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

Michael S. Gilmore[a,b,1], Marcus Rauch[a,b,2,3], Matthew M. Ramsey[a,b,2,4], Paul R. Himes[a,b,5], Sriram Varahan[c], Janet M. Manson[a,b,6], Francois Lebreton[a,b], and Lynn Ernest Hancock[c]

[a]Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA 02114; [b]Department of Microbiology and Immunobiology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA 02114; and [c]Department of Molecular Biosciences, University of Kansas, Lawrence, KS 66045

Edited by Roy Curtiss III, Arizona State University, Tempe, AZ, and approved April 24, 2015 (received for review January 13, 2015)

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 streptograms 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 oral 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 microniche 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 uncompetitive 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.

Author contributions: M.S.G. designed research; M.R., M.M.R., P.R.H., S.V., and J.M.M. performed research; S.V., J.M.M., and L.E.H. contributed new reagents/analytic tools; M.S.G., M.R., M.M.R., F.L., and L.E.H. analyzed data; and M.S.G., M.R., M.M.R., and L.E.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. Email: michael_gilmore@meei.harvard.edu.

[2]M.R. and M.M.R. contributed equally to this work.

[3]Present address: Janssen Prevention Center, London NW1 0NH, United Kingdom.

[4]Present address: Department of Microbiology, The Forsyth Institute, Cambridge, MA 02142.

[5]Present address: Department of Biology, University of Louisville, Louisville, KY 40292.

[6]Present address: CodonMedical, San Bruno, CA 94066.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1500553112/-/DCSupplemental.

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. 1*A*) or OG1X proliferates (Fig. 1*B*). 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*-acetylneuraminic acid, D-fucose, D-galactosamine, D-panose, D-galactose, GalNAc, and as a control, D-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).

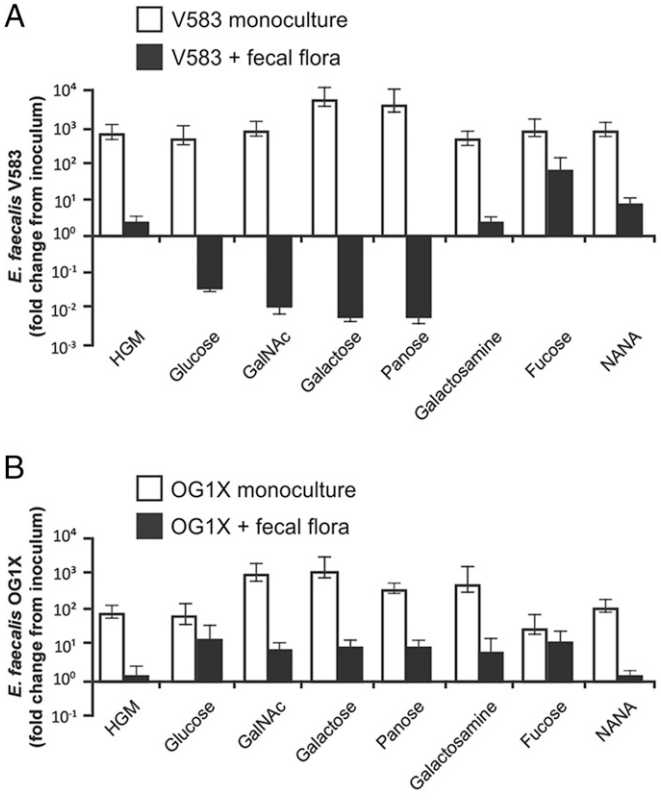

Fig. 1. Pathogenic but not commensal *E. faecalis* are killed in fecal consortia. (*A*) *E. faecalis* strain V583 and (*B*) OG1X after growth in monoculture or with added healthy human fecal flora were enumerated on Bile Esculin Azide agar containing antibiotics as appropriate. All cultures were performed in triplicate, and error bars represent the SD of the mean. NANA, *N*-acetylneuraminic acid.

This killing was observed, despite the fact that V583 grew well in monoculture in the presence of these carbon sources. In contrast, OG1X was observed to grow under all coculture conditions (Fig. 1*B*). This result was interpreted to mean that glucose, GalNAc, galactose, and panose increased the abundance of flora antagonistic to V583 or that these sugars selectively altered the metabolism of V583 (and not OG1X), rendering it susceptible to killing.

