Enterococcal biofilm—A nidus for antibiotic resistance transfer?

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INTRODUCTION

The continued increase in antibiotic resistance among human and animal pathogens is a threat to public health. Multiresistant organisms such as vancomycin resistant enterococci (VRE) have emerged as major threats to human health, particularly in healthcare settings worldwide (Faron et al., 2016; Hung et al., 2019; Uçkay et al., 2017) as well as in the environment (Huijbers et al., 2015), and Enterococcus faecalis (Efs) is an extremely common gut commensal of animals (including humans), (Lebreton et al., 2014). In the last decade, a fundamental reappraisal of how bacteria grow under environmental conditions has taken place (reviewed in detail by Haruta & Kanno, 2015). It is now clear that many bacteria exist as part of complex communities attached to surfaces, embedded in polymeric matrices of their own devising known as biofilm (reviewed in detail by Flemming et al., 2016). Biofilms were first identified in aquatic environments, such as rock surfaces in streams, but have now been recognized as major contributors to infection (Ch'ng et al.,...
Clinically, biofilms are found in a wide range of disease states, from indwelling medical devices and urinary tract infections to diabetic ulcers and the lungs of cystic fibrosis (CF) patients. Biofilms allow colonization of a variety of inanimate materials as well as forming directly on body surfaces thereby facilitating chronic infections such as post-surgical infections, endocarditis, otitis media, etc. Their significant clinical impact has recently been comprehensively reviewed by Schulze et al. (2021). The regulation of the physiological processes of biofilms is poorly understood (O’Toole & Wong, 2016; Santos-Beneit, 2015). Moreover, despite antibiotic resistance transfer mechanisms being described for enterococci growing in planktonic culture, our understanding of how efficiently these mechanisms function in biofilm is limited (Van Acker et al., 2014).

Various hypotheses have been put forward regarding what comprises a biofilm matrix and its cellular components, including the development of a multifaceted structure comprising adherent organisms (Dunny et al., 2014). Biofilm models of ‘development’ and biological function were initially based upon research carried out on Pseudomonas aeruginosa and P. fluorescens although the limitations of extrapolating these to Gram positive organisms have long been recognized (Monds & O’Toole, 2009). With regards to enterococci, models of development are still not fully understood, and an understanding of biological functions is just beginning to emerge (Gilmore et al., 2014; Kim et al., 2020). Biofilms have been shown to play a role in some enterococcal nosocomial infections, for example, providing a location for the attachment of a population of bacteria to a heart valve during endocarditis (O’Toole et al., 2000). The initial colonizing isolate produces anchoring sites through the release of DNA and polymeric substances, paving the way for later additions of new members using the DNA as an attachment site (Mohamed & Huang, 2007; O’Toole et al., 2000). The step-wise addition of new members to the microcolony biofilm means that late colonizers can consist of bacteria that could never form biofilm at the specific site due to issues with nutrient availability and oxygen saturation (Whitchurch et al., 2002). Barnes et al. (2017) who studied the colonization of EFs, noted that the rescue of mutant phenotypes by parent or other mutant strains within a pool had the potential to complicate the findings of biofilms studies in vivo. Moreover, rescue attempts in a cooperative environment may see the rise of cheaters that do not contribute as has been seen in other bacterial communities (Pollak et al., 2016).

Having several attachment sites in a given biofilm microcolony (biotic or abiotic surface, EPS or even directly to proteins on bacterial cell surfaces) may allow more pathogenic species of bacteria to enter the biofilm, creating continual sites for chronic, systemic infection (Gill et al., 2005; O’Toole et al., 2000; Vuong et al., 2004). Mature biofilms are usually harder to eradicate due to increased surface area of attachment, formation characteristics and the polymicrobial nature of the biofilm itself (Boles et al., 2004; Rochex et al., 2008). Where enterococci are concerned, the expression of the enterococcal surface protein (ESP), a cell wall-associated protein, has shown improved adhesion and therefore biofilm formation (Toledo-Aranas et al., 2001). Studies by Kristich et al. (2004) and Tendolkar et al. (2004) concluded that ESP must act in coordination with various factors involved in enterococcal biofilm formation, and its presence can improve biofilm formation. Hence, enterococcal biofilm is now described as multifactorial in nature (Dunny et al., 2014; Garg et al., 2017). Additionally, enterococcal gelatinase contributes to biofilm during infection by hydrolysing host tissues (collagen, fibrinogen and fibrin) into derivatives (gelatin, various peptides and amino acids) and is recognized as a key virulence factor (Hancock & Perego, 2004). Mediated through the Fsr quorum response, gelatinase provides both nutrients and anchoring sites for the development of biofilm through aiding in the production of aggregation substance (Fisher & Phillips, 2009; Thurlow et al., 2010). See Figure 1 for an overview of enterococcal biofilm formation and maturation.

