Pheromone killing of multidrug-resistant *Enterococcus faecalis* V583 by native commensal strains

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Multidrug-resistant *Enterococcus faecalis* possess numerous mobile elements that encode virulence and antibiotic resistance traits as well as new metabolic pathways, often constituting over one-quarter of the genome. It was of interest to determine how this large accretion of mobile elements affects competitive growth in the gastrointestinal (GI) tract consortium. We unexpectedly observed that the prototype clinical isolate strain V583 was actively killed by GI tract flora, whereas commensal enterococci flourished. It was found that killing of V583 resulted from lethal cross-talk between accumulated mobile elements and that this cross-talk was induced by a heptapeptide pheromone produced by native *E. faecalis* present in the fecal consortium. These results highlight two important aspects of the evolution of multidrug-resistant enterococci: (i) the accretion of mobile elements in *E. faecalis* V583 renders it incompatible with commensal strains, and (ii) because of this incompatibility, multidrug-resistant strains sharing features found in V583 cannot coexist with commensal strains. The accumulation of mobile elements in hospital isolates of enterococci can include those that are inherently incompatible with native flora, highlighting the importance of maintaining commensal populations as means of preventing colonization and subsequent infection by multidrug-resistant strains.

*Enterococcus* | vancomycin | antibiotics | resistance | fitness cost

The human gastrointestinal (GI) tract is a highly competitive environment colonized by a diverse microbial population (1). Diversity is shaped by factors such as vertical transmission, immune selection, diet, and other influences, including antibiotic therapy (2–4). Enterococci are ancient members of GI tract consortia of everything from humans to insects (5–7). Commensal enterococci have reduced genomes (∼2.7 Mb) (8) and despite their success in this highly competitive environment, possess auxotrophies for amino acids, vitamins, and other micronutrients (9). Paralleling the broad application of antibiotics in healthcare and animal husbandry, multidrug-resistant (MDR) enterococci emerged as leading causes of hospital infection (7, 10, 11). Approximately one-third are now vancomycin-resistant (VRE) (6).

Genomes of pathogenic MDR enterococci are replete with mobile genetic elements (8, 11). *Enterococcus faecalis* V583, the first VRE *Enterococcus* isolated in the United States (12), is additionally resistant to high levels of aminoglycosides, macrolides, lincosamides, and streptogram B and has an enlarged 3.36-Mb genome that includes six plasmids or plasmid-like elements (three integrated into the chromosome), seven prophages, and numerous other insertions (ISs), including a 138-kb pathogenicity island (PAI), transposons, and 38 IS elements (13, 14). This accumulation contrasts sharply with OG1RF, a well-characterized, commensal-like isolate (15, 16) that has a 2.7-Mb genome containing only a prophage previously identified as being ubiquitous in the species (17) and a remnant of a nonantibiotic resistance transposon of unknown functionality (18). Hospital-adapted MDR *E. faecalis* isolates generally lack a functional clustered regularly interspaced short palindromic repeats locus, which seems to have facilitated mobile element accretion (19).

MDR enterococci colonize the patient after perturbation of the native flora by antibiotic treatment, including therapies with little or no antienterococcal activity (6, 20–22). The *E. faecalis* V583 PAI and other mobile elements confer the ability to produce new virulence factors, produce colonization traits, and use novel metabolites (14). This observation raises the question as to whether pathogenic MDR enterococci occupy the same niche as native commensal enterococci (20).

At minimum, the simple maintenance of 600 kb of foreign DNA on mobile elements in V583 (13) would be predicted to impose a burden in a highly competitive environment where there is no offsetting antibiotic selection. We hypothesized that some or most of these mobile elements were obtained in an competitive antibiotic-affected environment, resulting in little penalty for an accompanying fitness cost. If true, the large complement of mobile DNA in V583 could compromise its ability to proliferate in a competitive, native GI tract consortium and possibly, fundamentally alter its interaction with other members of that consortium. To determine whether *E. faecalis* V583 behaved in ways different from a commensal strain (using for comparison the commensal-like nonantibiotic-resistant OG1), we compared

**Significance**

Multidrug-resistant enterococci are leading causes of hospital infection. The antibiotic-perturbed patient gut serves as a staging ground—small numbers of resistant hospital strains colonize and then, greatly amplify in the colon. Little is known of the colonization principles involved—whether hospital strains are competitive or noncompetitive with commensal enterococci or whether mobile elements comprising over 25% of the genome of the former impose significant fitness costs. We unexpectedly found that the prototype vancomycin-resistant *Enterococcus faecalis* strain V583 was actively killed by fecal organisms, and we traced that to pheromone production by commensal enterococci that trigger lethal mobile element cross-talk. This work highlights the importance of maintaining commensal enterococci in the gut of the hospitalized patient.

