Multidrug-Resistant Enterococci Lack CRISPR-cas

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ABSTRACT Clustered, regularly interspaced short palindromic repeats (CRISPR) provide bacteria and archaea with sequence-specific, acquired defense against plasmids and phage. Because mobile elements constitute up to 25% of the genome of multidrug-resistant (MDR) enterococci, it was of interest to examine the codistribution of CRISPR and acquired antibiotic resistance in enterococcal lineages. A database was built from 16 Enterococcus faecalis draft genome sequences to identify commonalities and polymorphisms in the location and content of CRISPR loci. With this data set, we were able to detect identities between CRISPR spacers and sequences from mobile elements, including pheromone-responsive plasmids and phage, suggesting that CRISPR regulates the flux of these elements through the E. faecalis species. Based on conserved locations of CRISPR and CRISPR-cas loci and the discovery of a new CRISPR locus with associated functional genes, CRISPR3-cas, we screened additional E. faecalis strains for CRISPR content, including isolates predating the use of antibiotics. We found a highly significant inverse correlation between the presence of a CRISPR-cas locus and acquired antibiotic resistance in E. faecalis, and examination of an additional eight E. faecium genomes yielded similar results for that species. A mechanism for CRISPR-cas loss in E. faecalis was identified. The inverse relationship between CRISPR-cas and antibiotic resistance suggests that antibiotic use inadvertently selects for enterococcal strains with compromised genome defense.

IMPORTANCE For many bacteria, including the opportunistically pathogenic enterococci, antibiotic resistance is mediated by acquisition of new DNA and is frequently encoded on mobile DNA elements such as plasmids and transposons. Certain enterococcal lineages have recently emerged that are characterized by abundant mobile DNA, including numerous viruses (phage), and plasmids and transposons encoding multiple antibiotic resistances. These lineages cause hospital infection outbreaks around the world. The striking influx of mobile DNA into these lineages is in contrast to what would be expected if a self (genome)-defense system was present. Clustered, regularly interspaced short palindromic repeat (CRISPR) defense is a recently discovered mechanism of prokaryotic self-defense that provides a type of acquired immunity. Here, we find that antibiotic resistance and possession of complete CRISPR loci are inversely related and that members of recently emerged high-risk enterococcal lineages lack complete CRISPR loci. Our results suggest that antibiotic therapy inadvertently selects for enterococci with compromised genome defense.
~90% of archaeal and ~45% of bacterial genomes possessing convincing CRISPR loci (24). Of the two CRISPR loci discovered in the E. faecalis OG1RF genome, one possesses the associated cas nuclease genes (CRISPR1-cas) and one is an orphan locus lacking cas genes (CRISPR2) (17). The hospital-adapted V583 strain possesses only the orphan locus CRISPR2, lacking the functional cas genes required for CRISPR defense (25).

Because of the potential for limiting entry of mobile elements, it was of interest to determine whether a correlation exists between the presence of CRISPR loci and the emergence of antibiotic-resistant enterococcal lineages. By examining enterococcal draft genomes to develop consensus information on the occurrence of CRISPR loci, and expanding the study to a historical collection of isolates that extends coverage through antibiotic and preantibiotic eras, we found a strong correlation between the absence of CRISPR-cas loci and the emergence of MDR enterococcal strains. We hypothesize that widespread antibiotic use has selected for enterococcal strains able to readily acquire novel traits—those with compromised genome defense—ultimately leading to the emergence of enterococcal lineages replete with antibiotic resistances and other mobile traits.

RESULTS
CRISPR distribution in 16 E. faecalis draft genomes. We recently collaborated in an effort to sequence the genomes of 28 enterococci, including 16 E. faecalis strains representing deep phylogenetic nodes in the E. faecalis multilocus sequence typing (MLST) dendrogram (26). CRISPRfinder (27) was used to identify putative CRISPR loci in the 16 draft E. faecalis genomes. The orphan CRISPR2 locus, consisting only of palindromes and spacers, was identified in all 16 E. faecalis genomes and was invariably located between homologues of the E. faecalis V583 CRISPR2 locus and the OG1RF CRISPR1-cas and CRISPR2 loci were previously reported (17, 25). Red bars and text denote novel DNA sequences present in V583. Primer sets used in CRISPR profiling are labeled with uppercase letters: A, CRISPR1-cas flanking primers; B, CRISPR1-cas csn1 screening primers; C, CRISPR2 screening primers; D, CRISPR3-cas flanking primers; E, CRISPR3-cas csn1 screening primers. The figure is not drawn to scale.
TABLE 1  E. faecalis CRISPR spacer identities to mobile genetic elements

