Enterococcus faecalis Strains with Compromised CRISPR-Cas Defense Emerge under Antibiotic Selection for a CRISPR-Targeted Plasmid

Wenwen Huo,a Valerie J. Price,a Ardalan Sharifi,a Michael Q. Zhang,a ©Kelli L. Palmera

aDepartment of Biological Sciences, The University of Texas at Dallas, Richardson, Texas, USA

Wenwen Huo and Valerie J. Price contributed equally to this work. Author order was determined alphabetically.

ABSTRACT Enterococcus faecalis is a Gram-positive bacterium that natively colonizes the human gastrointestinal tract and opportunistically causes life-threatening infections. Multidrug-resistant (MDR) E. faecalis strains have emerged that are replete with mobile genetic elements (MGEs). Non-MDR E. faecalis strains frequently possess CRISPR-Cas systems, which reduce the frequency of MGE acquisition. We demonstrated in previous studies that E. faecalis populations can transiently maintain both a functional CRISPR-Cas system and a CRISPR-Cas target. In this study, we used serial passage and deep sequencing to analyze these populations. In the presence of antibiotic selection for the plasmid, mutants with compromised CRISPR-Cas defense and enhanced ability to acquire a second antibiotic resistance plasmid emerged. Conversely, in the absence of selection, the plasmid was lost from wild-type E. faecalis populations but not E. faecalis populations that lacked the cas9 gene. Our results indicate that E. faecalis CRISPR-Cas can become compromised under antibiotic selection, generating populations with enhanced abilities to undergo horizontal gene transfer.

IMPORTANCE Enterococcus faecalis is a leading cause of hospital-acquired infections and disseminator of antibiotic resistance plasmids among Gram-positive bacteria. We have previously shown that E. faecalis strains with an active CRISPR-Cas system can prevent plasmid acquisition and thus limit the transmission of antibiotic resistance determinants. However, CRISPR-Cas is not a perfect barrier. In this study, we observed populations of E. faecalis with transient coexistence of CRISPR-Cas and one of its plasmid targets. Our experimental data demonstrate that antibiotic selection results in compromised E. faecalis CRISPR-Cas function, thereby facilitating the acquisition of additional resistance plasmids by E. faecalis.

KEYWORDS CRISPR-Cas, Enterococcus faecalis, antibiotic resistance, horizontal gene transfer, plasmids

Enterococcus faecalis is a Gram-positive bacterium that natively colonizes the human gastrointestinal tract and opportunistically causes life-threatening infections (1–4). Antibiotic resistance is a major concern for treatment of these infections. E. faecalis can acquire antibiotic resistance through horizontal gene transfer (HGT), mediated primarily by plasmids, transposons, and integrative conjugative elements (5–7). One of the most clinically relevant forms of HGT in E. faecalis is afforded through the pheromone-responsive plasmids. The pheromone-responsive plasmids are narrow-host-range conjugative plasmids that disseminate antibiotic resistance and virulence genes among E. faecalis organisms (5, 8–10) and mobilize resistance genes to other pathogens (2, 11–14).

Some E. faecalis strains encode CRISPR-Cas systems. We have demonstrated that these systems reduce the spread of antibiotic resistance plasmids among E. faecalis strains in vitro and in vivo in the murine intestine (15–19). CRISPR-Cas systems confer sequence-specific
genome defense against mobile genetic elements (MGEs) (20, 21). Type II CRISPR-Cas systems occur in *E. faecalis* (22–24). They consist of a CRISPR array and CRISPR-associated (*cas*) genes. In *E. faecalis*, the CRISPR is an array of 36-bp repeat sequences interspersed with 30-bp sequences referred to as spacers. When cells are challenged with an MGE, some incorporate a short segment (protospacer) of the invading MGE genome into the CRISPR as a novel spacer (20, 25). By this mechanism, the CRISPR serves as a heritable molecular memory of MGE encounters. Short sequence motifs adjacent to protospacers, called protospacer-adjacent motifs (PAMs), as well as the Cas9, Cas1, and Cas2 nucleases are important for adaptation (26–31). The CRISPR is transcribed and processed into small RNAs called CRISPR RNAs (crRNAs); one crRNA corresponds to one spacer (32). If an MGE possessing the protospacer and PAM enters the cell, the Cas9 nuclease is directed to the MGE genome by the crRNA. Cas9 cleaves the invading MGE, generating a double-stranded DNA (dsDNA) break, thereby inhibiting its entry (33–36).

Several of our studies (17, 18) have focused on the CRISPR3-Cas locus of the *E. faecalis* strain T11RF, which natively lacks horizontally acquired antibiotic resistance genes (37, 38). *E. faecalis* T11RF is closely related to the model multidrug-resistant *E. faecalis* strain V583 but lacks the ~620 kb of horizontally acquired genome content that V583 possesses (38). T11RF has a CRISPR-Cas system, while V583 does not (24). In general, multidrug-resistant *E. faecalis* strains lack CRISPR-Cas. The *E. faecalis* T11RF CRISPR-Cas possesses 21 spacers. Spacer 6 is identical to the *repB* gene of the model pheromone-responsive plasmid pAD1. pAD1 encodes a hemolytic bacteriocin referred to as cytolysin and the virulence factor aggregation substance (8, 39). We previously demonstrated that T11RF CRISPR3-Cas interferes with acquisition of the pAD1 derivative pAM714 (17), which encodes erythromycin resistance via *ermB* on Tn917 (40, 41). Deletion of *cas9* or spacer 6 in *E. faecalis* T11RF resulted in up to a 150-fold increase in pAM714 acquisition in *in vitro* mating with *E. faecalis* OG1SSp(pAM714) donors (17). This increase in conjugation frequency was not observed for the pheromone-responsive plasmid pCF10, which is not natively targeted by the T11RF CRISPR3 spacers (17). These experiments confirmed that CRISPR3-Cas is a sequence-specific antiplasmid defense system in *E. faecalis* T11RF.

Despite *E. faecalis* T11RF CRISPR3-Cas acting as a barrier to pAM714, up to 10^5 T11RF (pAM714) transconjugants still arose in *in vitro* conjugation reactions (17). In this study, we investigated the fate of these transconjugants in serial passage experiments with and without selection for the plasmid.