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their growth in the presence of healthy human fecal flora under a number of conditions. Not only did the healthy flora inhibit V583 (and not OG1), but the fecal consortium actively killed the MDR strain. With a view toward limiting the outgrowth of VRE in the GI tract of hospitalized patients, we determined the basis for this antagonism.

Results

MDR E. faecalis V583 Is Killed When Cultured with Healthy Human Flora. To determine whether MDR V583 was compromised in its ability to compete among healthy human GI tract flora, we compared its growth with that of model commensal OG1X [a sister clone of OG1RF that possesses a selectable streptomycin resistance marker (15)]. To simulate environmental cues and carbon sources that may occur in the GI tract (23), we developed an M9 minimal salts-based medium (24) that included 1% crude hog gastric mucin (HGM9; Sigma). Fig. 1 shows that, in HGM9 monoculture, either V583 (Fig. 1A) or OG1X proliferates (Fig. 1B). However, healthy flora derived from pooled human fecal samples imposed competitive limitation on each one.

To probe the relationship between the E. faecalis being compared and members of the fecal consortium, various carbohydrates were added, including some utilizable by E. faecalis and others utilizable only by members of the fecal consortium. These carbohydrates included N-acetyleneuraminic acid, D-fucose, N-galactosamine, N-panosamine, N-galactose, GalNAc, and as a control, N-glucose. To our surprise, we observed the active, selective killing of MDR V583 but not OG1X in HGM9 to which glucose, N-acetylgalactosamine, galactose, or panose had been added (Fig. 1).

Genetic Basis for V583 Susceptibility to Killing. To determine why V583 was inhibited by commensal E. faecalis but OG1RF and other strains were not, we examined differences between their genomes. As noted, V583 harbors ∼600 kb mobile element DNA that is absent from OG1RF (16, 18). The largest V583 mobile element is the 138-kb PAI (13, 14). We recently reported the generation of OG1RF/V583 hybrids possessing from 285 to 858 kb of V583 chromosomal DNA, including the PAI and flanking sequences that encompass it (30), in the OG1RF background. To test whether susceptibility to killing was encoded by the PAI or the considerable length of V583 sequence on either side, E. faecalis Pan7 was spotted onto soft agar lawns containing V583 or hybrids. Tc5 (an OG1RF hybrid possessing the V583 PAI plus 147 kb of additional flanking V583 sequence) or Tc12 (an OG1RF hybrid that harbors the PAI plus an additional 720 kb of flanking V583 genome sequence) (30). As shown in Fig. 3A, neither the PAI nor the large portions of V583 chromosomal DNA surrounding it confer sensitivity to Pan7-mediated killing.

Other mobile elements unique to V583 include its plasmids. A plasmid-cured derivative, V19 (31), was tested. Unlike V583, V19 grew well in coculture with Pan7 (Fig. 3B). This result implicated the involvement of one or more plasmids in the V583 susceptibility phenotype. Therefore, plasmids pTEF1 and pTEF2
Fig. 2. Commensal enterococci kill MDR *E. faecalis* V583. (A) Preculture of the fecal consortium in varying levels of erythromycin eliminated the ability of the fecal consortium to kill V583. (B) Erythromycin pretreatment of the fecal consortium also eliminated native enterococci from the consortium, whereas *Enterobacteriaceae* remained relatively unchanged. (C) Tetracycline pretreatment of the fecal consortium does not eliminate V583 killing and enhances killing, even in the absence of added galactose. (D) Tetracycline pretreatment of the fecal consortium enriched the presence of native enterococci in the consortium and inhibited *Enterobacteriaceae*, showing that they are not involved in V583 elimination and suggesting that the former may be involved. (E) Three commensal *E. faecalis* strains (OG1RF, FA2-2, and Pan7; isolated from the healthy fecal consortium) induce zones of clearing in lawns of V583. (F) Deletion of *rpoN* in the chromosome of V583 does not affect susceptibility to killing by commensal enterococci, such as Pan7.

