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Colonization of the mammalian intestinal tract by enterococci

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Enterococci are colonizers of the mammalian gastrointestinal tract (GIT) and normally live in healthy association with their human host. However, enterococci are also major causes of healthcare-acquired infections, prompting the US Centers for Disease Control and Prevention to declare vancomycin-resistant enterococci (VRE) a serious threat to public health. Because of both intrinsic and acquired antibiotic resistance, enterococci proliferate in the GIT during antibiotic therapy, leading to dissemination and disease. The recognition that colonization of the GIT is a pre-requisite for enterococcal infections has prompted research to study mechanisms used by enterococci to colonize this niche. This review discusses major findings of recent research to understand GIT colonization by enterococci using diverse experimental models, each of which exhibits unique strengths. This work has revealed enterococcal transcriptional reprogramming in the GIT, contributions of specific enterococcal genes encoded by the core genome to GIT colonization, the impact of genome plasticity, and roles for intra-species and inter-species interactions in modulation of GIT colonization.

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Introduction

Bacteria seeking to colonize the mammalian GIT face formidable challenges from both the host and from competing microbes. These include constant expulsion of intestinal contents via peristalsis, assault by antimicrobials produced by the host and bacterial competitors, and intense competition for nutrients by the trillions of bacteria co-inhabiting the GIT. Thus, it seems likely that bacteria adept at stably inhabiting the GIT have evolved sophisticated mechanisms to overcome these challenges.

Through millions of years of evolution, enterococci have become highly adapted colonizers of the digestive tract [1]. The success of enterococci as intestinal commensals is reflected by the fact that they colonize the digestive organs of diverse organisms ranging from insects to humans [2,3]. Although enterococci are core members of the healthy human GIT microbial consortium, they only represent a minority (<1%) of the intestinal microbiota [1]. Upon antibiotic therapy of the host, however, enterococci exploit their intrinsic and acquired antibiotic resistance to proliferate in the GIT [4••], leading to domination of the GIT community and dissemination to extra-intestinal organs where they may cause life-threatening infections [5•]. Hence, enterococci lead a dual lifestyle, in which their proficiency at GIT colonization positions them to effectively exploit ecological dysbiosis resulting from iatrogenic intervention, facilitating a transition to a pathogenic state. Consistent with this, enterococci are among the leading causes of hospital acquired infections [6,7]. Hence, understanding the mechanisms enterococci use to colonize the GIT will likely suggest innovative new therapeutic strategies or targets with the potential to 'decolonize' the GIT and prevent enterococcal infections. Additionally, given that enterococci are such successful GIT commensals, it seems likely that some of the colonization strategies used by enterococci may be shared with other commensals. Investigating enterococcal colonization may therefore reveal common mechanisms that shape assembly of the GIT microbiota. However, the genetic and molecular basis for GIT colonization by enterococci remains poorly understood. Most studies of GIT colonization to date have focused on the clinically relevant enterococci, *Enterococcus faecalis* (Ef) and *Enterococcus faecium* (Efm).

Three types of experimental mouse models have been used to investigate GIT colonization by enterococci. The first involves pre-treatment of mice with antibiotics to deplete their GIT microbiota and reduce colonization resistance, enabling robust enterococcal colonization of the GIT. This model thus mimics the phenomenon of antibiotic-induced enterococcal proliferation that

facilitates pathogenesis in humans. The second strategy exploits germ-free animals for GIT colonization. Enterococci are among the first organisms to colonize the newborn's GIT [8,9], so the use of germ-free animals may mimic colonization dynamics of the neonatal GIT. More recently, an experimental model that achieves colonization of the antibiotic-naïve GIT in conventional mice was developed [10••], enabling investigation of the factors that promote colonization of the unperturbed GIT. Below we summarize insights into enterococcal GIT colonization that have emerged from studies using each of these models.

Nutritional adaptation

The genomes of enterococci lack the machinery for biosynthesis of several amino acids and vitamins [1,11]. Enterococci thus rely on acquisition of nutrients from their environment to outcompete other microbes. Comparative analysis of Efm clinical and commensal isolates revealed that a four-gene cluster, encoding a putative mannose/fructose/sorbose family phosphotransferase system (PTS), is enriched in clinical Efm isolates from human and veterinary infections [12], suggesting this PTS promotes colonization of the antibiotic-perturbed GIT. PTSs are specialized, substrate-specific transport systems that mediate carbohydrate uptake. Deletion of *ptsD*, which encodes the predicted transporter, impaired colonization of the GIT in antibiotic-perturbed mice [12]. Paradoxically, elimination of the ability of an Ef strain to use ethanolamine (an abundant nutrient in the GIT) enabled the ethanolamine-deficient mutant to outcompete an otherwise wild-type strain in the antibiotic-perturbed mouse GIT [13]. Although the underlying mechanisms remain to be elucidated, these studies highlight the importance of nutrient acquisition in colonization of the GIT.