<table>
<thead>
<tr>
<th>Strain</th>
<th>CRISPR and spacer no.</th>
<th>Sequence identity</th>
<th>Representative Blast hit</th>
<th>Area of identity in Blast hit</th>
<th>Identical Blast hits</th>
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</thead>
<tbody>
<tr>
<td>ATCC 4200</td>
<td>1-4</td>
<td>29/30</td>
<td>Enterococcus phage ϕEF24C</td>
<td>EFp gp114 hypothetical protein</td>
<td>E. faecalis pCF10</td>
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<tr>
<td></td>
<td>2-4</td>
<td>29/30</td>
<td>V583 prophage 6</td>
<td>EF2813 tail tape measure protein</td>
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<tr>
<td></td>
<td>2-5</td>
<td>30/30</td>
<td>V583 prophage 6</td>
<td>EF2836_EF2387 intergenic region</td>
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<tr>
<td></td>
<td>2-6</td>
<td>29/30</td>
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<td>EF_B0043 sb-6</td>
<td>E. faecalis pCF10</td>
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<tr>
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<td>29/30</td>
<td>E. faecalis pMG2200</td>
<td>ORF54 hypothetical protein BAH02364.1</td>
<td>E. faecalis pBE99; pY114</td>
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<td>1-3</td>
<td>30/30</td>
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<td>gp54-gp55 intergenic region</td>
<td>Enterococcus phage ϕFL2A</td>
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<td></td>
<td>29/30</td>
<td>Enterococcus phage ϕFL3B</td>
<td>gp57-gp58 intergenic region</td>
<td>Enterococcus phage ϕFL3A; ϕFl1</td>
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<td>gp39-gp40 intergenic region</td>
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<td>1-8</td>
<td>29/30</td>
<td>V583 VR1-integrated plasmid</td>
<td>EF0133-EF0134 intergenic region</td>
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<td>EF2539-EF2540 intergenic region</td>
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<td>EF1486 endolysin</td>
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<td>EF2836_EF2387 intergenic region</td>
<td>E. faecalis pCF10</td>
</tr>
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</table>

* Table 1 indicates that CRISPR locus (1, 2, or 3) is followed by the spacer number is shown. CRISPR spacers are numbered in consecutive order from left to right as shown in Fig. 1.

* Sequence identity is shown as the number of base pairs with sequence identity in GenBank/total number of base pairs in spacer.

* V583 variable regions (VR) are from a report by McBride et al. (10).

* This spacer is identical to ATCC 4200 CRISPR2 spacer 5.

* This spacer is identical to ATCC 4200 CRISPR2 spacer 6.

4200, X98, Eisol, DS5, and D6) possessed CRISPR1-cas loci, always located between homologues of the V583 ORFs EF0672 and EF0673 (Fig. 1).

The putative Cas proteins encoded by these loci are highly conserved, as are the palindromic repeat sequences (see Tables S1 and S2 in the supplemental material). As for E. faecalis OG1RF CRISPR1-cas, these loci are of the Nmeni subtype, a CRISPR-cas organizational structure found only in vertebrate-associated com-

mensals and pathogens (28). For E. faecalis OG1RF CRISPR1-cas, these ORFs are more conserved than others: five strains (ATCC4200, D6, T8, Fly1, and T11) possess spacers with identity to pheromone-responsive plasmids, genes other than others: five strains (ATCC4200, D6, T8, Fly1, and T11) possess spacers with identity to pheromone-responsive plasmids, and acquired antibiotic resistance. These elements include the pheromone-responsive type plasmids pAD1/pTEF1 and pCF10/pTEF2, enterococcal phage and prophage, and plasmids integrated within the E. faecalis V583 genome (Table 1). Identities to certain mobile elements were more common than others: five strains (ATCC4200, D6, T8, Fly1, and T11) possess spacers with identity to pheromone-responsive plasmids, and five strains (ATCC4200, D6, T8, Fly1, and AR01/DG) possess spacer sequences with identities to different regions of E. faecalis V583 prophage 6.