**BIOFILM: NOVEL REGULATION SYSTEMS AND ANTIBIOTIC RESISTANCE TRANSFER**

The search for regulatory systems central to enterococcal biofilm formation and the regulatory systems that control them has been an area of active research (Grand et al., 2019, 2020; Manias & Dunny, 2018; Zheng et al., 2017). Chilambi et al. (2020) have further extended our understanding of these complex and diverse regulatory systems by reporting on the evolution of EFs in immunocompromised patients finding that vancomycin-resistant strains adapted during colonization and mutations accumulated that contributed to increased biofilm formation. There has been considerable effort put into defining EFs biofilm regulation in the hope of identifying a central control point that might become a novel antimicrobial drug target. While there are no outstanding candidates thus far, we are beginning to appreciate that EFs biofilm represents a complex environment that supports unexpected amounts of metabolic activity and evidence for its role in supporting horizontal gene transfer is steadily accumulating.

Information on how enterococci control biofilm has started to emerge recently with transcriptomic studies beginning to catalogue the regulatory systems involved. The report by Lim et al. (2017) was one of the first to compare the transcriptome of biofilm and planktonic grown cells, and they found an abundance of adherence-associated proteins upregulated in the biofilm state. Significantly, they also noted that genes involved with plasmid replication and genetic exchange were also upregulated, suggesting...
that the biofilm could be an environment that favours horizontal gene transfer. Sirvertsen et al. (2018) proposed an enterococcus cassette chromosomal (ECC) element, which acts as a focus for genetic exchange and contributes to the large variation of accessory genes found in *Enterococcus faecium* (Efm) and may aid in adaption to new environments. Suriyanarayanan et al. (2018) applied a proteomic approach to selected clinical strains of Efs. They highlighted the central role of metabolic processes, biosynthetic processes and transport systems in biofilm grown cultures, for example, proteins associated with the shikimate kinase pathway were upregulated in a strong biofilm former, while proteins associated with secondary metabolites were downregulated. Metabolic pathway and gene ontology analyses showed higher levels of metabolic activity in a weak biofilm former. There is no clear consensus at the moment as to the most important genes involved in biofilm formation by Efs. What is becoming clearer is that the extensive genetic heterogeneity associated with this organism can be reflected in functional diversity of biofilm and a differential response to various environmental stressors.

The interplay between antibiotic resistant bacteria and biofilm has become better understood in recent years although, as recently as 2016, Stalder and Top argued that much more effort is needed to understand the physicochemical and biological mechanisms involved in gene transfer in this environment. The notion that biofilm is simply a physical barrier that impedes drug access to the cells has now largely been dispelled as overly simplistic. The area has been recently reviewed by Abe et al. (2020), who concluded that biofilm is a hot spot for horizontal gene transfer in aquatic environments. They noted the contribution of the classical mechanisms of conjugation, transduction and transformation along with a novel membrane vesicle-mediated exchange. The latter was found to be a widespread mechanism of antibiotic resistance gene transfer although it seems to be primarily associated with Gram negative organisms.

Nagasawa et al. (2020) working with *Streptococcus mutans* biofilm showed that stress caused by sub-MIC levels of antibiotics stimulated biofilm formation and this contributed to higher levels of horizontal gene transfer. This theme is reflected in the enterococci where a number of investigators have demonstrated a direct link between conjugation elements, biofilm formation and virulence (Bhatt et al., 2015; Parthasarathy et al., 2020; Schmitt et al., 2018). While the molecular mechanisms underlying HGT in biofilm are beginning to be elucidated there has been a shift in focus to define the impact of the process in vivo. One area that is receiving increased attention is the role of biofilm on microplastics, which have been noted as a major environmental pollutant. There is now compelling evidence that bacteria can colonize and grow on microplastics, which may have implications for the bioavailability of antibiotics and other compounds. This area is still in its infancy, but further research is needed to fully understand the impact of biofilm on microplastics and the potential implications for human health and the environment.
evidence that microplastics selectively enrich antibiotic resistance genes. Recent work by Wang et al. (2020) indicated that the relative abundance of integron-integrase genes was greater on biofilm-microplastics, potentially suggesting a higher level of horizontal gene transfer. Yang et al. (2020) reviewed current knowledge of these microbial niches, concluding that they have, so far, unknown consequences for microbial ecology and environmental processes in aquatic ecosystems. A recent report by Pazos et al. (2020) has provided the first demonstration of biofilm-mediated association of enterococci with microplastics in a polluted ecosystem. Therefore, the need to understand the physiological processes within biofilm has never been greater, and it will only be delivered with a combination of experimental approaches encompassing molecular biology, microscopy and bacterial physiology.

