viability Assay, as described by Stiefel et al. (152). Additionally, the fluorescent DNA-binding dye Acridine Orange could be implemented to detect the totality of bacterial cells within biofilms, as also described by Stiefel et al. (152). Combining the data of these experiments may be of interest given the role of persister cells within biofilms (101; 151), which may not be detected by the viability assay but should be detected by Acridine Orange, as well as generally being able to compare the amount of metabolically-active cells within a biofilm to the whole bacterial

cell population of the biofilm. Stiefel at al. also determined that using fluorescein isothiocyanate (FITC) was an optimal means to detect amounts of protein with a biofilm, which could also be utilized in future work (26; 152). An important distinction to note for these methods is that they were all conducted with biofilms grown in 96-well plates and that biofilms were vortexed for 10 minutes to presumably detach them before fluorescence or luminescence intensity were measured, which was done after staining (152). This common aspect of these methods' protocols may provide limitations within itself, and precludes use of these methods to measure biofilm cell viability and matrix protein, polysaccharide, and eDNA concentrations of biofilms grown on Aclar membranes. However, this may very well be a better alternative to the method we used in our work. Additionally, measurement of the carbohydrate and eDNA composition of biofilms with the Total Carbohydrate Assay Kit and Quanti-iT PicoGreen dsDNA Assay Kit, as we used in our work, may be more accurate when conducted with biofilms grown in 96-well plates.

In addition to employing different means to measure biofilm composition, we were also interested in testing different conditions under which the biofilms were formed, as we did with different media conditions in Figs. 3 and 4. Therefore, we looked at substrate type and the absence or presence of shear force on biofilm formation for OG1RF and VA1128. We observed OG1RF and VA1128 to accumulate biofilm biomass at different rates and with varying amounts when grown on Aclar in TSB^{-D} over 48 hours (Fig. 10A and B). The kinetics of OG1RF and VA1128 biofilm biomass accumulation were different on Aclar compared to biofilm formation in 96-well polystyrene plates (Fig. 3B). Frank et al. could not recapitulate an effect observed in previous work of Ballering

et al., in which biofilm formation was attenuated in a strain lacking a newly-determined biofilm genetic determinant in the OG1RF background (13; 59). The only obvious difference between the two experiments conducted was the substrate the biofilms were grown on, in which Ballering et al. grew biofilms on cellulose membranes and Frank et al. grew biofilms on Aclar membranes. As such, Frank et al. suggested that the differences observed between the two experiments may be attributable to chemical differences of the substrates (59). The work presented in this study appears to corroborate this suggestion; altogether, these bodies of work provide stronger evidence that biofilm phenotypes can differ, contingent upon the environment in which the biofilms are formed.

Upon probing OG1RF and VA1128 biofilms with fluorescent molecules to indicate live *E. faecalis* cells, and viewing these biofilms by confocal microscopy, we formed several key conclusions. The architecture of the biofilms formed by the strains over 48 hours was quite dissimilar. Both strains' cellular density increased over the 48 hour duration, as one would expect, but VA1128's cellular density increased much more than in OG1RF (Fig. 12). This observation was accompanied by greater variation in architectural structures over the duration of biofilm formation for VA1128 and contrasted with a general lack of architectural variation over the course of biofilm formation for OG1RF (Fig. 12). An additional difference was the observation that VA1128 biofilm formation began with cells adhering to the substrate in chains, while individual cells of OG1RF attached to the substrate (Fig. 12). We do not know why VA1128 has a propensity to form chains. Increased cell chaining was reported in an OG1RF *gelE* deletion mutant (174). Increased cell chaining was also reported in an OG1RF *epaOX*

deletion mutant, as well as a change in cellular shape from oval to spherical, which was reported in micrographs from scanning electron microscopy (SEM) (38). As both of these genes are associated with biofilm formation in *E. faecalis*, it may be of interest to test if these genes are present in VA1128. It may also be of interest to conduct SEM with VA1128 to observe the strain's cellular shape to see if it may comparable to the *epaOX* deletion mutant. A separate study also observed the OG1RF *epaOX* deletion mutant to display considerable divergent biofilm architecture compared to the parent strain at 24 hours, defined by what the authors termed as "ultrastructured microcolonies" that formed due to chaining and clumping of the bacterial cells (39). This biofilm architecture was also observed to be associated with decreased biofilm stability, which the authors observed and measured as increased turbidity of the liquid phase of the biofilm cultures and washes of the biofilm-coated membranes, as this increased turbidity would correspond to detached biofilm (39). In our study, VA1128 formed biofilms with architecture that contained more clumps than OG1RF, particularly at 24 and 32 hours (Fig. 12), yet not as drastically as the *epaOX* mutant (39). Although we did not record it, the liquid phase of biofilm cultures of VA1128 grown on Aclar membranes were often, if not always, visibly more turbid than the biofilm cultures of OG1RF under the same conditions in our study. Dale et al. suggested that the ultrastructured microcolony architectural phenotype contributed to these biofilms being more susceptible to antibiotics (39). Based on this, it would be interesting to test how VA1128 biofilms respond to antibiotics compared to other strains, such as OG1RF.