E. faecalis CRISPR-cas and acquired antibiotic resistance. Examining the entire genomes of 16 E. faecalis strains allowed us...
to determine that, when present, CRISPR loci always occurred at conserved chromosomal positions: the CRISPR1-cas locus occurred between EF0672 and EF0673 homologues, CRISPR2 between EF2063 and EF2061 homologues, and CRISPR3-cas between EF1760 and EF1759 homologues. Based on this information, we extended the analysis of E. faecalis CRISPR by examining 29 additional isolates, including strains with isolation dates prior to the mid-1970s (Fig. 2; for additional isolation dates of these strains, see Table S1). Other antibiotic resistances (tetL and tetM, ermB, aac6'-aph2'', cat, blaZ, and vanA and vanB) were previously profiled (10). A single asterisk indicates that gentamicin resistance is conferred by a 3'-5'-aminoglycoside phosphotransferase in this strain; double asterisks denote E. faecalis strains for which draft or complete genome sequences are available.

![CRISPR-cas and acquired antibiotic resistance in a historical collection of E. faecalis strains.](image)

**FIG 2** CRISPR-cas and acquired antibiotic resistance in a historical collection of E. faecalis strains. E. faecalis strains are listed by date of isolation, from oldest to most recent. Acquired antibiotic resistance is shown in red, and CRISPR-cas presence is shown in green. Antibiotic resistance (tetracycline [tetL and tetM], erythromycin [ermB], gentamicin [aac6'-aph2''], chloramphenicol [cat], ampicillin [blaZ], and vancomycin [vanA and vanB]) was previously profiled (red squares) (10). A single asterisk indicates that gentamicin resistance is conferred by a 3'-5'-aminoglycoside phosphotransferase in this strain; double asterisks denote E. faecalis strains for which draft or complete genome sequences are available.
CRISPR content for *E. faecalis* V583, OG1RF, and HH22, as previously reported (17, 25), was included in the analysis.

All 48 *E. faecalis* strains possess an orphan CRISPR2 locus (see Table S1 in the supplemental material). In contrast, CRISPR-cas distribution varies among antibiotic-sensitive and antibiotic-resistant strains (Fig. 2). One-third of the *E. faecalis* strains in our collection (16/48) possess a complete CRISPR-cas locus (CRISPR1-cas or CRISPR3-cas), and all but two of these either lack acquired antibiotic resistance genes or possess tetM, a gene commonly disseminated by the self-mobilizable conjugative transposon Tn916 (29, 30). Strikingly, of the MDR *E. faecalis* strains in this collection (defined as resistant to two or more antibiotics), all except two lack CRISPR-cas loci (n = 22), including all vancomycin-resistant strains (n = 8) (Fig. 2).