ANTIBIOTIC RESISTANCE IN ENTEROCOCCI

Enterococcus is an ancient, resilient and versatile genus able to survive under harsh conditions (Lebreton et al., 2014). This has greatly contributed to their success in the health care environment and the pathogenicity of enterococci in human disease has recently been reviewed in detail by Fiore et al. (2019). Enterococci are some of the most common healthcare associated pathogens (Hung et al., 2019; Kreidl et al., 2018) and drug-intensive practices such as the selective decontamination of the gut, which have shown limited clinical effectiveness, have been linked to rising VRE rates. A meta-analysis performed by DiazGranados et al. (2005) suggested that patients with bacteraemia caused by vancomycin-resistant enterococci were more likely to die than those with vancomycin-sensitive enterococci, and this has been backed up by other meta-analyses. Recently, Dubler et al. (2020), examining patients with end-stage liver disease, suggested that it is the underlying severity of the disease that predicts the outcome rather than vancomycin resistant Enterococcus faecium (VREfm). Nonetheless, and irrespective of the direct action of VRE during infection, these investigators recognized a central role for vancomycin resistance in driving the use of alternative antibiotics and contributing to selection pressure in favour of linezolid-resistant isolates. Enterococci possess several intrinsic resistance phenotypes such as resistance to penicillins, aminoglycosides and cephalosporins (Hollenbeck & Rice, 2012) and are ideally placed to acquire antimicrobial resistance (AMR) genes owing to selective pressure from antimicrobial residues present in the wide range of environments they are known to inhabit (Bonten et al., 1998; Fisher & Phillips, 2009).

The most prevalent multidrug-resistant enterococci are Efm and Efs (Arias et al., 2010; Moellering, 1992; Molechan et al., 2019). In 2008, over 50% of all identified pathogenic Efm were multidrug resistant according to a study by Hidron et al. (2008). In the same study, Hidron et al. (2008) identified that 40% of medical devices associated infections were due to vancomycin and ampicillin resistant Efm only. Efs is less commonly resistant to vancomycin and is the primary causative agent for human endocarditis (Murdoch et al., 2009). This trend has remained for more than two decades with recent surveillance reporting MDR Efm incidence rates between 25% and 59.1% (HPSC, 2018). Efs and Efm are the most clinically relevant but other infective enterococci including E. durans, E. avium, E. gallinarum and E. casseliflavus have also being identified (Ahmed & Baptiste, 2018; Gordon et al., 1992).

ENTEROCOCCAL PATHOGENS: E. FAECIUM VERSUS E. FAECALIS

A significant factor for the rise in prominence of enterococcal infections is their growing, multidrug-resistance (MDR) linked to their overall genome plasticity and efficacy in acquiring additional resistance determinants (Bender et al., 2016; Hegstad et al., 2010). However, the ability of Efm to benefit from a broad exchange of genetic determinants (Gao et al., 2018) contrasts with Efs, which exhibits a more limited range of genetic inputs (Leavis et al., 2006). Importantly, many of the frequent clinical isolates of Efm are resistant to four or more antibiotics including vancomycin (Arias & Murray, 2012; Zhong et al., 2019). Work by Ekwanzala et al. (2020) identified two main multilocus sequence types (ST’s), namely ST40 and ST179, which constituted 50% of isolated vancomycin resistant Enterococcus faecalis (VREfs). These ST’s are commonly isolated from animals, humans and the environment worldwide (Zheng et al., 2017; Zischka et al., 2015). They have been found to carry a pathogenicity island, and they display isolate specific plasmid and phage patterns. Likewise, all isolated VREfs ST40 strains were predicted to be putative human pathogens and contained considerable genomic diversity in terms of mobile genetic elements (MGEs). Of the small number of VREfm isolated in the study, the three ST’s, ST80, ST203 and ST1446, were also isolated elsewhere (Hammerum et al., 2017) and ST203 and ST80 proved to be most prevalent. As previously highlighted, the strains found in this study are part of clonal complex 17 (CC 17) and represent the majority of VREfm strains causing infections in hospital worldwide (Lee et al., 2019). Momba and co-workers in their examination of
resistome determinants of both Efs and Efm revealed a treestructure based on STs (Ekwanzala et al., 2020). They found that CRISPR-cas systems were only found in six vancomycin resistance E. faecalis (VREfs) genomes and none of the vancomycin resistance E. faecium (VREfm) genomes contained a functional CRISPR-cas system although the CRISPR sequences were present. Of these systems, only ST40 and ST16 VREfs contained functional Type IIA CRISPR-cas systems. None of the ST179 VREfs contained functional CRISPR, and all of them were, therefore, dormant or orphan CRISPR.

In enterococci, the genomes forming monophylegenetic groups support previous results of speciation of enterococci based on the groESL locus (Sanderson et al., 2019; Zaheer et al., 2012). The diversity in wastewater strains may be a reflection of their origin from clinical, companion animal or agricultural sources. However, Efm and Efs are still the predominant species in wastewater likely due to the continuous input of faecal matter. The number of genes related to the mobilome increases with genome size in Efs and Efm, and this would suggest that the mobilome is a significant factor in the evolution of these bacteria in wastewater contributing to genomic expansion and diversity. There is more genetic diversity in vancomycin-resistant Efs (Leavis et al., 2006) than Efm (Gao et al., 2018). The lack of diversity in Efm and a preponderance of AMR genes in the mobilome suggests that Efm may be more specifically adapted to clinical environments (Zhong et al., 2019).