**RESULTS**

Serial passage experiments to study transconjugant evolution over time. We mated *E. faecalis* OG1SSp(pAM714) donors and T11RF or T11RF ∆*cas9* recipients for 18 h on agar plates without erythromycin selection (17). After 18 h, mating reaction products were recovered and plated on agar selective for transconjugants, donors, and recipients. These data were previously reported and demonstrated that CRISPR-Cas significantly reduces pAM714 acquisition by T11RF (17). We randomly selected 6 T11RF(pAM714) and 6 T11RF∆*cas9*(pAM714) transconjugants for further analysis in this study. The 6 T11RF(pAM714) transconjugants are referred to here as WT1 to WT6, and the 6 T11RF∆*cas9*(pAM714) transconjugants are referred to as ∆1 to ∆6. The transconjugant colonies were each resuspended in brain heart infusion (BHI) broth. Samples were removed from the colony resuspensions for serial dilution and plating to determine the percent erythromycin-resistant cells and for PCR and sequencing to determine the size and sequence of CRISPR3. These data are referred to as day 0 data. Next, each transconjugant colony resuspension was split equally into BHI broth and BHI broth with erythromycin and passaged daily for 14 days. At every passage, broth samples were removed to determine the percentage of erythromycin-resistant cells and the size (by agarose gel electrophoresis) and sequence (by Illumina sequencing) of the CRISPR3 amplicon. As a control, wild-type T11RF was also passaged for 14 days in BHI broth.

At day 0, the WT1 to WT6 populations, except for WT4, were a mix of erythromycin-sensitive (primarily) and erythromycin-resistant cells (Figure S1A). The ∆1 to ∆6 populations were composed of erythromycin-resistant cells (Figure S1A). The CRISPR3 arrays of all
populations were wild-type size (1.76 kb) based on PCR amplification and agarose gel electrophoresis analysis of products (Figure S1B).

Erythromycin resistance is lost from most WT but no Δcas9 populations during passage in nonselective medium. The frequency of erythromycin-resistant cells decreased in the WT1, WT2, WT3, WT5, and WT6 populations over the course of serial passage in BHI broth lacking erythromycin (Fig. 1a). In contrast, erythromycin resistance was stably maintained at high frequencies in the WT4 population and all of the Δ1 to Δ6 populations in the absence of erythromycin selection (Fig. 1a). The CRISPR3 arrays of all populations were of wild-type size based on PCR amplification and agarose gel electrophoresis analysis of products (Figure S1B).

![Graph](image)

**FIG 1** Antibiotic resistance phenotypes and qualitative assessment of CRISPR3 genotypes in serially passaged transconjugant populations. (a) Proportion of erythromycin-resistant CFU per milliliter (expressed as a percentage of total CFU per milliliter) remaining in *E. faecalis* populations over the course of passage without (left) and with (right) antibiotic selection. WT transconjugant populations are shown in green and red, and Δcas9 transconjugant populations are shown in black. (b) CRISPR3 amplicon size from early (day 1) and late (day 14) passages for six WT transconjugant populations and a representative Δcas9 transconjugant population (Δ4). The CRISPR3 region was PCR amplified using aliquots from passaged cultures as the template. As a control, T11RF without pAM714 was passaged for 14 days and the CRISPR3 locus amplified (T11RF'). P, positive control; T11RF, T11RF genomic DNA; L, DNA ladder.

We analyzed the WT4 population further because it was an outlier compared to the other WT populations. We sequenced the *cas9* coding region of the WT4 population from day 0 and identified a mutation resulting in an Ala749Thr substitution. Ala749 occurs within the RuvC nuclease domain and is conserved in the well-studied Cas9 of *Streptococcus pyogenes* (17). The Ala749Thr substitution may result in loss in Cas9 function, causing WT4 to phenocopy the Δ1 to Δ6 populations.
CRISPR-Cas mutants emerge in WT populations during passage with erythromycin.

We observed stable maintenance of erythromycin resistance in all WT1 to WT6 and ΔD1 to ΔD6 populations during passage with erythromycin selection (Fig. 1a). By day 14 of passage with erythromycin, CRISPR3 arrays in all WT populations except WT1 and WT4 were smaller than the wild-type T11RF control (Fig. 1b). The variation in array size arose sporadically over the 14 days, and each transconjugant had a unique pattern (Figure S2). Sanger sequencing of the CRISPR3 array amplicons from day 1 and day 14 erythromycin-passaged populations revealed that either CRISPR3 spacer 6 was deleted from the array or the spacer 6 region had poor sequence quality (Table 1). We refer to spacer 6 as S6 here and use this subscript nomenclature for the other 20 spacers of the T11RF CRISPR3 array (e.g., S1, S2, etc.).

In contrast to WT populations, CRISPR3 array sizes for the ΔD1 to ΔD6 populations were unchanged after passage in medium with erythromycin (Fig. 1b and Table 1). We chose the ΔD4 population as representative of the ΔD1 to ΔD6 populations for further analyses.

To investigate whether mutations were present outside the CRISPR3 array, we performed whole-genome Illumina sequencing on selected populations. We observed variation in the cas9 sequence of the WT1, WT2, and WT3 day 14 populations relative to the T11RF wild type (Table 2). All of the mutations led to nonsynonymous changes (Table 2). Some of the sequenced populations possessed variations in one or more additional genes (see Table S2 in the supplemental material). No mutations were identified in the S6 protospacer or the PAM region of the repB gene in pAM714. However, we did identify a mutation within repB, not associated with the protospacer or PAM, in the WT2 population (Table S2). We observed no evidence of Tn917 movement from pAM714 into the T11 chromosome, as all reads overlapping the ends of Tn917 also overlapped the pAD1 reference sequence.

CRISPR3 alleles with spacer deletions are present in most WT populations after serial passage with erythromycin selection. To attain greater resolution of CRISPR3 alleles, we used Illumina sequencing to analyze CRISPR3 amplicons. We analyzed the CRISPR3 amplicons obtained for the WT1 to WT6 and ΔD4 populations after day 1 and day 14 of serial passage with erythromycin. We also analyzed the wild-type T11RF control passaged in BHI broth. We achieved an average of 16 million reads for our amplicons (Table S3).