We used a combination of statistical analyses to evaluate the hypothesis that *E. faecalis* strains with CRISPR-cas possess significantly fewer acquired antibiotic resistance genes than those lacking CRISPR-cas. We first tallied acquired antibiotic resistance genes for each strain and performed a one-tailed Wilcoxon rank sum test to address the null hypothesis that there is no difference in the distributions of acquired antibiotic resistance genes between strains that possess CRISPR-cas and strains that lack CRISPR-cas. Because antibiotic resistance genes are often coacquired on elements conferring resistance to multiple antibiotics, we performed the Wilcoxon rank sum test using progressively stricter models for coacquisition, in which potentially coacquired genes were counted as resistance to one antibiotic, instead of two (see Data Set S1 in the supplemental material). We found that, irrespective of the model used, the null hypothesis could be rejected (P < 0.001), indicating that the distributions of acquired antibiotic resistance significantly differ between CRISPR-cas-positive and -negative *E. faecalis* strains. Probability values did not change for a model in which tetM and ermB, which can be codisseminted by Tn916 and related elements (30), are coacquired (P values of 0.0003 if acquired jointly and 0.0004 if acquired separately) (Data Set S1). If aac6’-aph2’’ and blaz are also assumed to be coacquired, as was suggested by the patterns of occurrence of antibiotic resistance in 106 *E. faecalis* strains (10), the P value increases nominally to 0.0005 (Data Set S1). Finally, if ermB and vancomycin resistance are assumed to be coacquired (10), in addition to tetM/ermB and aac6’-aph2’’/
coacquisition, the \( P \) value increases to 0.0008 (Data Set S1), remaining highly significant. We additionally used a one-tailed Fisher exact test with a \( 2 \times 2 \) contingency table comparing cooccurrences of CRISPR-cas and antibiotic resistance to address the null hypothesis that CRISPR-cas presence is unrelated to antibiotic resistance. The \( P \) value of 0.007 is highly significant (\( P < 0.01 \)), which led us to reject the null hypothesis (Data Set S1). The results of these analyses demonstrate that a statistically significant relationship between the presence or absence of CRISPR-cas and acquired antibiotic resistance exists.

To examine the phylogenetic distribution of CRISPR-cas loci in \( E. faecalis \), we generated an MLST dendrogram of the strains used for CRISPR-cas analysis (10) (Fig. 3). CRISPR-cas loci are discontinuously distributed across this MLST dendrogram, and CRISPR-cas distribution varies even between strains of a single sequence type (e.g., ST21 and ST40 lineages) (Fig. 3). In other lineages, including ST6 (a CC2 lineage; 6 strains), ST9 (a CC9 lineage; 4 strains), and CC8 (8 strains) lineages, CRISPR-cas loci are uniformly absent. All three lineages are associated with MDR (9, 10); vancomycin resistance and \( \beta \)-lactamase production first emerged in the United States in ST6 strains of CC2 (10), and the CC2 and CC9 lineages are highly associated with nosocomial infections and hospital endemicity (9). Loss of CRISPR-cas in founders of these lineages may have precipitated their success in acquiring traits facilitating hospital adaptation.

**CRISPR-cas in \( E. faecium \).** No CRISPR loci were identified in seven recently reported \( E. faecium \) draft genomes (31). We therefore examined the CRISPR locus content in eight additional \( E. faecium \) genomes (26). The \( E. faecium \) strains originated from human clinical samples (strains represented by numbers in Fig. 4 and 5) and from the feces of healthy human volunteers (Com12 and Com15). All were isolated in the early to mid-2000s. We identified an \( E. faecium \) CRISPR-cas locus (EfmCRISPR1-cas) in three genomes (Fig. 4). This locus is found between homologues of EfaeDRAFT_1858 and EfaeDRAFT_1857 from the previously sequenced clinical isolate \( E. faecium \) DO draft genome (GenBank accession no. ACJY00000000), which itself lacks CRISPR (25). EfmCRISPR1-cas possesses a palindromic repeat sequence that is divergent from those of the \( E. faecalis \) CRISPR-cas loci (see Fig. S1 in the supplemental material). The four predicted Cas proteins encoded by EfmCRISPR1-cas are consistent with the Nmeni subtype (Table S4). However, EfmCRISPR-cas differs from the \( E. faecalis \) Nmeni subtype by the presence of an ORF of unknown function 5’ to the csn1 gene which is conserved and unique to the three strains possessing EfmCRISPR-cas (Fig. 4). A Blastn comparison of the 20 EfmCRISPR1-cas spacers to sequences in the NCBI nonredundant nucleotide database reveals identity between two spacers and previously identified clostridial and lactococcal phages (Table 2).

MLST phylogeny and acquired antibiotic resistance profiles were determined for the eight \( E. faecium \) strains. Four strains belong to the hospital-adapted CC17, being either ST17 or double-locus variants of ST17 (Fig. 5; see Table S5 in the supplemental material). As expected for CC17 strains (1), all are MDR. Three of the CC17 strains possess \( vanA \), encoding vancomycin resistance, while the fourth CC17 strain constitutes a novel sequence type variant and lacks \( vanA \). The remaining four strains belong to other sequence types and lack acquired antibiotic resistance genes (Fig. 5). Although the \( E. faecium \) analysis was smaller in sample size than that for \( E. faecalis \), the distribution of EfmCRISPR1-cas in these genomes supports the conclusion that MDR enterococcal strains generally lack CRISPR-cas; in this case, three of four MDR strains lack EfmCRISPR1-cas, and these three strains are vancomycin-resistant members of the hospital-adapted CC17 lineage.