The success of Efm and Efs evolving as multiresistant nosocomial pathogens is associated with their ability to acquire and share adaptive traits, including antimicrobial resistance genes encoded by MGEs. Mikalsen et al. (2015) investigated this mobilome in successful hospital associated genetic lineages of Efs and Efm. Although the high level of inter-species horizontal gene transfer (HGT) must be acknowledged, the considerable species-specificity of these MGEs indicates a separate vertical evolution of MGEs within each species, and for Efs within each ST. Genetic modelling comparing whole genome sequences suggests two clades in Efm strains (clade A and B), where clade A includes Efm associated with human infections from CC17, as opposed to clade B that contains strains of non-hospital human origin (Galloway-Peña et al., 2012; Lebreton et al., 2014; Palmer et al., 2012). Efs seems to be less origin and/or host-restricted as dominant clones are shared between hospitals and the community although some CCs, including CC2, CC40 and CC87 show clear over-representation in hospital-associated infections (Kuch et al., 2012). Mikalsen et al. (2015) also identified a lack of Tn916 family conjugative transposons in Efm compared to Efs, in common with most reports of this transposon family in Efs. There was also a strong correlation between the presence of Tn916 targets and tetM in Efs that was not found in the Efm strains.

Efs and Efm cannot be distinguished morphologically but different genome structures have been identified between the two species (Gan et al., 2020). Another distinct difference between Efs and Efm is their interaction with bacteriophage; hence, the molecular mechanisms used by phages to infect Efs and how Efs overcomes phage infection to become resistant are important species differentiators. Chatterjee et al. (2020) identified bacterial genes essential for infection with bacteriophage VPE25. They screened a low-complexity transposon (Tn)-mutant library of E. faecalis OG1RF for phage resistance (Dale et al., 2015). In addition to the VPE25 receptor (Duerkop et al., 2016), transposon sequencing revealed novel Efs genes necessary for phage adsorption and optimum intracellular phage DNA replication and transcription.

When a phage infects a bacterium, it incorporates spacer into the CRISPR array within the bacterial chromosome and occasionally plasmids (Sanderson et al., 2020). The spacers are expressed as CRISPR RNAs (crRNAs) and provide a surveillance mechanism for descendant cells and guide the CRISPR/Cas system to enable cleavage of the protospacer sequence in the phage genome. The cleaved phage genomes are then cannibalized and can no longer support productive phage infection (Barrangou, 2015; Tao et al., 2018). Functional CRISPR/Cas arrays were detected in 13 Efs genomes, with all but one also containing a prophage. The lack of a functional CRISPR/Cas array was associated with multidrug resistance in Efm. Thus, genes related to phage and CRISPR/Cas arrays could potentially serve as environmental biomarkers. Genome analysis of the phage pointed to the absence of genes associated with lysogeny, suggesting that this may be more of a factor associated with Efm isolates. However, Melo et al. (2019) isolated and characterized two novel enterococcus phages, the siphovirus vB_EfaS-Zip (Zip) and the podovirus vB_EfaP-Max (Max) for application during wound treatment. Both phages demonstrated lytic behaviour against Efs and Efm suggesting that more work needs to be done to elucidate the interactions between phages and Efs and Efm.

**PLASMID-BASED CONJUGATION IN ENTEROCOCCI**

There are three primary conjugative systems known in enterococci—pheromone-responsive plasmids (recently reviewed by Sterling et al., 2020), broad host range plasmids (sometimes referred to as “pheromone-independent conjugation”) and ICE elements or conjugative transposons of which the first discovered was Tn916 in E. faecalis DS16 (Tables 1 and 2). Tn916 was originally recognized...
as a transposon because of its ability to insert at multiple sites on the co-resident plasmid pAD1 (Franke & Clewell, 1981; Gawron-Burke & Clewell, 1982). Regardless of the system, cell-to-cell contact is needed for the plasmid and the mobilized genetic elements to be transferred.

One of the most well-studied mechanisms of HGT in enterococci is pheromone-responsive plasmid transfer in E. faecalis (Dunny & Berntsson, 2016; Hirt et al., 2018; Panesso et al., 2005). The availability of proficient horizontal gene transfer mechanisms amongst enterococci (has been reviewed

### Table 1: Antibiotic resistance genes commonly found on enterococcal plasmids

| Gene | Phenotype | AMR | Mobile element |
|------|-----------|-----|----------------|
| vanA | D-Ala-D-Lac ligase | Vancomycin | pS177(a), pWZ1668(b), pTW9(c), pWZ7140(b), pWZ909(b), pF856(d), p5753cA(e), pZB18(f) |
| vanB | D-alanine-d-lactate ligase | Vancomycin | pVEF1(g), pVEF3(b), pIP186(f), pMG2200(h), pVEF2(e) |
| vanZ | Teicoplanin resistance protein | Teicoplanin | pDO2(k), pS177(a), pWZ1668(b), pTW9(c), pWZ7140(b), pWZ909(b), pF856(d), pVEF1(g), pVEF3(b), pIP186(f), p5753cA(e) |
| aadE | Aminoglycoside 6-adenyltransferase | Streptomycin | pDO2(k), pS177(a), pWZ1668(b), pTW9(c), pWZ7140(b), pWZ909(b), pF856(d), pRUM(f) |
| ermB | rRNA adenine N-6-methyltransferase | Erythromycin | pS177(a), pWZ1668(b), pTW9(c), pWZ7140(b), pWZ909(b), pF856(d), pRUM(f) |
| aphA | Aminoglycoside 3′-phosphotransferase | Kanamycin | pDO2(k), pS177(a), pF856(d) |
| pRE25(m)(j) | Aminoglycoside phosphotransferase type III | aminoglycosides | pDO2(k), pRE25(m) |
| cat | Chloramphenicol acetyltransferase | chloramphenicol | pDO2(k), pRE25(m), pCPP5(n), pRUM(f), pEF-01(o) |
| tetL | MFS family major facilitator transporter, tetracycline: cation symporter | Tetracycline | pDO1(k), pM7M2(p), pAMAlpha1(q) |
| tetM | Tetracycline resistance protein | Tetracycline | pM7M2(p), pCFlo(f) |
| tetP | Tetracycline resistance protein | Tetracycline | pDO1(k) |
| sace | Streptothricin acetyltransferase | Streptothricin | pDO2(k) |