To identify specific CRISPR3 alleles in the amplicon sequencing, we created a pool of references by sliding a contiguous and nonoverlapping window of 96 bp along the CRISPR3 reads.
reference, which allows each reference to contain exactly two adjacent spacers connected by one repeat. The sliding window starts at 30 bp upstream of the first repeat and ends at 30 bp downstream of the terminal repeat. In total, 22 references were generated to represent wild-type alleles. Then, we manually constructed artificial CRISPR3 reference sequences for every possible spacer deletion and rearrangement event with a length of 96 bp. In total, 462 references were constructed to represent all mutant alleles. We then mapped CRISPR amplicon reads to wild-type and mutant references (see Figure S3). The mapping was performed with stringency to allow unique mapping only. After mapping, the number of mapped reads per 96-bp reference was calculated and normalized to the total number of mapped reads per all references. To further evaluate the normalized reads per reference, we calculated z-score using means and standard deviations from the T11RF day 1 and day 14 mapping results. The higher the z-score, the more abundant reads per reference. The significance of z-score was calculated using a t-test with a degree of freedom of 483. By applying a P-value cutoff of 0.05, we identified the most abundant CRISPR3 alleles in each population (Fig. 2 and Table 1). Of the five T11RF transconjugant populations analyzed, all other than WT1 possessed at least one significantly enriched mutant CRISPR3 allele after 14 days of passage with antibiotic selection, and each of those alleles lacked S6 (Table 1).

One drawback of our method is that it is difficult to set a P-value cutoff. Here, we used the canonical definition of 0.05. However, the normalized reads per reference for wild-type alleles is expected to be much higher than that from mutant alleles, and in fact, it is much higher in control samples (T11RF control on day 1 and day 14). Such variation results in large standard deviation values, which skew the calculation of z-scores and hence cause underestimation of the abundance of mutant alleles. We predict that more mutant alleles existed in day 14 WT transconjugant samples, which is supported by an examination of the normalized reads per reference plot (Figure S4), but we are uncertain about their significance as measured by P value. Supporting our approach, control samples yielded results largely as expected. In control samples (T11RF and the representative Δcas9 transconjugant), only wild-type alleles were significantly abundant, with P values of <0.05. However, we also observed uneven distribution among different 96-bp references representing wild-type alleles, which resulted in a “loss of spacer16-17” call for the day 14 Δcas9 transconjugant at the P value of 0.057. When looking at the normalized reads per reference heat map (Figure S4), we believe that spacer16-17 was still largely intact within the Δcas9 transconjugant population, the abundance of which is about 1,000 times greater than that from a mutant 96-bp reference. In short, our statistical approach has strengths and weaknesses, which are discussed here.

Functional assays confirm that CRISPR-Cas defense was compromised after antibiotic selection. Our sequencing analyses suggested that the WT transconjugants passaged with erythromycin had become deficient for CRISPR-Cas defense either by deletion of spacer 6 or inactivation of Cas9 by a mutation. For a functional assessment, we tested whether the transconjugant populations could still defend against sequences targeted by T11RF CRISPR3-Cas. To do this, we utilized the pheromone-responsive plasmid pCF10 (42), which is not natively targeted by the T11RF CRISPR3-Cas system. We previously demonstrated that pCF10 transfer into wild-type and Δcas9 T11RF strains is equivalent (17). In this study, we modified pCF10 to be targeted by the T11RF CRISPR3-Cas system. We generated three derivatives of E. faecalis OG1SSp(pCF10), each with an insertion of a T11RF CRISPR3 spacer (S1, S6, or S7) and CRISPR3 consensus PAM in the pCF10 uvrB gene (Fig. 3a). Disruption of uvrB does not impact pCF10 conjugation (17). We then compared conjugation of wild-type pCF10 and these derivatives into the control T11RF population that had been passaged in BHI broth for 14 days. As expected, conjugation frequencies were significantly lower for all pCF10 derivatives bearing CRISPR3 targets than for the wild-type pCF10 (Fig. 3b).

We then evaluated pCF10 transfer into the WT5 day 14 populations. For WT5 passaged without erythromycin, we observed results similar to those for the T11RF control (Fig. 3b), demonstrating that the CRISPR3-Cas system in this population is still functional. For WT5 passaged with erythromycin, the conjugation frequency of only the pCF10 derivative bearing S6 was reduced relative to that of wild-type pCF10 (Fig. 3b). This is consistent with
FIG 2 Detection of mutant CRISPR3 array alleles using amplicon sequencing. Heat maps show the mutant alleles with significant abundance ($P < 0.05$) present at day 1 and day 14 of T11RF control (a) and (Continued on next page)
the deletion of $S_6$ and $S_7$ in this population (Fig. 2) and demonstrates that the sequencing data are accurate and not the result of PCR amplification bias. We note that we observed a $3 \log$ higher conjugation frequency of pCF10 into this population than the WT5 population passaged without erythromycin (Fig. 3b). This is due to comparatively low donor numbers (averages of $7.1 \times 10^6$ and $5.6 \times 10^9$ CFU/mL, respectively; raw transconjugant and donor numbers for Fig. 2 are presented in Data Set S1). pAM714 encodes the bacteriocin cytolysin (8). We previously reported that $E. faecalis$ with pAM714 kills $E. faecalis$ cells that lack pAM714 (18). The probable explanation is that the WT5 population passaged with erythromycin, which has a very high carriage rate of pAM714 (Fig. 1), killed OG1SSp(pCF10) donors using cytolysin.

We also evaluated pCF10 transfer into the WT4 day 14 populations. WT4 was unique among the 6 WT transconjugants in that it maintained erythromycin resistance at high frequencies in the absence of erythromycin selection (Fig. 1a). We identified a probable loss-of-function mutation in the RuvC domain-encoding region of $cas9$. The WT4 populations did not interfere with any of the three pCF10 derivatives bearing CRISPR3 targets (Fig. 3b). This is consistent with a loss of CRISPR-Cas function in the WT4 populations conferred by a loss-of-function mutation in $cas9$.

**Analysis of $E. faecalis$ genomes identifies a strongly supported instance of in situ CRISPR-Cas compromise.** Our data demonstrate that loss-of-function mutations in CRISPR-Cas arise that promote plasmid acquisition (for WT4) or plasmid maintenance (for WT2, -3, -5, and -6) in $E. faecalis$. We used genome data available for $E. faecalis$ to identify potential instances where this may have occurred in nature. We focused on published multidrug-resistant (MDR) $E. faecalis$ strains that possess CRISPR-Cas, reasoning that CRISPR-Cas function may have
been compromised, allowing for the accumulation of acquired resistance genes. More specifically, we narrowed our focus to strains that encode CRISPR-Cas as well as resistance to the last-line antibiotic vancomycin or linezolid, which is often plasmid borne. Using these strict criteria, we analyzed the literature and identified one strongly supported occurrence of CRISPR-Cas compromise. The MDR *E. faecalis* strain KUB3006, for which a completely closed genome is available (43), possesses 4 plasmids, including one that encodes linezolid resistance. However, it also encodes CRISPR3-Cas (43). The *cas9* gene is frameshifted, and the frameshift occurs within the codon for one of the two *Cas9* active sites that we previously experimentally confirmed in *E. faecalis* (17). We conclude that CRISPR3-Cas function is likely to be compromised in this strain, which requires experimental confirmation.