Investigation of the enterococcal transcriptional response during GIT colonization revealed additional evidence of nutritional adaptation. Lindenstrauss *et al.* inoculated the GIT of germ-free mice with Ef and analyzed the enterococcal transcriptome by RNA-seq [14], revealing that the majority of induced genes are involved in nutrient transport or metabolism (e.g. multiple PTSs, and genes for glycerol metabolism). The results also revealed downregulation of the notable virulence proteins SprE (a serine protease) and the GelE metalloprotease. These findings suggest that, upon introduction to the sterile GIT, enterococci repress virulence gene expression and rely on import of nutrients present in this environment to persist.

To assess changes in Ef gene expression during colonization of the unperturbed GIT, a previously described Recombinase-based *in vitro* Expression Technology (RIVET) system [15] was applied in the antibiotic-free colonization model [10••]. The screen identified 114 Ef promoters that were induced in the GIT (unpublished data). Similar to the Lindenstrauss study [14], genes involved in nutrient transport and energy metabolism were the major functional categories identified as induced in the GIT.

Genome plasticity and antimicrobial production

Comparative genomics of Efm isolates from diverse sources revealed a phylogenetic split into 2 clades—a hospital-associated clade (clade A) and a community-associated clade (clade B), which exhibit substantial differences in the content of their genomes [16–18]. Clade B strains outcompete clade A strains when co-cultured *in vitro* and when competed with each other in an antibiotic-treated mouse GIT colonization model [19]. Although this study did not identify the specific gene(s) responsible, it is clear that differences in genome content can influence competitive fitness of enterococcal lineages to impact GIT colonization.

The presence of chromosomally integrated bacteriophages is variable among enterococcal isolates. Duerkop *et al.* [20•] reported that a particular composite bacteriophage found in Ef V583 (a VRE isolate) confers a competitive advantage over Ef lacking the phage during colonization of the antibiotic-treated mouse GIT. Lysogens of an Ef strain that previously lacked the phage (resistant to phage infection) no longer exhibited a competitive defect, indicating that phage production in the GIT can impact the dynamics of enterococcal colonization in the GIT.

Naturally occurring plasmids contribute to the plasticity of enterococcal genomes and thus the evolution of hospital-adapted multidrug-resistant enterococci [21]. In addition, plasmids can influence GIT colonization fitness. For example, a study by Rice *et al.* [22] found that acquisition of a plasmid carrying a hyaluronidase gene enhances GIT colonization by Efm in an antibiotic-treated mouse model, and this trait was transferable to other Efm strains; however, the specific plasmid gene(s) conferring this phenotype were not identified. Gilmore *et al.* [23] reported inhibition of VRE by commensal enterococci in an *ex vivo* culture system through a mechanism in which commensal enterococci induced lethal cross-talk between mobile genetic elements resident in the VRE. These findings suggest that accretion of mobile elements (typically encoding antibiotic resistance) in multi-drug resistant enterococcal isolates render them incompatible with commensal enterococci, suggesting by extension that VRE occupy distinct niches in the GIT from commensal enterococci.

Similar to other microbial ecosystems, commensals in the GIT enhance their competitive fitness by producing antimicrobials to inhibit the growth of competing bacteria [24]. A subset of these antimicrobials are bacteriocins,

often genetically encoded on conjugative plasmids. Analyzing colonization of the unperturbed mouse GIT, Kommineni *et al.* [10••] observed that the bacteriocin-encoding plasmid pPD1 enhances the ability of Ef to colonize the GIT. Carriage of pPD1 allowed newly introduced Ef strains to displace preexisting enterococcal populations, including VRE. Importantly, the pPD1-mediated colonization advantage required the resident bacteriocin synthesis operon. Thus, this study demonstrated a role for naturally occurring plasmids in promoting GIT colonization, and provided evidence that plasmid-encoded bacteriocins could potentially be leveraged in therapeutic strategies intended to reduce the GIT burden of multidrug resistant enterococci [25].