Note: Information used for table acquired using PubMed microbial gene database queries with reference to *E. faecalis* and *E. faecium* where appropriate. References: Halvorsen et al. (2011)(a), Zhu et al. (2010)(b), Unpublished NCBI Reference Sequence: NC_014726.1(c), Szakacs et al. (2014)(d), NCBI Reference Sequence: NC_013317.1(e), NCBI Reference Sequence: NC_016967.1(f), Sletvold et al. (2007)(g), Sletvold et al. (2008)(h), Sletvold et al. (2010)(i), Zheng et al. (2009)(j), Qin et al. (2012)(k), Unpublished NCBI Reference Sequence: NZ_KP842560.1(l), Schwarz et al. (2001)(m), Liu et al. (2014)(n), Liu et al. (2012)(o), Li et al. (2011)(p), Francia and Clewell (2002)(q), Hirt et al. (2005)(r).

### Table 2: Enterococcal associated antimicrobial resistance containing transposons and their associations with other organisms

| Transposon | Categorization | Function (genotype) | Host range |
|------------|---------------|---------------------|------------|
| Tn916      | Conjugative   | Tetracycline (TetM) | *Enterococcus, Staphylococcus, Streptococcus, Lactococcus, Lactobacillus, Bacillus, Clostridium, Leuconostoc, Listeria, Mycoplasma, Actinobacillus, Acholeplasma, Acinetobacter, Alcaligenes, Butyribivora, Citrobacter, Erysipelothrix, Escherichia, Fusobacterium, Granulicatella, Haemophilus, Neisseria, Pseudomonas, Thermus, Ureaplasma, Veillonella, anaerobes |
| Tn917      | Tn3           | Erythromycin (ErmB) | *Enterococcus, Staphylococcus, Streptococcus, Lactococcus, Bacillus, Listeria, Paenibacillus |
| Tn1546     | Tn3           | Vancomycin (vanA)  | *Enterococcus, Bacillus, Staphylococcus, Oeskorvia, Streptococcus, Rhodococcus, Arcanobacterium haemolyticum, Paenibacillus |
| Tn1547     | composite     | Vancomycin (vanB1) | *Enterococcus |
| Tn5281     | composite     | Gentamycin (aac-6'/aph-2") | *Enterococcus, Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma |

Note: Information used for table acquired using PubMed microbial gene database queries with reference to *E. faecalis* and *E. faecium* where appropriate.
in detail by Weaver, 2019) and the AMR genes associated with pheromone-responsive plasmids are known to transfer with high efficiency (Hirt et al., 2002, 2018). The system is driven by specific short chain peptide pheromones encoded chromosomally. When these specifically bind to ‘donor’ strains, which harbour conjugative plasmids, they induce aggregation substance production (Waters et al., 2003; Waters & Dunny, 2001). These plasmid-containing donors also produce a competing inhibitor peptide that prevents conjugation occurring between strains carrying the same plasmid. Aggregation substance is a membrane associated surface-protein that induces clumping of donors and recipients significantly, increasing the efficiency of bacterial plasmid conjugation (Yagi et al., 1983), as seen in Figure 2. This process of HGT occurs primarily amongst Efs strains, but interspecies transfer has also been recorded with vancomycin (vanA) resistance being moved from Efm to Efs (Conwell et al., 2017; McCarron et al., 2019). Tetracycline resistance transfer has been demonstrated on the pheromone responsive plasmid pCF10, which has served as a model of the system for many years (Christie et al., 1987) as it has a type 4 secretion system (T4SS) (Rehman et al., 2019). These efficient pheromone responsive plasmids have shown limited replication outside the Enterococcus genus with transfer to Streptococcus gordonii being the only recognized intrageneric transmission (Mansfield et al., 2017). There is evidence for plasmids driving the evolution of specific pathogenic lineages among enterococci (Arredondo-Alonso et al., 2020). Therefore, anything that facilitates HGT could potentially contribute to new pathogenic strains. In addition to VREs, there is evidence of commensal Efm harbouring and passing on a plasmid encoding 10 resistances. The plasmid, pEF37BA, was created from the recombination of Erysipelothrix rhusiopathiae chromosomal ZJ multiresistance gene cluster with the Efm’s pM7M2 plasmid. This recombinant plasmid was successfully passed to another strain of Efm as well as Listeria welshimeri (Morrone et al., 2019).