**DISCUSSION**

In this study, we investigated the fates of *E. faecalis* transconjugants that acquired a CRISPR-targeted plasmid, using serial passage with and without antibiotic selection for the plasmid.

We observed that 5 of 6 wild-type T11RF(pAM714) transconjugants lost the plasmid over the course of antibiotic-free serial passage, while T11RF Δcas9 (pAM714) transconjugants maintained the plasmid. This is indicative of active CRISPR-Cas defense in the T11RF(pAM714) transconjugant populations. An important caveat is that erythromycin is a bacteriostatic antibiotic, and we did not restreak transconjugant colonies before starting the serial passage experiment; therefore, we cannot be certain that plasmid-free T11RF organisms surviving on the transconjugant-selective agar were not carried over with the initial transconjugant colonies that were picked. At least two non-mutually exclusive explanations for our observations are possible. The first is that T11RF cells lacking pAM714 (either carried over or sporadically arising) outcompete T11RF(pAM714) cells. This competitive effect is due to the growth defect of cells that simultaneously possess CRISPR-Cas and one of its plasmid targets, as we have previously reported (15, 16). The second is that CRISPR-Cas “scrubs” pAM714 from T11RF over the course of antibiotic-free serial passage. In Figure S1, the WT4 colony stood out as containing essentially all erythromycin-resistant cells, while the other WT transconjugant colonies did not. As determined in this study, WT4 is a *cas9* loss-of-function mutant. These results seem to favor the second explanation, since carryover of erythromycin-sensitive cells may have equally affected the randomly selected WT4 colony compared to other randomly selected WT colonies. Regardless, this could be further investigated by repeating the experiment and streaking the initial transconjugant colonies prior to serial passage.

On the other hand, when the same T11RF(pAM714) transconjugants were passaged with antibiotic selection, the plasmid was maintained, as expected, but variants emerged that lacked the spacer targeting the plasmid. In some cases, multiple sequential spacers were deleted, and we demonstrated that this resulted in loss of defense against multiple plasmids (specifically, pAM714 and derivatives of pCF10; see the results for WTS in Fig. 3). This supports our CRISPR amplicon sequence analysis by demonstrating that spacer losses identified by sequencing corresponded with loss of functional CRISPR-Cas defense. Variations in *cas9* were also observed in some antibiotic-passaged populations but were not experimentally investigated, so their impacts on CRISPR-Cas function remain undefined. We also identified one very strongly supported example of CRISPR-Cas compromise in an independent *E. faecalis* isolate (KUB3006) with a closed genome. Other studies have identified spacer deletion events occurring in transformants or transconjugants of CRISPR-targeted plasmids (44–46), providing more evidence that loss-of-function mutations in CRISPR-Cas contribute to antibiotic resistance plasmid dissemination in bacterial populations.

Our work occurs in the broader context of work on “self-targeting” spacers in CRISPR-Cas systems, which have been studied for some time across different types of CRISPR-Cas in different species (47). Self-targeting spacers typically refer to those with sequence complementarity to targets within the host genome. This phenomenon was mostly observed during phage infection and plasmid invasion (48), but self-targeting spacers against nonmobile elements have also been reported (49). Different hypotheses have been posited for their maintenance in microbial genomes, including alteration of target sites via DNA repair (50–52), large deletion
of the target sites (53), alteration of the PAM sequence (54), loss of spacers (16, 55) or mutations in cas genes (53). The “self-targeting” outcome is dependent on the fitness cost and environmental selective pressure (56). The conflict between active CRISPR-Cas and self-targeting spacers plays a role in shaping bacterial evolution, including altering the population-level genetic diversity (57–59), remodeling of pathogenicity islands (60), and modulation of metabolic pathways (61). Our work sought to investigate how this conflict can be resolved in E. faecalis, as entry and maintenance of pAM714 in wild-type T11RF create a self-targeting situation that theoretically must be resolved to prevent persistent stress from DNA damage at the targeted pAM714 repB site. In essence, this is the basis of CRISPR-Cas gene editing, and in a previous study, we took advantage of this property to implement CRISPR-Cas9 gene editing in E. faecalis (15). Here, a recombination template to repair DNA damage (in this case, damage to pAM714 repB) was not provided to the cells. We observed (in general) that the self-targeting CRISPR spacer was retained and the target lost in populations passaged without antibiotic selection for the target. On the other hand, CRISPR spacer and/or potentially overall CRISPR-Cas function was lost, and the target retained, in populations passaged with antibiotic selection for the target. In other studies, these self-targeting spacers have been engineered to promote the loss of genomic islands and other mobile elements in bacteria (53, 60, 62). Our results demonstrate that selection for the targeted element impedes this process, which is relevant to the design and implementation of CRISPR-based antimicrobials that “cure” E. faecalis of antibiotic resistance genes (19, 63). Our results suggest that these systems will need to be introduced and utilized either before or after antibiotic therapy, not during.

Overall, we posit that the interplay of CRISPR-Cas, plasmids, and antibiotic selection should be further investigated to understand the role of CRISPR-Cas in the antibiotic resistance crisis. Particularly important will be experimental designs that better replicate the in vivo setting, where antibiotic resistance plasmids disseminate, for, e.g., the gastrointestinal tract, where multispecies biofilms are present and cell densities, mutation rates, CRISPR-Cas activities, and antibiotic concentrations are likely to vary. More specifically, it remains unclear how frequently CRISPR-Cas spacer deletion or loss-of-function mutants arise in vivo under antibiotic selection for self-targeted plasmids. In a prior study, we assessed the in vivo functioning of T11RF CRISPR-Cas against pAM714 in a mouse intestinal colonization model where OG155sp(pAM714) donors and T11RF or T11RFΔcas9 recipients were introduced (18). CRISPR-Cas defense against pAM714 appeared robust—no T11RF(pAM714) transconjugants stably colonized the mouse intestine above our limit of detection (<10^2 CFU/g feces). Conversely, T11RFΔcas9(pAM714) transconjugants were stably present in most mice (up to ~10^2 CFU/g feces). The key question we did not answer in that study is whether T11RF (pAM714) transconjugants with spacer 6 deletions or other loss-of-function mutations in CRISPR-Cas would have emerged if we had treated the mice with erythromycin. This remains an outstanding question to be addressed.