Cell envelope integrity and antimicrobial resistance

A recent study by Lebreton *et al.* [26••] tracing the evolution of the *Enterococcus* genus argues that the success of these organisms as GIT commensals is tightly linked to the evolution of a robust cell envelope. This study found that the origins of the *Enterococcus* genus coincided with the terrestrialization of their hosts, and that this transition required the development of a hardened cell envelope to facilitate survival under harsh physico-chemical conditions encountered during transmission from host to host on land. Given that many intestinally produced antimicrobial agents target the cell envelope, this hardened cell envelope also contributes to antimicrobial resistance, suggesting that maintenance of envelope integrity is a key determinant of GIT colonization. In *E. faecalis*, IreK, a transmembrane Ser/Thr kinase in the PASTA kinase protein family, is critical for cell envelope integrity and resistance towards antimicrobials that target the cell wall [27,28]. Deletion of *ireK* in Ef resulted in a profound GIT colonization defect in antibiotic-naïve mice [29], consistent with the hypothesis that maintenance of enterococcal cell envelope integrity is a key driver of efficient GIT colonization. However, resistance to individual intestinal antimicrobials is not, in itself, critical for GIT colonization, as Ef mutants singularly defective in resistance to either bile acids or lysozyme retained the ability to colonize [29].

The enterococcal polysaccharide antigen (Epa) is a rhamnose-containing cell-wall polysaccharide whose functions are not fully understood. Epa was shown to be important for cell shape, resistance to phage-induced lysis, biofilm formation and virulence in mice [30]. Although a core *epa* locus is conserved across enterococci, the full *epa* locus varies in organization and gene content among strains, suggesting biochemical variation of the polysaccharide [17]. Analysis of the genome content of a subset of virulent Ef clinical isolates revealed the enrichment of *epaX* (an 'accessory' *epa* gene) in these hospital-associated Ef isolates. Deletion of *epaX* altered the composition of Epa polysaccharide, increased susceptibility to the bile acid cholate, and impaired GIT colonization in antibiotic-treated mice, suggesting that strain-specific biochemical features of Epa can influence colonization of the GIT [31].

Biofilm formation and physical interactions with the intestinal environment

Peristalsis poses a challenge to intestinal colonization. To overcome this challenge, one hypothesis proposes that commensals have developed mechanisms to adhere to the mucus layer [32,33]. The turnover rate of the mucus layer is slower than the peristalsis-driven transit time for intestinal contents, suggesting that mucus binding would be advantageous during intestinal colonization [34]. In addition, some researchers proposed that commensals associate with mucosal structures as part of biofilms [32,33,35]. Microscopic examination of intestinal sections from germ-free mice colonized with Ef revealed biofilm microcolonies throughout the GIT [36]. Ef microcolonies were found to abut the intestinal epithelial cells, indicating that these structures form at the base of the inner mucus layer. It is unclear if this phenomenon is specific to the monoassociation mouse model, or if it can be generalized to mice colonized with a complex GIT community. Others have reported the presence of bacteria adjacent to the epithelium at a limited number of sites along the GIT, although not specifically in the form of a biofilm [37,38]. Whether enterococcal microcolonies persist in the complex microbiota remains unknown; if so, these structures could represent a reservoir from which enterococci could continuously seed the GIT lumen. Formation of biofilms in the GIT by enterococci could also have important implications on the ability of these organisms to exchange genetic material and tolerate antibiotics in this environment.

Using antibiotic-treated mouse models, several studies have identified genes that influence enterococcal biofilm formation *in vitro* as contributors to GIT colonization. Deletion of *ebrB*, which encodes an AraC family transcriptional regulator required for biofilm formation, results in a modest decrease in GIT colonization by Efm [39]. EbrB was also found to be required for expression of Esp, a cell wall anchored protein required for biofilm formation. Previous studies had indicated that Esp, although enriched among clinical Ef and Efm isolates, does not influence intestinal colonization by either organism [40,41], suggesting that the contributions of EbrB to GIT colonization is independent of its regulation of Esp.

Disruption of the *bop* (biofilm on plastic) locus, which contains putative maltose metabolism genes, results in a moderate delay in GIT colonization by Ef [42]. *bop* genes influence biofilm formation, but their specific effect depends on growth conditions. Mutants lacking the *bopABC* genes form more biofilms than wild type cells

in the presence of glucose, while they are unable to produce biofilms in the presence of maltose. Although the reason for these opposing phenotypes remains unclear, this study suggests that the ability to form biofilms can influence GIT colonization by enterococci, and by extension that the nature of available nutrients may impact biofilm-mediated colonization.