[using pTEF2Spc (30) to provide a selectable spectinomycin resistance marker] were reintroduced into V19 (or as a control, introduced into OG1RF). V19(pTEF1) did not show susceptibility to coculture-mediated inhibition, but V19(pTEF2Spc) was found to be highly susceptible (Fig. 3B).

Whereas OG1RF and Pan7 were able to kill V583 or V19(pTEF2), transconjugants OG1RF(pTEF2) and Pan7(pTEF2) had lost the ability to induce killing (Fig. 3C). The plasmid acquisition-dependent loss of effector/killing activity was reminiscent of the known plasmid acquisition-associated loss of pheromone production by *E. faecalis* (32–34). If pTEF2 suppressed expression of the killing factor in a manner analogous to suppression of pheromone production in its host, then it stood to reason that the plasmid-free variant of V583 lacking pTEF2, strain V19, itself might now be capable of killing V583. We found that this was, in fact, the case (Fig. 3D). Interestingly, unlike the V19(pTEF2) transconjugant, OG1RF(pTEF2) and Pan7(pTEF2) remained resistant to killing (*SI Materials and Methods* and Fig. S1). This observation suggested that pTEF2 contributed to susceptibility but by itself, was insufficient to completely confer the susceptible phenotype.

**pTEF2-Dependent Killing Is Mediated by a Pheromone.** Enterococcal pheromones are generated by processing and release of short lipoprotein signal peptide fragments (33, 35). One step in the processing is mediated by membrane metalloprotease Eep (35). If pheromone production by Pan7 and other commensal *E. faecalis* was involved in killing, then an *eep* mutant should be defective as an effector/killing strain. In fact, *E. faecalis* FA2-2Δeep (36) showed partially diminished killing of V583 (Fig. 4A). In a screen of *mariner* IS mutants in FA2-2 for loss of killing activity, an additional mutant was found that possessed a transposon IS in ORF EF0688 as recently reported (37). This mutant was even more defective in killing than the *eep* mutant, suggesting that the putative ATP binding cassette transporter was involved in the pheromone production pathway. Because ef0688 and ef0689 were predicted to reside on the same transcript, a specific deletion of reading frames EF0688 and EF0689 was generated, FA2-2Δ*efAB*, and verified as having a more than fivefold decrease in killing activity (Fig. 4A).

The known pheromone receptors TraC and PrgZ, which are encoded on plasmidea-responsive plasmids pAD1 (38) and pCF10 (39), specifically bind and facilitate pheromone uptake (40). A homolog was identified by BLASTP (41) on pTEF2 EFB0004 (traC-2). To test its role in conferring the susceptibility phenotype, we constructed an in-frame deletion of traC-2 in pTEF2 in V583. As predicted, the traC-2 null mutant showed decreased susceptibility to Pan7 killing (Fig. 4B), further implicating pheromones.

**Identification of the Effector Pheromone.** To identify the hypothetical pheromone responsible for killing, we developed a library of synthetic peptides corresponding to all predicted pheromone sequences inferred from the *E. faecalis* genome sequence (13). Pheromone peptides are derived from the seven to nine amino acids preceding the signal peptide cleavage site cysteine of lipoproteins (35, 42). Based on the V583 genome (13), 81 oligopeptides inferred from lipoprotein leader sequences were synthesized (Table S1) and tested for inhibition of V583. Three inhibited V583 and also, V19 in a pTEF2-dependent manner (Fig. 4B). One in particular (NH3-VAVLVLGA-COOH) possessed activity in picomolar concentrations, being >104-fold more active than the only other two and showing inhibitory activity. This highly active peptide matched a pheromone previously identified as cOB1 (43). To confirm that cOB1 was responsible for inhibition of V583, the sequence was scrambled, retested, and found to have lost all inhibitory activity (Table S1). To confirm that cOB1 production by the effector strain was responsible for killing, the precursor lipoprotein encoding cOB1, *ef2496*, was deleted from the chromosome of *E. faecalis* FA2-2. As predicted, FA2-2Δ*ef2496* lost the ability to inhibit V583 (Fig. 4C), and the inhibitory phenotype was restored by repair of the *ef2496* reading frame, (Fig. 4D), proving...
that cOB1 production by the effector was necessary and sufficient to mediate V583 killing.