MATERIALS AND METHODS

Strains, reagents, and routine molecular biology procedures. Bacterial strains and plasmids used in this study are listed in Table 3. E. faecalis strains were grown in BHI broth or on agar plates at 37°C unless otherwise noted. Antibiotics were used for E. faecalis at the following concentrations: erythromycin, 50 μg/mL; chloramphenicol, 15 μg/mL; streptomycin, 500 μg/mL; spectinomycin, 500 μg/mL; rifampicin, 50 μg/mL; fusidic acid, 25 μg/mL; tetracycline, 10 μg/mL. Escherichia coli strains used for plasmid propagation and were grown in lysogeny broth (LB) or on agar plates at 37°C. Chloramphenicol was used at 15 μg/mL for E. coli. PCR was performed using Taq (New England Biolabs) or Phusion (Fisher Scientific) polymerase. Primer sequences used are in Table S1. Routine Sanger sequencing was carried out at the Massachusetts General Hospital DNA core facility (Boston, MA). E. faecalis electrocompetent cells were made using the lysozyme method as previously described (64).

Generation of pCF10 derivatives. To insert the T11 CRISPR3 spacer 1 (S1), S2, and S3 sequences and CRISPR3 PAM (TTGTA) into pCF10, 47-bp and 39-bp single-stranded DNA oligonucleotides were annealed to each other to generate dsDNA with restriction enzyme overhangs for BamHI and PstI. The annealed oligonucleotides were ligated into the pLT06 derivative pWH107, which includes sequence from pCF10 uvrB, to insert these sequences into the uvrB gene of pCF10 by homologous recombination. A knock-in protocol was performed as previously described (17).

Conjugation experiments. E. faecalis donor and recipient strains were grown in BHI overnight to stationary phase. A 1:10 dilution was made for both donor and recipient cultures in fresh BHI broth and incubated for 1.5 h to reach mid-exponential phase. A mixture of 100 μL donor cells and 900 μL recipient cells was pelleted and plated on nonselective BHI agar to allow conjugation. After 18 h of incubation, the conjugation
mixture was scraped from the plate using 2 mL 1× phosphate-buffered saline (PBS) supplemented with 2 mM EDTA. Serial dilutions were prepared from the conjugation mixture and plated on BHI agars selective for transconjugants or donors. After 24 to 48 h of incubation, CFU per milliliter was determined using plates with 30 to 300 colonies. The conjugation frequency was calculated as the concentration (CFU per milliliter) of transconjugants divided by the concentration (CFU per milliliter) of donors.

**Serial passage.** Transconjugant colonies were suspended in 50 μL BHI broth. The 50-μL suspension was used as follows: 3 μL was used for PCR to confirm the integrity of the CRISPR array, 10 μL was inoculated into plain BHI broth, another 10 μL was inoculated into selective BHI broth for plasmid selection, and another 10 μL was used for serial dilution and plating on selective medium to enumerate the initial number of plasmid-containing cells in the transconjugant colonies. Both cultures were incubated for 24 h, followed by 1:1,000 dilution into either fresh plain BHI or fresh selective BHI. At each 24-h interval, 3 μL of each culture from the previous incubation was used for PCR to check CRISPR array integrity, and 10 μL was used for serial dilution and plating on agars to determine CFU per milliliter for total viable cells and plasmid-containing cells. The cultures were passaged in this manner for 14 days; cryopreserved culture stocks were made daily in glycerol. To use the day 14 cultures, an overnight broth culture was used as the template in PCR using Phusion polymerase with CR3_seq_F/R primers (Table S1). The PCR products were purified using the Thermo Scientific PCR purification kit (Thermo Scientific). Genomic DNA was isolated using the phenol-chloroform method (65). The purified PCR amplicons and genomic DNA samples were sequenced using 2 × 150-bp paired-end sequencing chemistry by Molecular Research LP (MR DNA, Texas).

**Whole-genome sequencing analysis.** T11 supercontig and pAD1 plasmid contig references were downloaded from NCBI (T11, accession numbers NZ_GG688637.1 to NZ_GG688649; pAD1, accession numbers AB007844, AF394225, AH011360, L01794, L19532, L37110, M84374, M87836, U00681, X17214, X62657, X63585, and M11180 [Tn917]). Reads were aligned to these references using default parameters in CLC Genomics Workbench (Qiagen), where ≥50% of each mapped read has ≥80% sequence identity to the reference. Variations occurring with ≥35% frequency at positions with ≥10× coverage between our samples and the reference contigs were detected using the Basic Variant Detector tool. At the same time, local realignment was performed, followed by Fixed Ploidy variant detection using default parameters and variants probability ≥90% in CLC Genomics Workbench. The basic variants and fixed ploidy variants were combined for each sequencing sample and subjected to manual inspection. The variants that were detected in the T11 genome from all samples were inferred to be variants in our parent T11 stock and were manually removed. The variants that were detected in the pAD1 genome from all transconjugant samples were inferred to be variants in our pAM714 stock and hence were also manually removed. Next, variants within the CRISPR3 array were removed, as we analyzed CRISPR3 alleles using a different approach (amplicon deep sequencing; see below). All variants detected from all populations were manually checked for coverage depth to eliminate the detection bias. The variants detected in all samples are shown in Table S2. To detect the insertion site of Tn917, the mapped reads on reference M11180 were inspected. Reads immediately adjacent to the 5′- and 3′-ends of Tn917 shared consensus sequences which were later used for BLAST analysis against T11 and pAD1 (Figure S5). No evidence for Tn917 insertions other than in the expected position in pAM714 was detected.

**Analysis of CRISPR3 amplicon sequencing.** To analyze the amplicon sequencing, we first created a pool of references with 96 bp to represent both wild-type and mutant CRISPR3 alleles. In the wild-type reference
pool, each 96-bp reference contains two adjacent spacers connected by one direct repeat (5'-Sx-R-Sy-3'), where x is from 1 to 20. To include the leader end and terminal end, the wild-type reference tool also contains 30 bp upstream of the first repeat (5'Sx) and 30 bp downstream of the terminal repeat (3'Sy), generating two additional wild-type references: 5'-Sx-R-Sy-3' and 5'-T-R-ST-3'. In total, 22 references were created to represent the wild-type CRISPR3 allele.