Loss of Commensal Enterococci from the Fecal Consortium Correlates with Loss of V583 Killing. In an attempt to enrich or deplete a putative V583 antagonist from the fecal consortium, we pretreated the consortium for 24 h with either erythromycin or tetracycline (Fig. 2). Erythromycin eliminated the galactose-enhanced V583 antagonism (Fig. 2*A*), which correlated with the loss of native enterococci from the fecal consortium (Fig. 2*B*). In contrast, V583 killing by the fecal consortium was unaffected and even enhanced, such that it no longer required galactose augmentation by tetracycline treatment (Fig. 2*C*), conditions that also enriched the native enterococci in the fecal consortium (Fig. 2*D*).

Because measures that inhibit native enterococci in the flora reduce V583 inhibition and measures that increase native enterococcal numbers enhance V583 inhibition and because bacteriocin production by enterococci is common and well-known (25), we selectively plated the GI tract flora onto Bile Esculin Azide agar with tetracycline to isolate implicated, potentially bacteriocin-producing, tetracycline-resistant enterococcal strain(s) resident within the consortium. The colonies obtained were uniform, and a representative colony was chosen for additional study. It was found by 16S sequencing to be *E. faecalis* and termed strain Pan7. Pan7 and as controls, the well-characterized *E. faecalis* laboratory strains OG1X (15) and FA2-2 (26) [sequenced plasmid-free strains that represent diverse points across the species phylogeny (17); both were not previously known to express bacteriocin activity] were spotted onto soft agar lawns of V583. Fig. 2*E* shows zones of inhibition around Pan7 and surprisingly, controls as well. If the V583 inhibition observed was bacteriocin-dependent, it was because of a previously unreported activity expressed by all *E. faecalis* strains tested. *E. faecalis* susceptibility to class IIa bacteriocins involves RpoN-dependent expression of a mannose phosphotransferase system operon (27, 28). To determine whether V583 sensitivity to *E. faecalis* inhibition was RpoN-dependent, V583Δ*rpoN* (29) was tested for resistance to Pan7-mediated inhibition. It was observed to be as sensitive as WT V583 (Fig. 2*F*), indicating that susceptibility was unrelated to the RpoN-dependent mechanism.

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 Tc3 (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. 3*A*, 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. 3*B*). This result implicated the involvement of one or more plasmids in the V583 susceptibility phenotype. Therefore, plasmids pTEF1 and pTEF2