Identification of the Second V583 Locus Required for the Susceptible Phenotype.

Identification of a heptapeptide that inhibited V583 gave us a tool to isolate cOB1-resistant mutants of V583. To ensure that resistance did not derive from the simple curing of pTEF2, a chloramphenicol-resistant derivative of pTEF2 (pTEF2Cm) (30) was introduced into plasmid-cured strain V19. As shown in Fig. 4B, cOB1-resistant mutants, selected in the presence of 15 μg/mL chloramphenicol, occurred at an unexpectedly high frequency of $2.4 \times 10^{-6}$. One arbitrarily selected mutant, V19(pTEF2)^PR, was subcultured in the absence of cOB1 selection and found to be stably resistant to cOB1 killing. The genome of V19(pTEF2)^PR was sequenced and aligned to V583 (13). The main difference was the mutation of a 32,719-bp IS-like element annotated as a plasmid remnant (base pairs 131,647–164,366 encompassing ORFs EF0127 to EF0166) (Fig. S2) (17). This region includes genes predicted to encode a relaxase (EF0143), recombinase (EF0166), surface exclusion protein (EF0146), and aggregation substance (EF0149) along with a number of conserved hypothetical...
Other E. faecalis Strains Harbor Portions of the IS-Like Element That Correlates with Susceptibility to cOB1. We recently generated a large number of draft genome sequences for a diverse collection of E. faecalis (https://olive.broadinstitute.org/projects/work_package_6) and queried them for integration of an element similar to that lost by V19(pTEF2). Thirteen E. faecalis strains of diverse multilocus sequence types possessed varying portions. Plasmid pTEF2Cm was introduced into each strain by filter mating, and 10 became sensitive to the cOB1 pheromone. Alignment of the genomes of the pheromone-susceptible and -nonsusceptible isolates (Fig. S3) allowed us to narrow the region of the inserted element that sensitizes a strain to pheromone-mediated killing in the presence of pTEF2. All cOB1-susceptible strains possessed at least genes EF0151–EF0165, implicating them or sequences encompassed in the cOB1 susceptibility phenotype. Direct deletion of DNA encoding EF0143–EF0160 rendered V583 resistant to cOB1 killing (Fig. S4), further narrowing the genes involved. Specific deletion of either a putative relaxase (EF0143) or a putative amidase (EF0160), however, did not confer resistance.

Discussion
Little is actually known about the nature of Enterococcus existence in native ecologies. In hospitals, patients acquire MDR E. faecalis and Enterococcus faecium in their GI tract consortia after admission, mainly from contaminated surfaces (6). Colonization is facilitated by destabilization of the patient’s native microbiota by antibiotic treatment, a main predisposing factor (6). A few orally acquired cells are well-positioned to greatly expand into antibiotic-vacated habitats of the gut, which serves as a staging ground for infection. The precise nature of the food web in which the auxotrophic needs are satisfied for either commensal enterococci or hospital-endemic MDR strains is currently unknown. Understanding the fundamental principles of colonization could provide new insights for preventing downstream, antibiotic refractory infection.

We found that prototype MDR E. faecalis V583 possessed limited ability to grow in a consortium of GI tract flora that included commensal enterococci. The incompatibility between V583 and commensal enterococci was traced to the production of plasmid pTEF2 and an IS-like element resident in V583–plasmid pTEF2 and an IS-like element resident within the chromosome (Fig. 5). This incompatibility suggests that the accretion of these elements by V583 occurred in the relative absence of commensal enterococci. The susceptibility of V583 to killing by commensal strains provides strong evidence that they rarely occur in the same niche and that V583 has proliferated in habitats in which commensal strains are excluded—because of nutritional requirements, lack of colonization traits, such as those encoded by the PAI, or antibiotic elimination.