To detect CRISPR3 spacer deletions and rearrangements, we manually created an additional 462 references with 96-bp in length to represent mutant CRISPR3 alleles. Each mutant reference contains two spacers connected by a direct repeat, where the reference was not already represented in the wild-type reference pool. Schematically, the mutant references can be expressed by 5'-spacer[x]-repeat-spacer[y]-3' (5'-5'Sx-R-Sy-3') where y ≠ x. The selection of spacer[x] is from S1 to Sy, while the selection of spacer[y] is from Sx to Sy. Additionally, we assume that if spacer[y] is Sx, then the repeat would be terminal repeat TR, generating 5'-Sx-T-R-ST-3' (x is from 0 to 21). In total, 462 references of 96 bp were created.

Sequencing reads were first clipped into 75-bp fragments to enhance mapping efficiency, allowing for retrieval of maximal sequence information. The clipped reads were used to map to the pool of 96-bp references using stringent mapping parameters in CLC Genomics Workbench (Qiagen). The stringent mapping parameters require 100% of each mapped read to be ≥95% identical to one unique reference. Thus, the sequencing reads from different CRISPR alleles will be distinguished. The number of mapped reads per each reference was calculated and normalized to the total number of mapped reads to all references, generating normalized reads per reference. The total number of mapped reads to pools of wild-type references and mutant references are summarized in Table S3.

To visualize the normalized reads per reference, a heat map was generated (Figure S4). On the heat map, each row name represents 5'-Sx (x is from 0 to 20) and each column name represents R-Sy-3' (y is from 1 to 21) or TR-Sy-3'. The diagonal cells are empty due to the assumption of no repetitive spacers (or 5'-Sx-R-Sy-3'). Intuitively, the cells adjacent to the diagonal cells on the upper side represent the wild-type pool of references, 5'-Sx-R-Sy-3' where y = x + 1 (Figure S3). The cell color represents normalized reads per reference (white to red) to black corresponds to lowest to intermediate to highest abundance.

To evaluate the statistical significance of normalized mapped reads, we calculated z-score using the mean and standard deviation calculated from the T11RF control. Combining the normalized reads per reference from day 1 and day 14 T11RF control, we obtained an average number of normalized reads per reference of 0.002 or 2% with standard deviation of 0.0096 or 9.6%. These numbers were used to calculate z-score for normalized reads per reference for all samples. To assess the significance, the z-score was further transformed into a P value. A P value of 0.05 was used as a cutoff for significance.

To visualize the P value and derive mutant alleles, a heat map was plotted using the method described above. The cells with P values of <0.05 were color coded. To derive the mutant allele, we assume that Sx and Sy are intact. The sequence of spacers was derived based on the idea of forward algorithm and Viterbi algorithm. Each P value was considered a conditional probability: PSx-Sy-R-Sy-3' = P(spacer[y]

For supplemental material, the sequencing data for amplicon and whole-genome sequencing analysis of transconjugant populations have been deposited in the NCBI Sequence Read Archive under accession no. PRJNA418345.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.01 MB.
SUPPLEMENTAL FILE 3, PDF file, 4.5 MB.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant R01AI116610 to K.L.P. and the Cecil H. and Ida Green Chair to M.O.Z.

We thank Chen Jia for consultation on data analysis methods.

REFERENCES

1. Lebreton F, Willems R, Gilmore MS. 2014. Enterococcus diversity, origins in n, and gut colonization. In Gilmore MS, Clewell DB, Ike Y, Shankar N (ed), Enterococci: from commensals to leading causes of drug resistant infection. Massachusetts Eye and Ear Infirmary, Boston, MA.
2. Kristich CJ, Djoric D, Little JL. 2014. Genetic basis for vancomycin-enhanced cephalosporin susceptibility in vancomycin-resistant enterococci revealed using counterselection with dominant-negative thymidylate synthase. Antimicrob Agents Chemother 58:1556–1564. https://doi.org/10.1128/AAC.02001-13.
3. Utley AH, George RC, Naidoo J, Woodford N, Johnson AP, Collins CH, Morrison D, Gilfillan AJ, Fitch LE, Heptonstall J. 1989. High-level vancomycin-resistant enterococci causing hospital infections. Epidemiol Infect 103:173–181. https://doi.org/10.1017/S0950268899000347.
4. Huycke MM, Sahn DF, Gilmore MS. 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg Infect Dis 4:239–249. https://doi.org/10.3201/eid0402.980211.
5. Clewell DB, Weaver KE, Dunny GM, Coque TM, Francia MV, Hayes F. 2014. Extrachromosomal and mobile elements in enterococci: transmission, maintenance, and epidemiology. In Gilmore MS, Clewell DB, Ike Y, Shankar N (ed), Enterococci: from commensals to leading causes of drug resistant infection. Massachusetts Eye and Ear Infirmary, Boston, MA.
6. Palmer KL, Kos VN, Gilmore MS. 2010. Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. Curr Opin Microbiol 13: 632–639. https://doi.org/10.1016/j.mib.2010.08.004.
7. Hegstad K, Mikalsen T, Coque T, Werner G, Sundsfjord A. 2010. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium. Clin Microbiol Infect 16:541–554. https://doi.org/10.1111/j.1469-0691.2010.03226.x.
8. 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 58:205–227. https://doi.org/10.1016/j.plasmid.2007.05.001.
9. Dunny GM. 2007. The peptide pheromone-inducible conjugation system of Enterococcus faecalis plasmid pAD1-encoded cyto- toxic toxin system and its relationship to lantibiotic determinants. J Bacteriol 176:7335–7344. https://doi.org/10.1128/jb.176.23.7335-7344.1994.

10. Jasni AS, Mullany P, Hussain H, Roberts AP. 2010. Demonstration of conjugative transposon (Tn5397)-mediated horizontal gene transfer between Clostridiun difficile and Enterococcus faecalis. Antimicrob Agents Chemother 54:4924–4926. https://doi.org/10.1128/AAC.00496-10.

11. Gilmore MS, Segarra RA, Booth MC, Bogie CP, Hall LR, Clewell DB. 1994. CRISPR-Cas and Plasmid Maintenance Applied and Environmental Microbiology June 2023 Volume 89 Issue 6 10.1128/aem.00124-23.