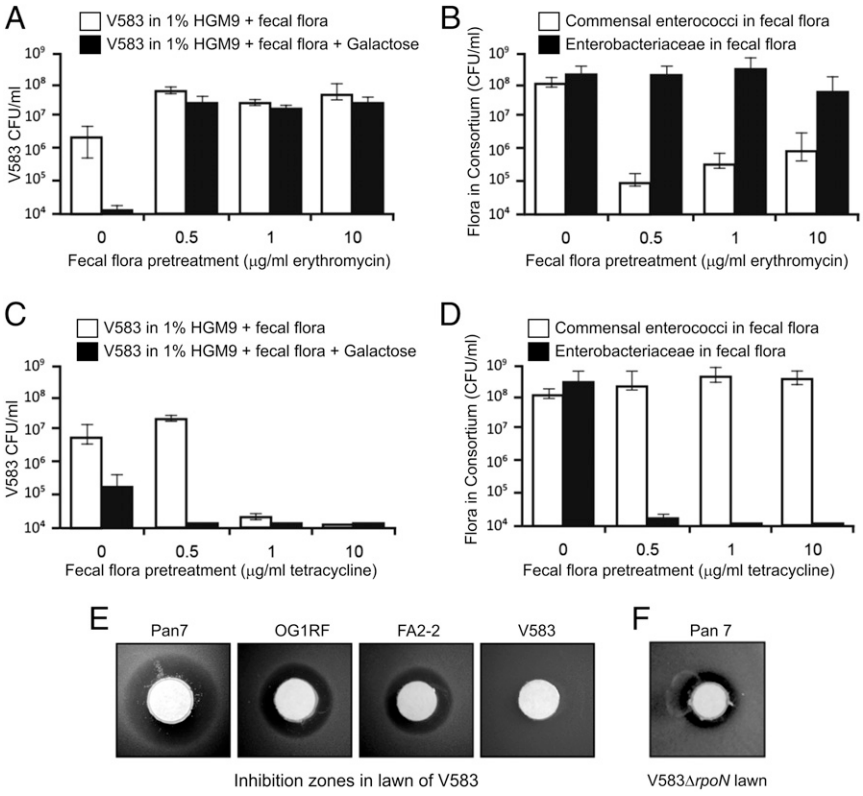

Fig. 2. Commensal enterococci kill MDR *E. faecalis* V583. (*A*) Preculture of the fecal consortium in varying levels of erythromycin eliminated the ability of the fecal consortium to kill V583. (*B*) Erythromycin pretreatment of the fecal consortium also eliminated native enterococci from the consortium, 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. 3*B*).

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. 3*C*). 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. 3*D*). 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* FA2-2Δ*eep* (36) showed partially diminished killing of V583 (Fig. 4*A*). 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*pptAB*, and verified as having a more than fivefold decrease in killing activity (Fig. 4*A*).

The known pheromone receptors TraC and PrgZ, which are encoded on pheromone-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. 4*A*), 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. 4*B*). One in particular (NH3-VAVLVLGA-COOH) possessed activity in picomolar concentrations, being $>10^6$-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. 4*A*), and the inhibitory phenotype was restored by repair of the *ef2496* reading frame, (Fig. 4*A*), proving