**FIGURE 2** Overview of enterococcal pheromone-based conjugation. (a) Plasmid containing enterococci secrete inhibitor sex pheromones (red extracellular peptide) into the extracellular environment to out compete against any inducer sex pheromones (green extracellular peptide). (b) When plasmid free enterococci sense a compatible plasmid containing bacteria, pheromone production is directed towards outcompeting the inhibitor production in the plasmid containing bacteria. Once a threshold has been reached and the inhibition mechanism has been overcome, binding of the pheromone occurs to the cell surface binding sites on the plasmid containing bacteria. (c) Induction of aggregation and the production of aggregation substance (yellow) occurs. (d) The plasmid containing bacteria clump together along with the plasmid free enterococci increasing surface area and allowing conjugation to occur (arrows). Created using BioRener.com with information from Dunny and Berntsson (2016)
Conjugative plasmids, including the broad host range Inc18 group (Kohler et al., 2018), which are not phero-
monedependent, are capable of transmitting antibiotic resistance across genus boundaries allowing the dissemi-
nation of antibiotic resistance to other Gram-positive bacte-
eria (Palmer et al., 2010). When the genome of Efs OG1RF, one of the first and most intensively studied isolates was
sequenced, no foreign DNA, indicative of horizontal gene transfer (HGT) was detected (Bourgogne et al., 2008). Huo
et al. (2015) identified a type II restriction modification system within OG1RF conferring a 5-methylcytisine motif
that protects it from non-self-DNA integration.

In contrast to the limited number of HGT events, OG1RF has been apparently incorporated over time; other
enterococci can and do accumulate genetic information through a variety of methods. Conjugative transposons,
which are best represented by the Tn916 family, are mostly integrated in the chromosome. Their movement results
in a non-replicative circular intermediate that is able to transfer conjugatively, followed by stable insertion into the
genome of the recipient cell. The evidence so far is lim-
ited but points to segments of DNA greater that 100kb,
which appear to have been “acquired” from an unrelated
source. In some cases, these ubiquitous and quite diverse
elements appear to be able to conjugate (Guglielmini et al.,
2011; Wozniak & Waldor, 2010). They frequently bear mul-
tiple determinants for integrase and insertion sequences,
as well as genes that resemble those involved in conjuga-
tive transfer. Putative oriT sites, relaxase determinants and
plasmid-like conjugation genes—sometimes even similar
to those found in Tn916—have also been identified. The
presence of such determinants has given rise to the term
integrative conjugative elements (ICEs), although direct
demonstration of such transfer has not always been pos-
sible, with the additional presence in ICEs of genes that
facilitate survival or the ability to take advantage of a new
environment such as biofilms formed in vivo is common,
with determinants that encode antibiotic resistance and
virulence, being a good example (Tan et al., 2020).

Transposons are important genetic elements in the ge-
nomes of many enterococci, often encoding strain specific
virulence and resistance phenotypes (Kristich et al., 2014).
There are three main categories of enterococcal trans-
posons: composite transposons, Tn family transposons
and conjugative transposons (Table 2). In terms of van-
comycin phenotypes, VanA and VanB are common in Efm
and Efs, but other Van genes predominate in less-common
clinically relevant species (VanC in E. casseliflavus and E.
gallinarum) (Ahmed & Baptiste, 2018). The Tn1546 trans-
poson carries a VanA gene cluster encoding resistance to
vancomycin and teicoplanin (Bjorkeng et al., 2013). VanB
is made up of subtypes (VanB1-B3) (Dahl et al., 1999). The
most common, the VanB2 subtype (Hanrahan et al., 2000)
is linked to a Tn5382-like conjugative transposon. Large
(90-250kb) chromosomal elements or conjugative plas-
mids facilitate the intra- and inter-species transfer of vanB
(Dahl & Sundsfjord, 2003).