12. Showsh SA, De Boever EH, Clewell DB. 2001. Vancomycin resistance plas- mid in Enterococcus faecalis that encodes sensitivity to a sex pheromone also produced by Staphylococcus aureus. Antimicrob Agents Chemother 45:2177–2178. https://doi.org/10.1128/AAC.45.7.2177-2178.2001.

13. Tsvetkova M, VanrauJC, Lambert T. 2010. Analysis of the mobilization functions of the vancomycin resistance transposon Tn5149, a member of a new family of conjugative elements. J Bacteriol 192:702–713. https://doi.org/10.1128/JB.00680-09.

14. Zhu W, Murray PR, Huswic JK, Jernigan JA, McDonald LC, Clark NC, Anderson KD, McDougal LK, Hageman JC, Olsen-Rasmussen M, Frace M, Alangaden GJ, Chenoweth C, Zervas MJ, Robinson-Dunn B, Schreckenberger PC, Reller LB, Rudrik JT, Patel JB. 2010. Dissemination of an Enterococcus Incorporate vanA plasmid associated with vancomycin-resistant Staphylococcus aureus. Antimicrob Agents Chemother 54:4334–4340. https://doi.org/10.1128/AAC.01185-10.

15. Hullahali K, Rodrigues M, Nguyen UT, Palmer K. 2018. An attenuated CRISPR-Cas system in Enterococcus faecalis permits DNA acquisition. mBio 9:e00414-18. https://doi.org/10.1128/mBio.00414-18.

16. Hullahali K, Rodrigues M, Palmer KL. 2017. Exploiting CRISPR-Cas to manipulate Enterococcus faecalis populations. Elife 6:e26664. https://doi.org/10.7554/eLife.26664.

17. Price VJ, Huo W, Sharif A, Palmer KL. 2016. CRISPR-Cas and restriction-modification act additively against conjugative antibiotic resistance plasmid transfer in Enterococcus faecalis. mSphere 1:e00064-16. https://doi.org/10.1128/mSphere.00064-16.

18. Price VJ, McBride SW, Hullahali K, Chatterjee A, Duerkop BA, Palmer KL. 2019. Enterococcus faecalis CRISPR-Cas is a robust barrier to conjugative antibiotic resistance dissemination in the murine intestine. mSphere 4:e00464-19. https://doi.org/10.1128/mSphere.00464-19.

19. Rodrigues M, McBride SW, Hullahali K, Palmer KL, Duerkop BA. 2019. Conjugative delivery of CRISPR-Cas9 for the selective depletion of antibiotic-resistant enterococci. Antimicrob Agents Chemother 63:e01454-19. https://doi.org/10.1128/AAC.01454-19.

20. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero M, Horvath P, Doudna JA. 2007. CRISPR provides acquired resistance against viruses in pro- karyotes. Science 315:1709–1713. https://doi.org/10.1126/science.1138140.

21. Marraffini LA, Sontheimer EJ. 2008. CRISPR interference limits horizontal gene transfer in Staphylococcus aureus. Antimicrob Agents Chemother 52:7113. https://doi.org/10.1128/AAC.00496-10.

22. Bourgogne A, Garin A, Qin X, Singh RV, Sillanpaa J, Yerapragada S, Ding Y, Dungan-Rocha S, Buhay C, Shen H, Chen G, Williams G, Muzny D, Maadani A, Fox KA, Gioia J, Chen L, Shang Y, Arias CA, Nallapareddy SR, Zhao M, Prakash VP, Choudhury S, Jiang H, Gibbs RA, Murray BE, Highlander SK, Weinstock GM. 2008. Large scale variation in Enterococcus faecalis illustrated on its chromosome and optrA-plasmid. Front Microbiol 9:2576.https://doi.org/10.1371/journal.pone.01000582.

23. Palmer KL, Godfrey P, Griggs A, Kos VN, Zucker J, Desjardins C, Cerqueira G, Gevers D, Walker S, Wortman J, Fieldgarden M, Haas B, Birren B, Gilmore MS. 2012. Comparative genomics of enterococci: variation in Enterococcus fae- calis, clade structure in E. faecium, and defining characteristics of E. gallinarum and E. casseli. mBio 3:e00318-11.https://doi.org/10.1128/mBio.00318-11.

24. Weaver KE, Tettle DJ. 1994. Identification and characterization of an Enter- fococcus faecalis plasmid pAD1-encoded stability determinant which produces two small RNA molecules necessary for its function. Pnas 32:168–181. https://doi.org/10.1073/pnas.19941053.

25. Ike Y, Clewell DB, Segarra RA, Gilmore MS. 1990. Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in Enterococcus faecalis: Tn97 insertional mutagenesis and cloning. J Bacteriol 172:155–163. https://doi.org/10.1128/JB.172.1.155-163.1990.

26. Clewell DB, Tormich PK, Gavoron-Burke MC, Franke AE, Yagi Y, An FY. 1982. Mapping of Streptococcus faecalis plasmids pAD1 and pAD2 and studies relating to transposition of Tn97. J Bacteriol 152:1220–1230. https://doi.org/10.1128/JB.152.3.1220-1230.1982.

27. Dunny G, Yuhas M, Ehrenfeld E. 1982. Genetic and physiological analysis of conjugation in Streptococcus faecalis. J Bacteriol 151:1855–1859. https://doi.org/10.1128/JB.151.5.1855-1859.1982.

28. Urozda M, Sekizuka T, Matsu H, Suzuki K, Seki H, Saito M, Hanaki H. 2018. Complete genome sequence and characterization of linezolid-resistant Enterococcus faecalis clinical isolate KUB3006 carrying a cfr(B)-transposon. Antimicrob Agents Chemother 62:e02576-17. https://doi.org/10.1128/AAC.02576-17.

29. Liu W, Maniv I, Arain F, Wang Y, Levin BR, Marraffini LA. 2013. Dealing with the evolutionary downgrade of CRISPR immunity: bacteria and beneficial plasms. PLoS Genet 9:e1003844. https://doi.org/10.1371/journal.pgen.1003844.

30. Rao C, Chinn D, Emsinger AW. 2017. Priming in a permissive type I-C CRISPR-Cas system reveals distinct dynamics of spacer acquisition and loss. RNA 23:1525–1538. https://doi.org/10.1261/rna.062083.117.