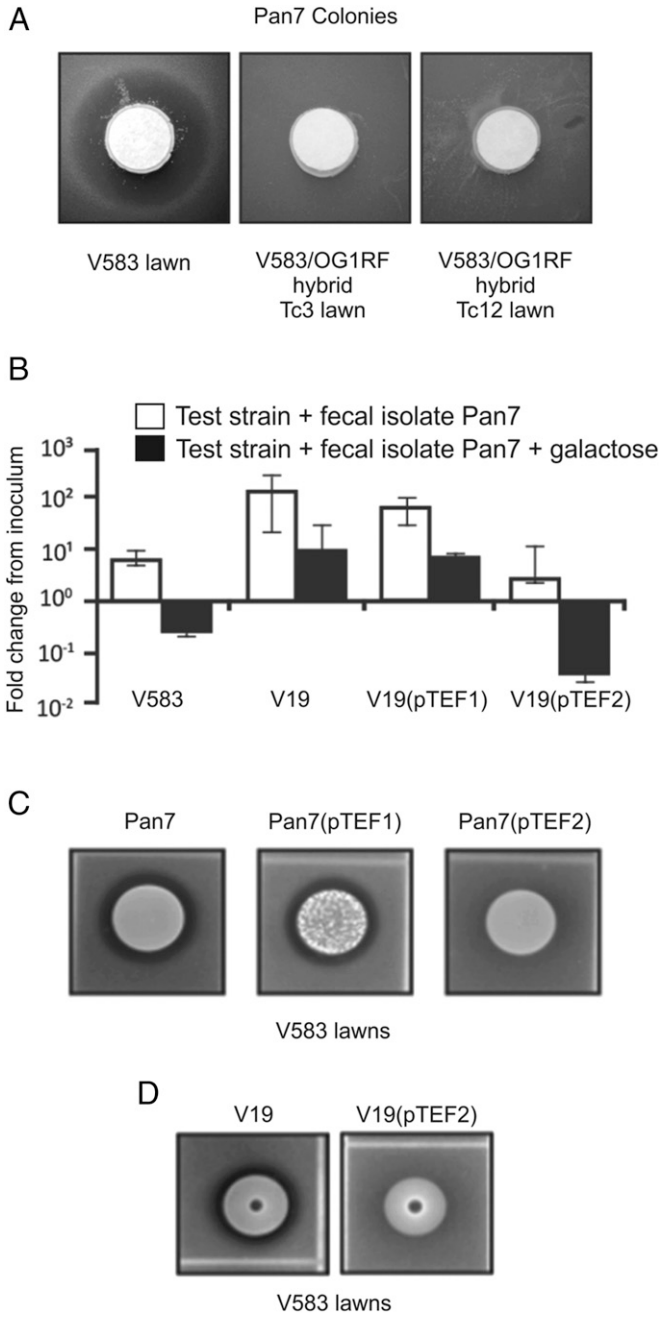

Fig. 3. Both killing and susceptibility to killing relate to the presence of pTEF2. (*A*) Zone of inhibition around *E. faecalis* Pan7 on V583 but not lawns of OG1RF/V583 hybrids containing the 138-kb V583 PAI and 147- (Tc3) or 720-kb (Tc12) additional flanking V583 chromosomal DNA in the OG1RF background. (*B*) Killing by fecal isolate Pan7 requires the presence of pTEF2 in the genetic background provided by the plasmid-cured V583 derivative, strain V19. A second pheromone-responsive plasmid present in V583, pTEF1, does not render V19 susceptible to killing. (*C*) In addition to conferring susceptibility to killing in V19, pTEF2 but not pTEF1, when transferred into commensal strain Pan7, inhibits its ability to kill V583. (*D*) Moreover, V19 (V583 cured of plasmids) becomes capable of killing V583, but that ability is lost on reacquisition of pTEF2.

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. 4*B*, cOB1-resistant mutants, selected in the presence of 15 μg/mL chloramphenicol, occurred at an unexpectedly high frequency of 2.4×10^{-6}. One arbitrarily selected mutant, V19(pTEF2)$^{\text{pheromone-resistant (PR)}}$, 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 excision from the mutant 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