The precise mechanism for cOB1 killing of V583 is the subject of ongoing study. Microarray experiments found high levels of cOB1-mediated induction (>100-fold) of many pTEF2 genes related to conjugal transfer and little change in chromosomal gene expression (Tables S2 and S3), giving no obvious clue to the interactions between pTEF2 and the chromosomally integrated IS-like element that is necessary for susceptibility. Recent work from the laboratory of Menard and Grossman (44) has shown that imprecise excision of the Bacillus subtilis integrative and conjugative element, ICEBsl1, from nonpreferred sites of integration leads to a loss in cell viability. The element studied here shares properties with ICEBsl1, including terminal repeats and genes related to movement. The fact that its imprecise excision may somehow be triggered in trans by pheromone-induced gene products of pTEF2 seems likely. However, we showed that this hypothetical excision is not mediated by the relaxase encoded within the IS-like element, EF0143, or the pTEF2 relaxase mutated in a previous study (30), because both mutants are sensitive to pheromone inhibition. It seems that other proteins encoded by pTEF2 act in trans to volatilize the chromosomal IS-like element on cOB1 induction or otherwise, result in effects that are toxic to the cell (Fig. 5).

Irrespective of mechanism, the incompatibility between V583 and commensal strains of E. faecalis provides clear support for the hypothesis that this MDR hospital strain and commensal enterococci do not occupy the same habitat (20) and begins to shed light on differences in the colonization properties of hospital and commensal lineages of enterococci. This incompatibility highlights the opportunity for therapies that preserve native enterococcal flora as a barrier to colonization by such hospital-adapted lineages.

Materials and Methods
Bacterial Strains and Growth of Enterococci in Fecal Consortia. Fecal samples from healthy volunteers were obtained and pooled using an institutional review board-approved protocol. Pan7, a nonhemolytic, tetracycline-resistant E. faecalis strain speicied by 16S rDNA sequence, was the most abundant Enterococcus in the pooled fecal consortium. Other strains used in this study are listed in Table S4. To approximate cues that occur in the GI tract, a medium was developed that included a commercially available crude extract of HGM (Pflazt & Bauer); 1% HGM9 was made by dissolving 1% (wt/vol) HGM in Davis minimal medium (24), pH 7.5. E. faecalis and fecal bacteria were grown in 1% HGM9 at 37 °C without shaking. A layer of autoclaved light mineral oil (Fisher Scientific) was added to cultures containing fecal bacteria to limit oxygenation.

Fitness of V583 and Mutants Assessed in Coculture. The effect of fecal flora on growth and proliferation of V583 was determined in 1% HGM9. Various carbohydrates were added to induce population shifts within the fecal population. Details of the coculture assay are provided in SI Materials and Methods.
Assessment of Killing Activity of Purified Peptides. Soft agar overlay assays were used to screen and visualize killing of various E. faecalis strains by commercial enterococci or purified pheromones as described in SI Materials and Methods. V19(pTET2), which showed the greatest sensitivity, was used in soft agar overlays to detect inhibition by purified pheromones. The 81 theorized pheromone peptides were synthesized initially at >75% purity (Lifetein, LLC) and resuspended in acetonitrile. Peptides exhibiting inhibitory activity were re-synthesized and HPLC-purified to >95% purity. Inhibitory activity was determined as a visible zone of clearing affected by where 1 μL 1-mg/mL solution of the peptide was placed. To determine comparative activities of peptides exhibiting activity, highly purified peptides were diluted twofold in acetonitrile, and 1-μL aliquots of each dilution were again spotted on the top agar lawn.

Generation of trac2, EF0143, EF0160, and EF0143–EF0160 Deletion Mutants in V583 and Deletion of EF2496 in FA2-2. Deletion of most of the coding sequences of trac2, EF0143, EF0160, and EF2496 in strain FA2-2, generating in-frame fusions of sequences near the start and stop codons of each, minimized polar effects essentially as described (45). A similar approach was used to delete sequences spanning from EF0143 to EF0160. Primers used for the construction of plasmid deletion constructs are listed in Table S5, and detailed information on construction of mutants is provided in SI Materials and Methods.

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