**Mechanism for CRISPR-cas variability.** It was previously proposed that deletion of a Streptococcus thermophilus Nmeni subtype CRISPR-cas may occur through recombination (32). Nmeni subtype CRISPR-cas loci possess a conserved but imperfect CRISPR repeat upstream of the \( cas \) operon, meaning that a Nmeni CRISPR-cas locus is flanked on both the 5’ and 3’ ends by repetitive sequences (28, 32). It is possible that recombination could occur at these sites, resulting in deletion (32). We analyzed the \( E. faecalis \) genome sequences corresponding to the locations where deletion of CRISPR1-cas or CRISPR3-cas would have occurred. Interestingly, \( E. faecalis \) strains lacking CRISPR-cas possess small, common sequences at these sites. \( E. faecalis \) V583 possesses 205 bp of unique sequence when aligned with the \( E. faecalis \) D6 CRISPR1-cas and flanking region and 53 bp of unique sequence compared to the Fly1 CRISPR3-cas region (Fig. 1). These 205-bp and 53-bp sequences are highly conserved in all \( E. faecalis \) strains.
strains lacking CRISPR1-cas or CRISPR3-cas (see Fig. S2 and S3 in the supplemental material). This would not be predicted for CRISPR-cas loss by homologous recombination and excision.

We recently reported the low-frequency mobilization and conjugal transfer of large regions of chromosomal DNA from *E. faecalis* V583 donors to *E. faecalis* OG1RF recipients, resulting in transconjugant strains with hybrid V583-OG1RF genomes (33). This transfer was found to be dependent on either of two pheromone-responsive plasmids resident in V583, pTEF1 or pTEF2, and occurred as the result of plasmid integration and chromosome mobilization. Chromosome-to-chromosome movement of the PAI, a vancomycin resistance transposon, capsule genes, and other V583 genes to OG1RF recipients was observed. It is important to note that OG1RF does not possess CRISPR spacers with identity to pTEF1, pTEF2, or other V583 sequences (17), and in these experiments, CRISPR1-cas did not block incoming DNA from V583. Because the OG1RF CRISPR1-cas locus occurs ~41.5 kb 3′ to the site of PAI incorporation, it was of interest to determine whether the CRISPR1-cas locus in PAI-containing transconjugants had been displaced by incoming DNA from V583 donors. We used five strains (TC1, TC3, TC4, TC5, and TC12) representing the five classes of PAI transconjugants (A to E) identified in our previous study (33). These transconjugants possess the V583 PAI and various amounts of flanking DNA, representing a total acquisition of 285 to 857 kb of V583 donor chromosome (33). The five PAI-containing transconjugants were screened for the occurrence of the OG1RF CRISPR1-cas by PCR. Strikingly, all five transconjugants lacked CRISPR1-cas and instead possessed at this locus the conserved 205-bp sequence found in the V583 donor (see Fig. S2 in the supplemental material). Thus, in a single conjugative transfer event, a novel hybrid strain that simultaneously becomes antibiotic resistant, acquires the PAI, and is rendered deficient for CRISPR-cas can be generated.