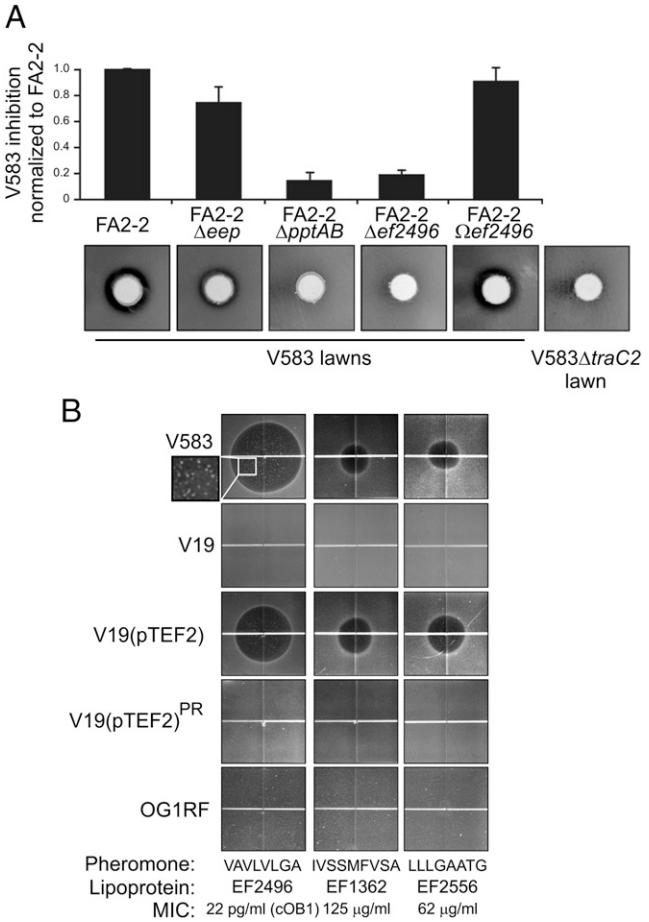

Fig. 4. V583 killing is mediated by production of pheromone cOB1 by commensal strains. (*A*) WT plasmid-free strain FA2-2 is capable of killing V583. This ability is reduced in a mutant strain defective in a membrane protease involved in processing lipoprotein signal peptides into pheromones (eep). It is nearly completely eliminated in a mutant lacking a new putative ATP binding cassette transporter encoded by *pptAB* (37). FA2-2, in which the gene encoding the lipoprotein that gives rise to pheromone cOB1, *ef2496*, is deleted, is no longer able to kill V583. This ability is restored in revertant FA2-2*Ωef2496*. Deleting putative pheromone receptor *traC2* from pTEF2 in V583 eliminates it susceptibility to killing, further implicating a pheromone. (*B*) Purified cOB1 pheromone (VAVLVLGA) specifically inhibits V583 [with an MIC (minimum inhibitory concentration) of 25 pM] but not its plasmid cured derivative V19. Of 81 pheromone peptides tested, only 2 others showed detectable activity with MICs ~10^6-fold higher than the MIC of cOB1. Susceptibility to pheromone killing was restored in V19 by the return of pTEF2. (*Inset*) Resistant V19(pTEF2) mutants, such as V19(pTEF2)^{PR}, were readily obtained in the zones of clearing in lawns exposed to cOB1.

proteins. An identical deletion was observed in 10 independently obtained resistant mutants.

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)^{PR}. 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 by commensal strains of the heptapeptide pheromone, cOB1, which induced lethal cross-talk between mobile elements 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, ICEBs1, from nonpreferred sites of integration leads to a loss in cell viability. The element studied here shares properties with ICEBs1, 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).

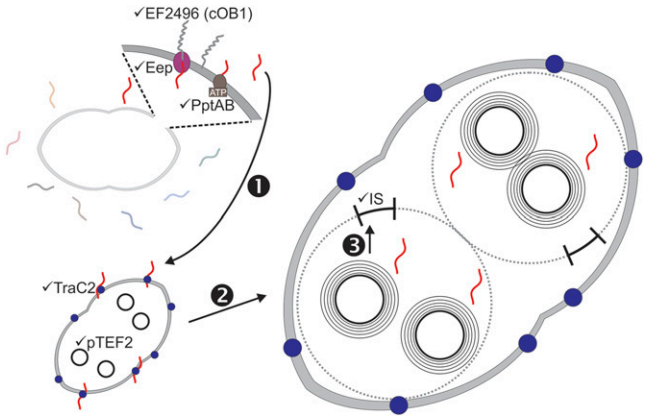

Fig. 5. Model for pheromone killing of V583. (*1*) Pheromones expressed by plasmid-free commensal *E. faecalis* are encoded within the signal peptide of lipoproteins, such as EF2496, which contain within the precursor sequence pheromone cOB1 (red wavy line). The signal peptides are processed, in part, by membrane protease Eep and pumped into the medium by a putative ATP binding transporter, PptAB, discovered here to our knowledge (more details in ref. 37). Functions shown in this work to contribute to the killing and susceptible phenotypes are identified by ✓. Enrichment of commensal enterococci in the fecal consortium (for example, by suppression of competitive microbes with tetracycline) enhanced V583 killing. (*2*) TraC2, a homolog to known pheromone receptors, presumably facilitates uptake of the cOB1 pheromone identified here as the killing effector molecule produced by commensal enterococci. (*3*) The main effect of cOB1 on V583 is to specifically induce transcription of plasmid pTEF2 genes over 100-fold. Cross-talk between the induced genes and an essential IS-like element on the chromosome of V583 and a number of other MDR *E. faecalis* strains resulted in V583 killing. All spontaneously resistant mutants of V583 had spontaneously lost the IS-like element.

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 speciated 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 (Pfaltz & 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*.

MICROBIOLOGY

Assessment of Killing Activity of Purified Peptides. Soft agar overlay assays were used to screen and visualize killing of various *E. faecalis* strains by commensal enterococci or purified pheromones as described in *SI Materials and Methods*. V19(pTEF2), 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 resynthesized 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 *traC*-2, EF0143, EF0160, and EF0143–EF0160 Deletion Mutants in V583 and Deletion of EF2496 in FA2-2. Deletion of most of the coding sequences of *traC*-2, 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*.