**ENTEROCOCCAL CONJUGATION ON SURFACES**

While bacteria have been shown to conjugate under plank-
tonic conditions, surface-associated conjugation, may be a
more likely mode of natural HGT due to the concentration
of bacteria on solid surfaces (Aminov, 2011; Angles et al.,
1993) (Table 3). In the same way, as transfer efficiencies
can differ between enterococci when it comes to growth in
planktonic or solid surface environments, the same could
be said of transfer of each type of conjugative element in
biofilm. In enterococci, the behaviour of conjugative plas-
mids and their transfer efficiently can vary whether the
reaction occurs in a broth or on a solid surface. There are
plasmids, such as pAMβ1, that transfer well on solid mat-
ing but have low transfer efficiency under broth mating
conditions (Reniero et al., 1992). The plasmids such as
pCF10 and pAD1 transfer with the use of a sex pheromone
signalling pathway, allowing for efficient gene transfer at
maximum rates of 10⁻¹ transconjugants (Christie et al.,
1987; Clewell et al., 1982). See Figure 3 for an overview
of the pCF10 conjugation system. Enterococci use pep-
tide pheromones to aggregate potential donor strains to
facilitate HGT (Clewell, 2011; Palmer et al., 2010) and
the cell-surface protein, encoded by the PrgB aggrega-
tion gene is located on all pheromone-inducible plasmids
(Palmer et al., 2010). Bacteria that contain a pheromone
responsive plasmid have their own pheromone produc-
tion inhibited by a plasmid produced binding protein (the
inhibitor – iCF10) (Clewell, 2011; Kozlowicz et al., 2006;
Palmer et al., 2010). This mechanism can be overcome by
the presence of un-inhibited pheromone at a median con-
centration 80-fold higher than the inhibitor, produced by
a plasmid free Enterococcus (Hirt et al., 2002; Lysakowska
et al., 2012). Once the inhibition system has been success-
fully out competed, downstream signalling activates the
production of aggregation substance causing the clump-
ing of the donor strain, making it receptive to conjugation
(Clewell, 2011; Lysakowska et al., 2012). This allows Efs
strains to conjugate with a donor strain at efficiencies up
to 10⁻¹ transconjugants per donor (Donelli et al., 2004;
Hirt et al., 2002). Conjugation has also been previously in-
stigated in two directional interspecies HGT of antibiotic
resistance to other enterococci, staphylococci and strepto-
cocci (Gomez et al., 2011; Palmer et al., 2010).

Bacterial biofilms have been postulated to be the loca-
tion of choice for such processes (Tuson & Weibel, 2013)
and although evidence is still accumulating in support of the importance of HGT in biofilms in enterococci, work by Król et al. (2013) showed widespread transferability in an *E. coli* biofilm. Madsen et al. (2012) argues that HGT is generally higher in biofilm communities compared to planktonic environments, but also suggests that successful introduction of plasmids into biofilm may require that plasmids are part of a biofilm at the very start of its development. Savage et al. (2013) showed that in *Staphylococcus aureus* conjugation frequencies were comparable for filter mating and biofilm (4.4 × 10⁻⁴ and 1.9 × 10⁻⁴, respectively) in comparison to planktonic culture (2.7 × 10⁻⁸). Van Meervenne and co-workers in their work with *Pseudomonas putida* and *Escherichia coli* showed a plasmid transfer ratio of 1/100 in filter mating (Van Meervenne et al., 2012), conditions in comparison to biofilm data collected by Van Meervene et al. (2014), which gave transfer ratios of between 2/100 and 1/10.

In terms of HGT in biofilm, there is a growing understanding that some important elements may be transferred in enterococcal biofilms in vivo. For instance, Abe et al. (2020) in a recent review of HGT in aquatic environments pointed to possible interconnections between HGT mechanisms and biofilms. However, it is unclear in most cases if the evidence of HGT in vivo is biofilm associated or not. There is little direct evidence of these types of studies having been carried out possibly due to the lack of appropriate experimental systems to investigate the phenomenon. Neela et al. (2009) reported that tet(M) was transferred from *Lactococcus garvieae* to human EfS but not to *E. coli*. In contrast, *Vibrio* spp. transferred tet(M) to *E. coli*, but not to EfS. These donors (*L. garvieae* and *Vibrio* spp.) are fish-pathogenic bacteria and, in vivo, these organisms would form biofilms on fish intestine, where the transfer of ARGs would occur. Some conjugative plasmids facilitate biofilm development by encoding biofilm-associated proteins. Notably, the pCF10 conjugative plasmid discussed above encodes three cell-wall-anchoring proteins (PrgA, PrgB and PrgC) that promote cell–cell adhesion at an early stage of biofilm formation (Bhatty et al., 2015).

While the literature on conjugative DNA transfer by enterococci and other bacteria is extensive, reports demonstrating these transfer events within biofilms are relatively scarce, reflecting the technical challenges of demonstrating the process in situ. Nonetheless, this area has been receiving increasing attention and a recent review by Abe et al. (2020) has shown that biofilm is an important site for horizontal gene transfer (HGT) in aquatic environments, and Conwell et al. (2021) have proposed a novel model to identify biofilm associated HGT using molecular imaging techniques. Abe et al. (2020) considered how HGT impacts on environmental processes and examined the major mechanisms for biofilm-associated HGT, including the membrane vesicle-mediated exchange. The latter process has been reviewed relatively recently by Domingues and Nielsen (2017). Prescott and Decho (2020) made the point that quorum sensing networks develop in biofilm and they are closely linked to bacterial flexibility and adaptability.

While next generation sequencing methodology is contributing extensive amounts of data on bacterial species, antibiotic resistance genes and mobile elements in biofilm, Abe et al. (2020) argue strongly for improved microscopic methods for direct visualization of biological processes within this complex matrix. It is becoming increasingly clear that the combination of experimental and bioinformatic approaches will be necessary to estimate the contribution of biofilm to emerging antibiotic resistance, and this will be an important parameter in determining how best to manage the risk from environmental hot spots.