Visual analysis of these strains' biofilms with fluorescent probes specific for carbohydrates, protein, and eDNA provided supporting evidence that biofilm architecture

is dissimilar in OG1RF and VA1128 biofilms, yet also provided evidence that the specific composition of these strains' biofilm matrices may be somewhat similar (Fig. 15, 16, and 17). For both strains, the majority of cells were co-localized with one or more stain between 4 and 24 hours, and the majority of cells stained with only Hoescht by 32 and 48 hours (Fig 15, 16, and 17). In particular, the stains corresponding to proteins and eDNA co-localized in punctate forms over the duration of biofilm formation, and the distribution of the matrix components became more homogenous with increased age of the biofilms (Fig. 15, 16, and 17). Our observation that biofilms of both *E. faecalis* strains contained puncta of co-localized proteins and eDNA is of particular interest, as protein-DNA complexes have been described in *Salmonella typhimurium* biofilms (64). The proteins in these complexes were specifically determined to be curli, a type of amyloid fiber that can be expressed in both pathogenic and commensal bacteria (64). Furthermore, this study demonstrated that these curli-DNA complexes in *S. typhimurium* biofilms were a potent trigger of autoimmunity, and this study additionally demonstrated that a commensal curli-expressing *Escherichia coli* strain was also a potent stimulator of autoimmunity (64). Other bacterial species, including the Gram-positive bacterium, *S. aureus,* are known to produce amyloids within their biofilms that may also contribute to the development of autoimmune disease (117). Although *E. faecalis* was until recently not one of these species, a 2020 study provided evidence that the N-terminal region of Esp forms amyloid-like fibers, which may serve as crucial building-blocks for *E. faecalis* biofilms (155). This recent data may suggest that these Esp amyloid-like structures could be among the proteins we observed to co-localize with eDNA in the biofilm matrices of OG1RF and VA1128. If so, these amyloid-DNA complex would be similar to that

observed in other bacterial species that promote autoimmunity. The relationship of *E. faecalis* biofilms, particularly keeping in mind the non-pathogenic role of *E. faecalis* as a common commensal resident within the human gut microbiome, and autoimmune disease may serve as a potent path of investigation for future studies.

Windham et al. conducted a similar set of fluorescence confocal microscopy experiments with *H. pylori* biofilms (177). They observed *H. pylori* to form biofilms that became more homogenous in architectural appearance with age, as we observed with OG1RF but not VA1128. They also observed *H. pylori* to form biofilms that became more homogenous in matrix composition with age, as we observed for both OG1RF and VA1128. This demonstrates how *E. faecalis* forms biofilms with properties that are both similar and dissimilar to that of other bacterial species and genera. Beyond this, these data further demonstrate the dissimilarities of biofilm properties that exist within distinct *E. faecalis* strains.

Overall, these data indicate how distinct *E. faecalis* strains can display biofilm properties that are characteristic of only a single strain. As resistance to antimicrobials remains an ever-increasing concern in bacteria, and the need to find targets to combat bacterial infections that do not promote antibiotic resistance becomes more pressing (14; 133), this knowledge warns that not all strains of a single bacterial species may contain the same targets or respond to an inhibitor in the same way. Future studies will need to elucidate a more mechanistic understanding of the differences in biofilm properties observed among the distinct *E. faecalis* strains. It would also be worthwhile to characterize additional strains in order to further understand how common these differences are. Specific avenues of investigation should include looking at other possible

components of biofilm matrices that were not tested for, such as lipids; studying biofilms of DS16, V583, MMH594, in further depth, as was conducted with OG1RF and VA1128; and treating biofilms of the different strains with the aforementioned dispersal agents and then conducting microscopic analyses for biofilm matrix components, similar to what was done in this thesis for OG1RF and VA1128. Beyond this, conducting immunological experiments to assay the immune response to different *E. faecalis* strains would provide a logical next step in this line of research. This may include, but is not be limited to, testing cytokine and chemokine production by macrophages after being exposed to biofilm supernatants, measuring phagocytosis by macrophages after being exposed to biofilm supernatants, and evaluating macrophage activation after being exposed to biofilm supernatants. These potential future studies may aid in furthering our understanding of the nuances of enterococcal biofilm diseases, how the immune response responds to or may even contribute to these diseases, and how better means to combat these diseases may be developed.

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