Sortase A (SrtA) is a membrane-associated enzyme that mediates anchoring of surface proteins to the enterococcal cell wall [43] and promotes biofilm formation [44,45]. Inspection of the genome of Ef OG1RF revealed that it encodes 21 predicted sortase-dependent cell wall-anchored proteins, including cell-surface adhesins such as Ace (mediates binding to extracellular matrix proteins [46]) and the Ebp pilus (a polymeric structure previously shown to mediate biofilm formation and adhesion to various host extracellular matrix proteins [47,48]). Hypothesizing that SrtA-dependent surface proteins could be important for binding to host molecules in the GIT, we found that an Ef mutant lacking SrtA was defective in adherence to mucin *in vitro*, and this defect could be traced specifically to loss of a combination of Ace and Ebp (unpublished data). Using the antibiotic-naïve mouse GIT colonization model, we found that enterococcal mutants (both Ef and Efm) lacking sortase were impaired at GIT colonization as well (unpublished data). Together, these data suggest that enterococci use their surface proteins to facilitate retention in the mucus layer during GIT colonization.

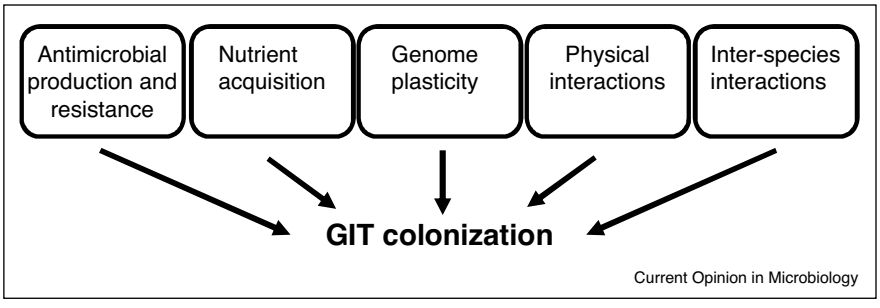
Microbiota and bacterial competition

The proliferation of GIT enterococci that occurs during antibiotic therapy reflects the elimination of microbial components that regulate the enterococcal population, either directly or through their interaction with the host mucosal immune system [5•,49•,50,51]. The longstanding view is that enterococci proliferate because of increased availability of nutrients and physical niche(s) in the context of a depleted microbiota. Recent research has provided a more detailed understanding of this phenomenon. In hospitalized patients placed on antibiotic therapy, colonization with bacteria belonging to the *Barnesiella* genus has a protective effect against domination by VRE [52]. Detailed analysis of microbial taxa associated with elimination of VRE in mice revealed that cooperative relationships between *Clostridum bolteae* and *Blautia producta* drive clearance of VRE [53•]. The presence of *C. bolteae* promotes colonization of *B. producta*, which directly suppresses the proliferation of VRE, although the specific mechanism by which this occurs is unclear. This data indicates that enterococcal populations in the GIT can be directly modulated by the presence of other members of the microbiota, although the extent to which this phenomenon can be generalized across commensal enterococcal lineages, and across hosts with diverse GIT communities, remains unknown.

Conclusion

Although much work remains to truly understand the mechanisms by which enterococci establish and maintain colonization of the mammalian GIT, the initial studies summarized here represent important initial steps towards that goal. These findings begin to paint an emerging picture of what is likely to be a sophisticated, multifactorial strategy employed by enterococci to ensure they maintain a foothold in the highly competitive environment of the GIT (Figure 1). Already we have evidence that enterococcal cell surface proteins, maintenance of cell envelope integrity, adaptation to available nutrient sources, and potentially formation of biofilms *in vivo* play key roles in promoting GIT colonization. Most of these functions are mediated largely by genes encoded in the core enterococcal genome, which is consistent with the concept that enterococci have evolved for millennia as GIT commensals, and remain ubiquitous GIT colonizers today. Hence, future investigations into the roles of core genes during GIT colonization are likely to be informative as we seek to understand the forces that shape enterococcal-microbiota-host interactions and dynamics. The role of genome plasticity as an important driver of GIT colonization should not be overlooked, however. Already we have evidence indicating that, for example, acquisition of accessory genes can alter the Epa surface

Figure 1



Multifactorial strategy for enterococcal GIT colonization.
Multiple traits encoded by both the core enterococcal genome as well as mobile genetic elements together influence GIT colonization.

polysaccharide with consequences for GIT colonization, and that various types of mobile genetic elements can also impact colonization. It is possible that such elements in the accessory genome are especially important for colonization in the dysbiotic setting of the antibiotic-treated GIT, although more comparative studies in antibiotic-naïve mice are necessary to definitively establish such a conclusion. Lastly, acquisition of the capacity to produce bacteriocins can provide a substantial colonization advantage sufficient to displace established enterococcal lineages from the GIT, a significant observation with obvious potential to be developed into new therapeutic strategies with the goal of preventing infections by antibiotic-resistant enterococci in the future.

Conflict of interest

The authors declare no conflict of interest.

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