1. Huttenhower C, et al.; Human Microbiome Project Consortium (2012) Structure, function and diversity of the healthy human microbiome. *Nature* 486(7402):207–214.
2. Ley RE, et al. (2008) Evolution of mammals and their gut microbes. *Science* 320(5883): 1647–1651.
3. Walter J, Ley R (2011) The human gut microbiome: Ecology and recent evolutionary changes. *Annu Rev Microbiol* 65:411–429.
4. Jernberg C, Löfmark S, Edlund C, Jansson JK (2010) Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* 156(Pt 11):3216–3223.
5. Martin JD, Mundt JO (1972) Enterococci in insects. *Appl Microbiol* 24(4):575–580.
6. Gilmore MS, Lebreton F, van Schaik W (2013) Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. *Curr Opin Microbiol* 16(1):10–16.
7. Van Tyne D, Gilmore MS (2014) Friend turned foe: Evolution of enterococcal virulence and antibiotic resistance. *Annu Rev Microbiol* 68:337–356.
8. Palmer KL, et al. (2012) Comparative genomics of enterococci: Variation in Enterococcus faecalis, clade structure in E. faecium, and defining characteristics of E. gallinarum and E. casseliflavus. *MBio* 3(1):e00318-11.
9. Niven CF, Sherman JM (1944) Nutrition of the Enterococci. *J Bacteriol* 47(4):335–342.
10. Sievert DM, et al. (2013) Antimicrobial-resistant pathogens associated with healthcare-associated infections: Summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infect Control Hosp Epidemiol* 34(1):1–14.
11. Lebreton F, et al. (2013) Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *MBio* 4(4):e00534-13.
12. Sahm DF, et al. (1989) In vitro susceptibility studies of vancomycin-resistant Enterococcus faecalis. *Antimicrob Agents Chemother* 33(9):1588–1591.
13. Paulsen IT, et al. (2003) Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. *Science* 299(5615):2071–2074.
14. Shankar N, Baghdayan AS, Gilmore MS (2002) Modulation of virulence within a pathogenicity island in vancomycin-resistant Enterococcus faecalis. *Nature* 417(6890): 746–750.
15. Gold OG, Jordan HV, van Houte J (1975) The prevalence of enterococci in the human mouth and their pathogenicity in animal models. *Arch Oral Biol* 20(7):473–477.
16. Dunny GM, Brown BL, Clewell DB (1978) Induced cell aggregation and mating in Streptococcus faecalis: Evidence for a bacterial sex pheromone. *Proc Natl Acad Sci USA* 75(7):3479–3483.
17. McBride SM, Fischetti VA, Leblanc DJ, Moellering RC, Jr, Gilmore MS (2007) Genetic diversity among Enterococcus faecalis. *PLoS ONE* 2(7):e582.
18. Bourgogne A, et al. (2008) Large scale variation in Enterococcus faecalis illustrated by the genome analysis of strain OG1RF. *Genome Biol* 9(7):R110.
19. Palmer KL, Gilmore MS (2010) Multidrug-resistant enterococci lack CRISPR-cas. *MBio* 1(4):e00227-10.
20. Gilmore MS, Ferretti JJ (2003) Microbiology. The thin line between gut commensal and pathogen. *Science* 299(5615):1999–2002.
21. Jordens JZ, Bates J, Griffiths DT (1994) Faecal carriage and nosocomial spread of vancomycin-resistant Enterococcus faecium. *J Antimicrob Chemother* 34(4):515–528.
22. Van der Auwera P, Pensart N, Korten V, Murray BE, Leclercq R (1996) Influence of oral glycopeptides on the fecal flora of human volunteers: Selection of highly glycopeptide-resistant enterococci. *J Infect Dis* 173(5):1129–1136.
23. Laux DC, Cohen PS, Conway T (2005) Role of the mucus layer in bacterial colonization of the intestine. Colonization of Mucosal Surfaces, eds Nataro JP, Cohen PS, Mobley HLT, Weiser JN (American Society for Microbiology, Washington, DC), pp 199–212.
24. Miller JH (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab Press, Plainview, NY).
25. Brock TD, Peacher B, Pierson D (1963) Survey of the bacteriocines of enterococci. *J Bacteriol* 86(4):702–707.