**DISCUSSION**

We examined 48 *E. faecalis* strains from a historical collection and 8 recent *E. faecium* isolates to determine the relationship between CRISPR and the emergence of multidrug resistance in enterococci. We found that CRISPR1-cas and CRISPR3-cas loci are variable among *E. faecalis* strains, but, interestingly, an orphan CRISPR2 locus occurred in all *E. faecalis* strains tested. Selective pressure for maintenance of CRISPR2 in a CRISPR-cas-deficient *E. faecalis* background is unclear, as is the origin of CRISPR2 (i.e., whether this locus is functionally and evolutionarily linked to CRISPR1-cas, or once independently possessed cas genes that have been lost). CRISPR1-cas and CRISPR2 repeat sequences are identical (17), and because Cas proteins interact with CRISPR repeat sequences following transcription (22), it is likely that CRISPR1-cas and CRISPR2 are functionally linked. Nevertheless, genome sequence analysis finds that CRISPR-cas-deficient *E. faecalis* strains lack any detectable csn1 or csn1-like gene, which is essential for protection mediated by a Nmeni subtype CRISPR-cas locus (20). The lack of required functional genes in strains with the CRISPR2 orphan locus, together with the abundance of antibiotic resistance genes in these strains, indicates that CRISPR2 alone does not confer immunity in *E. faecalis* hosts. Ultimately, a functional analysis of the CRISPR1-cas and CRISPR2 loci will be required to confirm this hypothesis.

By analyzing a large data set, we were able to detect identities between CRISPR spacers and sequences on known mobile elements, including pheromone-responsive plasmids and phage. Enterococci have a highly coevolved relationship with the narrow-host-range pheromone-responsive plasmids, which encode machinery to utilize extracellular signals produced by enterococcal cells to induce conjugation functions and to manipulate signal production in enterococcal cells in which they reside (34). The results of this study and our recent work (33) support a role for pheromone-responsive plasmids as important drivers of enterococcal genome plasticity, capable of promoting their own transfer, mobilizing chromosomally encoded antibiotic resistance and virulence determinants, and now also causing displacement of CRISPR-cas.

That *E. faecalis* CRISPR loci contain spacers with identity to enterococcal mobile elements, and the distribution of these spacers, suggests that certain elements, such as pheromone-responsive plasmids, are frequently encountered by *E. faecalis* and/or have a propensity to be incorporated into CRISPR loci as spacers. It is interesting that no CRISPR spacers have yet been identified with sequence identity to conjugative transposons such as Tn916, which are also vectors of antibiotic resistance in the enterococci (29). CRISPR elements have been shown experimentally to confer immunity to plasmids and phages (20–23), although many mechanistic details remain unknown. To our knowledge, there is no experimental evidence that CRISPR defense confers protection from conjugative transposons. The observation that the tetM gene, commonly disseminated by Tn916 and related conjugative transposons (29, 30), is present in *E. faecalis* strains possessing CRISPR-cas (Fig. 2) suggests that conjugative transposons may evade this defense. Spacers targeting the Inc18 plasmid family, plasmids that are significant for their role in the dissemination of vancomycin resistance genes from enterococci to MRSA (6, 7), are also absent. Tn916 and the Inc18 plasmid family are broad host range in nature (29, 30), and the notable lack of spacers with identity to these elements may reflect the relative rarity of interspecies transfer or the relative inefficiency of transfer of elements lacking mechanisms for effective pair formation, such as the pheromone-induced aggregation mechanism.

Of the 48 *E. faecalis* and 8 *E. faecium* strains examined in this study, 7 *E. faecalis* and 2 *E. faecium* strains lacked CRISPR-cas and
also lacked antibiotic resistance. Draft genome sequences are not available for six of these nine strains, so it remains a formal possibility that novel CRISPR-cas loci may be found in those strains. Alternatively, it may be that the absence of CRISPR-cas (particularly in strains isolated before, or early in, the age of antibiotics, such as E. faecalis D173 [isolated in 1939], ATCC 19433 [isolated before 1942], and T1 [isolated before 1950] [10]) facilitated acquisition of a mobile element carrying traits other than antibiotic resistance (for example, hemolysin/bacteriocin production and resistance or new metabolic properties), which provided a selective advantage.

Mobile elements often provide an accessory pool of genes that enhance survival in select environments. It is possible that elements such as the pheromone-responsive plasmids and putative enterococcal self-defense mechanisms such as CRISPR-cas act, respectively, to diversify and to stabilize the enterococcal genome, and that the dynamic between these opposing forces is important for the ultimate success of these microbes. Based on the data presented here, we speculate that modern antibiotic therapy has shifted this dynamic toward the facile acquisition of foreign elements conferring antibiotic resistance, among other things, decreasing genome stability/increasing plasticity, and enabling the colonization of new habitats, including the antibiotic-laden hospital environment.