### ENTEROCOCCAL BIOFILM—GAPS IN THE LITERATURE

While biofilm and its formation has been extensively investigated, enterococcal-specific biofilm characteristics...
and formation are less well-understood (Barnes et al., 2012). There are a limited number of reports in the literature relating to the understanding of enterococcal biofilm characteristics. Studies tend to focus on interventions to prevent or destroy enterococcal biofilm. Due to the low numbers of publications interspaced by years, there are no universally accepted standard methods for analysing biofilm in enterococci. Variations in assays of biofilm formation and characteristics for enterococci are frequently reported. This can even stretch as far as the absence of a standard biofilm formation medium and is discussed in detail, by Dunny et al. (2014), Colomer-Winter et al. (2019), Willett et al. (2021) and briefly by Kim et al. (2020).

The consensus from the literature appears to be that our understanding of biofilm formation capability as a function of the specific characteristics of growth, substrate and biofilm promoters is based on the limited model systems available at present (Fisher & Phillips, 2009; Gilmore et al., 2014; Goh et al., 2017); with time, and additional model systems, our understanding may change. There are also distinctive biofilm formation variations based on static or laminar flow growth conditions (Garrett et al., 2008). Optimization of biofilm biomass using these characteristics...
may have a negative impact on the functionality of bacteria in the biofilm state, as a model of persistent antibiotic resistant infection in vivo (Nguyen et al., 2011). There is a growing consensus that bacteria modulate their biofilm to adapt to changing conditions of stress; rather than producing biofilm in large quantities as a function of their pathogenicity or growth conditions (Cambronel et al., 2020; Garrett et al., 2008; Stewart et al., 2015).

As compared to pathogenicity studies, biofilm studies are for the most part assay based and can range from a measure of biofilm formation based on cell counting alone, the ratio of cells to biomass, or dry biomass alone (Creti et al., 2006; Hufnagel et al., 2004; Kristich et al., 2004; Rosa et al., 2006). There exists a drive for insight on the workings of enterococcal biofilm, as the environment is conducive for exchange of information, especially when coupled to the knowledge of intercellular signalling pathways such as the fsr and Acyl-homoserine lactone systems (McDougal et al., 2012; Parsek & Greenberg, 2000). It may be that HGT within a biofilm is inefficient as compared to laboratory methodologies (Cook et al., 2011). Understanding these processes will unlock the opportunity for a calculated approach, dealing with increasingly resistant opportunistic infections through effective treatment and preventative strategies.

In order to make relevant comparisons to pathogenic mechanisms, laboratory biofilm assays need to reflect the conditions that enterococci are exposed to during infection. Such parameters would include nutrient content, substrate composition and mechanical/chemical stress (Cambronel et al., 2020; Mohamed & Huang, 2007; Van Wamel et al., 2007). Biofilm assays that work on Gram negative, flagellated P. aeruginosa, which binds to most abiotic surfaces, are likely to be inappropriate when used in conjunction with Gram positive, non-flagellated E. faecalis, which binds to biotic surfaces (O’Toole et al., 2000; O’Toole & Kolter, 1998).

Several biofilm formation assays, using simple apparatus, are available; however, issues exist with biofilm assays, such as the polystyrene microplate assays, which have yet to be resolved. Leuck et al. (2014) revealed that enterococcal clinical isolates, which could from biofilm on porcine heart valves produced weak and variable biofilm on polystyrene microplates. They suggested that enterococcal ex vivo biofilm formation should be performed on relevant tissue substrates. Both collagen and gelatin have been investigated as a support for biofilm formation. Gelatin has been shown to provide significant improvements on biofilm formation as compared to polystyrene and glass alone (Bukhari, 2013), whereas collagen coating has been shown to increase the polysaccharide concentration of enterococcal biofilms (Ali et al., 2020). The results obtained with gelatin coated glass align with results from Bukhari (2013), whereby substrate improvements with tissue components (collagen based) improve enterococcal biofilm formation.

There are many devices described in the literature for the study of biofilm formation. The Calgary biofilm device can only be imaged with glass bottomed microplates (Ceri et al., 1999). Coupon based biofilm apparatuses such as the drip-flow biofilm reactor, rely on an insert that must be removed and processed, increasing chances of damage (Xu et al., 1998). In terms of biofilm, Leuck et al. (2014) stated that enterococcal ex vivo biofilm formation can often be weak as compared to using in vivo substrates or explanted tissue. The mechanical stresses applied to biofilm processing, such as washing with PBS carried out by Toledo-Aranal et al. (2001), applies sheer stress to biofilm cells. This is especially true when biofilm formation assays are carried out on abiotic surfaces (polystyrene) known to facilitate weak biofilm formation, as carried out by Nallapareddy et al. (2006). Therefore, there is a need to develop procedures that allow for the study of biofilm formation that limit damage to the biofilm itself.

**CONCLUSION**

Bacterial pathogens efficiently pass on antimicrobial resistance genes through contact mediated HGT. Resistant members of the enterococcal family can easily form biofilm and conjugate antibiotic resistance genes, such as vancomycin determinants. There is a clear need not only for appropriate assays to study biofilms themselves but also for specific purposes such as the study of transfer of antibiotic resistance in enterococcal biofilm.

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**CONFLICT OF INTEREST**

The authors confirm that they have no conflict of interest.

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