26. Clewell DB, et al. (1982) Mapping of Streptococcus faecalis plasmids pAD1 and pAD2 and studies relating to transposition of Tn917. *J Bacteriol* 152(3):1220–1230.
27. Héchard Y, Pelletier C, Cenatiempo Y, Frère J (2001) Analysis of sigma(54)-dependent genes in Enterococcus faecalis: A mannose PTS permease (EII(Man)) is involved in sensitivity to a bacteriocin, mesentericin Y105. *Microbiology* 147(Pt 6):1575–1580.
28. Dalet K, Briand C, Cenatiempo Y, Héchard Y (2000) The rpoN gene of Enterococcus faecalis directs sensitivity to subclass IIa bacteriocins. *Curr Microbiol* 41(6):441–443.
29. Iyer VS, Hancock LE (2012) Deletion of σ(54) (rpoN) alters the rate of autolysis and biofilm formation in Enterococcus faecalis. *J Bacteriol* 194(2):368–375.
30. Manson JM, Hancock LE, Gilmore MS (2010) Mechanism of chromosomal transfer of Enterococcus faecalis pathogenicity island, capsule, antimicrobial resistance, and other traits. *Proc Natl Acad Sci USA* 107(27):12269–12274.
31. Hanin A, et al. (2010) Screening of in vivo activated genes in Enterococcus faecalis during insect and mouse infections and growth in urine. *PLoS ONE* 5(7):e11879.
32. Clewell DB, Weaver KE (1989) Sex pheromones and plasmid transfer in Enterococcus faecalis. *Plasmid* 21(3):175–184.
33. An FY, Clewell DB (2002) Identification of the cAD1 sex pheromone precursor in Enterococcus faecalis. *J Bacteriol* 184(7):1880–1887.
34. Clewell DB, Francia MV, Flannagan SE, An FY (2002) Enterococcal plasmid transfer: Sex pheromones, transfer origins, relaxases, and the Staphylococcus aureus issue. *Plasmid* 48(3):193–201.
35. An FY, Sulavik MC, Clewell DB (1999) Identification and characterization of a determinant (eep) on the Enterococcus faecalis chromosome that is involved in production of the peptide sex pheromone cAD1. *J Bacteriol* 181(19):5915–5921.
36. Varahan S, Iyer VS, Moore WT, Hancock LE (2013) Eep confers lysozyme resistance to enterococcus faecalis via the activation of the extracytoplasmic function sigma factor SigV. *J Bacteriol* 195(14):3125–3134.
37. Varahan S, Harms N, Gilmore MS, Tomich JM, Hancock LE (2014) An ABC transporter is required for secretion of peptide sex pheromones in *Enterococcus faecalis*. *MBio* 5(5): e01726-14–e14.
38. Clewell DB (2007) Properties of Enterococcus faecalis plasmid pAD1, a member of a widely disseminated family of pheromone-responding, conjugative, virulence elements encoding cytolysin. *Plasmid* 58(3):205–227.
39. Dunny GM (2007) The peptide pheromone-inducible conjugation system of Enterococcus faecalis plasmid pCF10: Cell-cell signalling, gene transfer, complexity and evolution. *Philos Trans R Soc Lond B Biol Sci* 362(1483):1185–1193.
40. Leonard BA, Podbielski A, Hedberg PJ, Dunny GM (1996) Enterococcus faecalis pheromone binding protein, PrgZ, recruits a chromosomal oligopeptide permease system to import sex pheromone cCF10 for induction of conjugation. *Proc Natl Acad Sci USA* 93(1):260–264.
41. Altschul SF, et al. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25(17):3389–3402.
42. Dunny GM, Leonard BA (1997) Cell-cell communication in gram-positive bacteria. *Annu Rev Microbiol* 51:527–564.
43. Nakayama J, Abe Y, Ono Y, Isogai A, Suzuki A (1995) *Isolation and Structure of the Enterococcus faecalis Sex Pheromone, cOB1, That Induces Conjugal Transfer of the Hemolysin/Bacteriocin Plasmids, pOB1 and pYI1* (Japan Society for Bioscience Biotechnology and Agrochemistry, Tokyo), pp 703–705.
44. Menard KL, Grossman AD (2013) Selective pressures to maintain attachment site specificity of integrative and conjugative elements. *PLoS Genet* 9(7):e1003623.
45. Thomas VC, Thurlow LR, Boyle D, Hancock LE (2008) Regulation of autolysis-dependent extracellular DNA release by Enterococcus faecalis extracellular proteases influences biofilm development. *J Bacteriol* 190(16):5690–5698.