MATERIALS AND METHODS

Genomes, bacterial strains, and media. Draft genome data for 16 E. faecalis and 8 E. faecium strains were generated in collaboration with the Broad Institute (26) and accessed at the Enterococcus database website (http://www.broadinstitute.org/annotation/genome/enterococcus_faecalis/MultiHome.html). E. faecalis strains used for CRISPR profiling are shown in Fig. 2 and in Data Set S1 in the supplemental material and have previously been described with respect to isolation dates, antibiotic resistance profiles, and MLST data [10]. Colony PCR was performed as described above with primer pairs flanking the E. faecalis CRISPR1-cas region (For-5′-GCC ATG TTA GCT GAT ACA AC-3′ and Rev-5′-CGA ATA TGC CTG TGG TGA AA-3′); expected product size of 315 bp for strains lacking CRISPR1-cas and flanking the E. faecalis CRISPR3-cas region (For-5′-GAT CAC TAG GTT CAA TT C-3′ and Rev-5′-CAT CGA TTC ATT CCT CCA A-3′); expected product size of 224 bp for strains lacking CRISPR3-cas. E. faecalis strains for which CRISPR-cas loci were not detected in genomic analyses (Fig. 1) were similarly screened. E. faecalis OG1RF, Fly1, and V583 were included as positive and negative controls where appropriate. All PCR products were sequenced to confirm the absence of CRISPR loci.

E. faecalis CRISPR locus identification in draft genome sequences. CRISPR loci were identified in 16 E. faecalis and 8 E. faecium draft genomes (26) using CRISPRfinder (http://crispr.u-psud.fr/Server/CRISPRfinder.php) (27). Sequence data from the Broad Institute Enterococcus database were downloaded, and CRISPR repeat-spacer arrays were manually annotated in MacVector to confirm the presence of CRISPR. In E. faecalis strains Fly1, HIP11704, and T2, the CRISPR2 locus was detected by annotation of the intergenic region between homologues of the E. faecalis V583 ORFs EF0672–EF0673 and EF1760–EF1761, respectively. Colony PCR was performed as described above with primer pairs flanking the E. faecalis CRISPR1-cas region (For-5′-GCC ATG TTA GCT GAT ACA AC-3′) and a primer nested in CRISPR1-cas (Rev-5′-CTT CAC TAA CAA ATA GTA GTC TCC-3′). E. faecalis OG1RF was included as a positive control. Strains were additionally screened with CRISPR1-cas flanking primers as described above, and products were sequenced to confirm the absence of CRISPR1-cas.

Analysis of E. faecalis CRISPR distribution. To test whether the acquired antibiotic resistance gene contents of CRISPR-cas-positive and -negative strains significantly differed, data were analyzed by the nonparametric Wilcoxon rank sum test and the Fisher exact test. MLST data for E. faecalis strains have been previously reported (10), and an MLST-based phylogenetic tree of strains used in this study was generated using the E. faecalis MLST database (http://efaecalis.mlst.net/).

Analysis of E. faecium genomes. MLST of E. faecium strains was performed by concatenating sequences of the aak, atpA, dfl, gdh, gya, pckK, and pps loci and comparing them to known sequences in the E. faecium MLST database (35) (http://efaecium.mlst.net/). Single- and double-locus variants of ST17 were assigned to the CC17 lineage. Antibiotic resistance genes were identified by Blastp queries of the Broad Institute Enterococcus group database with reference enterococcal resistance proteins from the NCBI protein database. Accession numbers for the proteins used in the Blastp queries are as follows: for TetM (Enterococcus faecium), accession no. ADA62733; TetL (Enterococcus faecium), AA92527; VanA (Enterococcus faecium), ACC93633; VanB (Enterococcus faecium), AAQ12894; EmrB (Enterococcus faecium), AA866219; Cat (Enterococcus faecium), AA64429; BlaZ (Enterococcus faecalis HH22), AAA24777; and Ac6′-ApH 2′, P0A0C2. Genes encoding VanB, Cat, and BlaZ were not detected in draft E. faecium genomes in this database.

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