31. Harper CL, Sanchez MD, Sauvage E, da Cunha V, Clermont D, Ratsima Hariniaina E, González-Zorn B, Poyart C, Rosinski-Chupin I, Glaser P. 2012. The highly dynamic CRISPR1 system of Streptococcus agalactiae controls the diversity of its mobiles. Mol Microbiol 85:1057–1071. https://doi.org/10.1111/j.1365-2958.2012.08172.x.
Wimmer F, Beisel CL. 2019. CRISPR-Cas systems and the paradox of self-targeting spacers. Front Microbiol 10:3078. https://doi.org/10.3389/fmicb.2019.03078.

Staals RH, Jackson SA, Biswas A, Brouns SJ, Brown CM, Fineran PC. 2016. Interference-driven spacer acquisition is dominant over naive and primed adaptation in a native CRISPR-Cas system. Nat Commun 7:12853. https://doi.org/10.1038/ncomms12853.

Stem A, Keren L, Wurtzel O, Amitai G, Sorek R. 2010. Self-targeting by CRISPR: gene regulation or autoimmunity? Trends Genet 26:335–340. https://doi.org/10.1016/j.tig.2010.05.008.

Cui L, Bikard D. 2016. Consequences of Cas9 cleavage in the chromosome of Escherichia coli. Nucleic Acids Res 44:4243–4251. https://doi.org/10.1093/nar/gkw223.

Staclier AE, Turgeman-Grott I, Shriftman-Segal E, Allers T, Marchfelder A, Gophna U. 2017. High tolerance to self-targeting of the genome by the endogenous CRISPR-Cas system in an archaeon. Nucleic Acids Res 45:5208–5216. https://doi.org/10.1093/nar/gkw150.

Xu T, Li Y, Shi Z, Hemme CL, Li Y, Zhu Y, Van Nostrand JD, He Z, Zhou J. 2015. Efficient genome editing in Clostridium cellulolyticum via CRISPR-Cas9 nickase. Appl Environ Microbiol 81:4423–4431. https://doi.org/10.1128/AEM.00873-15.

Guan J, Wang W, Sun B. 2017. Chromosomal targeting by the type III-A CRISPR-Cas system can reshape genomes in Staphylococcus aureus. mSphere 2:e00403-17. https://doi.org/10.1128/mSphere.00403-17.

Dy RL, Pitman AR, Fineran PC. 2013. Chromosomal targeting by CRISPR-Cas systems can contribute to genome plasticity in bacteria. Mob Genet Elements 3:e26831. https://doi.org/10.4161/mge.26831.

Canez C, Selle K, Goh YJ, Barrangou R. 2019. Outcomes and characterization of chromosomal self-targeting by native CRISPR-Cas systems in Streptococcus thermophiles. FEMS Microbiol Lett 366:fnz105. https://doi.org/10.1093/femsle/fnz105.

Westra ER, Levin BR. 2020. It is unclear how important CRISPR-Cas systems are for protecting natural populations of bacteria against infections by mobile genetic elements. Proc Natl Acad Sci U S A 117:27777–27785. https://doi.org/10.1073/pnas.1915966117.

Rollie C, Chevallereau A, Watson BNJ, Chyou TY, Fradet O, McLeod I, Fineran PC, Brown CM, Gandon S, Westra ER. 2020. Targeting of temperate phages drives loss of type I CRISPR-Cas systems. Nature 578:149–153. https://doi.org/10.1038/s41586-020-1936-2.

van Houte S, Ekrath AK, Broniewski JM, Chabas H, Ashby B, Bondy-Denomy J, Gandon S, Boots M, Paterson S, Buckling A, Westra ER. 2016. The diversity-generating benefits of a prokaryotic adaptive immune system. Nature 532:385–388. https://doi.org/10.1038/nature17436.

Watson BNJ, Steens JA, Staals RHJ, Westra ER, van Houte S. 2021. Coevolution between bacterial CRISPR-Cas systems and their bacteriophages. Cell Host Microbe 29:715–725. https://doi.org/10.1016/j.chom.2021.03.018.

Vercoe RB, Chang JT, Dy RL, Taylor C, Gristwood T, Clulow JS, Richter C, Przybilski R, Pitman AR, Fineran PC. 2013. Cytotoxic chromosomal targeting by CRISPR/Cas systems can reshape bacterial genomes and expel or remodel pathogenicity islands. PLoS Genet 9:e1003454. https://doi.org/10.1371/journal.pgen.1003454.

Aklujkar M, Lovfey DR. 2010. Interference with histidyl-tRNA synthetase by a CRISPR spacer sequence as a factor in the evolution of Pelobacter carbinolicus. BMC Evol Biol 10:230. https://doi.org/10.1186/1471-2148-10-230.

Selle K, Klaenhammer TR, Barrangou R. 2015. CRISPR-based screening of genomic island excision events in bacteria. Proc Natl Acad Sci U S A 112:8076–8081. https://doi.org/10.1073/pnas.1508525112.

Palacios Araya D, Palmer KL, Duerkop BA. 2021. CRISPR-based antimicrobials to obstruct antibiotic-resistant and pathogenic bacteria. PLoS Pathog 17:e1009672. https://doi.org/10.1371/journal.ppat.1009672.

Bae T, Kozlovicz B, Dunny GM. 2002. Two targets in pCF10 DNA for PrgX binding: their role in production of Qa and prgX mRNA and in regulation of pheromone-inducible conjugation. J Mol Biol 315:995–1007. https://doi.org/10.1006/jmbi.2001.5294.

Manson JM, Keis S, Smith JMB, Cook GM. 2003. A blonal lineage of VanA-type Enterococcus faecalis predominates in vancomycin-resistant enterococci isolated in New Zealand. Antimicrob Agents Chemother 47:204–210. https://doi.org/10.1128/AAC.47.1.204-210.2003.

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 20:473–477. https://doi.org/10.1016/0003-9907(75)90236-6.

Perez-Casal J, Caparon MG, Scott JR. 1991. Mry, a trans-acting positive regulator of the M protein gene of Streptococcus pyogenes with similarity to the receptor pheromone-inducible conjugation. J Mol Biol 20:473–477. https://doi.org/10.1016/0022-2836(91)90029-A.

Efremova E, Pechman H, Beratis N, Przybilski R, Pitman AR, Fineran PC. 2013. Cytotoxic chromosomal targeting by CRISPR/Cas systems can reshape bacterial genomes and expel or remodel pathogenicity islands. PLoS Genet 9:e1003454. https://doi.org/10.1371/journal.pgen.1003454.

Host Microbe 29:715–725. https://doi.org/10.1016/j.chom.2021.03.018.