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METHODS

RecT Recombinase Expression Enables Efficient Gene Editing in *Enterococcus* spp.

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ABSTRACT *Enterococcus faecium* is a ubiquitous Gram-positive bacterium that has been recovered from the environment, food, and microbiota of mammals. Commensal strains of *E. faecium* can confer beneficial effects on host physiology and immunity, but antibiotic usage has afforded antibiotic-resistant and pathogenic isolates from livestock and humans. However, the dissection of *E. faecium* functions and mechanisms has been restricted by inefficient gene-editing methods. To address these limitations, here, we report that the expression of *E. faecium* RecT recombinase significantly improves the efficiency of recombineering technologies in both commensal and antibiotic-resistant strains of *E. faecium* and other *Enterococcus* species such as *E. durans* and *E. hirae*. Notably, the expression of RecT in combination with clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 and guide RNAs (gRNAs) enabled highly efficient scarless single-stranded DNA recombineering to generate specific gene-editing mutants in *E. faecium*. Moreover, we demonstrate that *E. faecium* RecT expression facilitated chromosomal insertions of double-stranded DNA templates encoding antibiotic-selectable markers to generate gene deletion mutants. As a further proof of principle, we use CRISPR-Cas9-mediated recombineering to knock out both sortase A genes in *E. faecium* for downstream functional characterization. The general RecT-mediated recombineering methods described here should significantly enhance genetic studies of *E. faecium* and other closely related species for functional and mechanistic studies.

IMPORTANCE *Enterococcus faecium* is widely recognized as an emerging public health threat with the rise of drug resistance and nosocomial infections. Nevertheless, commensal *Enterococcus* strains possess beneficial health functions in mammals to upregulate host immunity and prevent microbial infections. This functional dichotomy of *Enterococcus* species and strains highlights the need for in-depth studies to discover and characterize the genetic components underlying its diverse activities. However, current genetic engineering methods in *E. faecium* still require passive homologous recombination from plasmid DNA. This involves the successful cloning of multiple homologous fragments into a plasmid, introducing the plasmid into *E. faecium*, and screening for double-crossover events that can collectively take up to multiple weeks to perform. To alleviate these challenges, we show that RecT recombinase enables the rapid and efficient integration of mutagenic DNA templates to generate substitutions, deletions, and insertions in the genomic DNA of *E. faecium*. These improved recombineering methods should facilitate functional and mechanistic studies of *Enterococcus*.

KEYWORDS CRISPR, *E. faecium*, *Enterococcus*, VRE, biotechnology, genetics, recombineering

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The Gram-positive bacterial genus *Enterococcus* has diverse origins from the environment, food, animals, and humans (1–3). *Enterococcus faecium* has been linked to many aspects of human health, including both probiotic and pathogenic activities (1–3). For example, *E. faecium* remains a global public health concern for its ability to acquire vancomycin resistance and cause nosocomial infections (1–3). Yet commensal *E. faecium* has been discovered to modulate host immunity to promote protection against other enteric pathogens and decrease diarrheal severity, and it has been associated with enhanced immunotherapy efficacy (4–10). To better understand the mechanisms of both pathogenic and beneficial functions of *Enterococcus*, genetic-based studies of *Enterococcus* are needed to accelerate our comprehension of these functions. Although there are some existing tools to genetically manipulate and analyze enterococci (11, 12), the study of *E. faecium* in the laboratory is largely limited by inefficient reverse genetics.

To date, the generation of scarless genetic mutants in *Enterococcus* relies heavily on homologous recombination (11–14). Traditionally, this process involves cloning homologous templates hundreds of bases to a thousand bases long flanking the DNA edit of interest into an *Enterococcus*-compatible vector containing a counterselectable marker and/or a temperature-sensitive replication factor that is then transformed into *Enterococcus*. Transformants are screened for a single-crossover plasmid insertion before being screened for a double crossover in the gene of interest using a multistep selection-and-counterselection process (11–13, 15). While homologous recombination is a proven way to acquire genetic *Enterococcus* mutants, this process requires many screening steps and takes weeks to perform. Conversely, the development of clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 gene-editing methods offers new opportunities to improve scarless mutagenesis in bacteria (16). CRISPR-Cas9 is an endonuclease system that site-specifically cleaves genomic DNA using a homologous RNA guide (16). The Cas9 nuclease uses guide RNA (gRNA) consisting of both CRISPR RNA (crRNA) and an antisense transactivating crRNA (tracrRNA) for targeted DNA cleavage (17). In bacteria, efficient Cas9-guided genomic cleavage usually leads to cell death due to the lack of inherent DNA repair mechanisms to repair and escape Cas9 double-stranded DNA (dsDNA) breaks. Thus, CRISPR-Cas9 can be used alongside complementary gene-editing methods to select for mutants by targeting Cas9 to the wild-type (WT), unedited genomic sequence, thereby enriching for bacteria that mutated and escaped Cas9 chromosomal cleavage. Indeed, a recent study sought to ameliorate the homologous-recombination selection process using CRISPR-Cas9 counterselection in *E. faecium* (18). While this study was successful in demonstrating CRISPR-Cas9 as an effective method for mutant selection in *E. faecium*, the method described still relied on endogenous homologous recombination, which required multiple cloning steps of both a homologous template and gRNA on the same plasmid to generate a targeted mutation.

Recombineering is a gene-editing method in bacteria utilizing phage-derived recombinases to mediate the incorporation of single-stranded DNA (ssDNA) or dsDNA templates into bacterial genomes (19). Although recombineering is well established for model species, many bacteria, including *Enterococcus*, lack functional recombineering methods. More recently, recombineering has been combined with CRISPR-Cas-based technologies to counterselect for successfully scarless bacterial mutants in select species (17, 20). CRISPR-Cas-mediated recombineering is advantageous over homologous recombination due to its ability to incorporate short ssDNA oligonucleotide templates that are available from commercial vendors without the need to assemble the desired template through cloning. Recombineering can also be performed with blunt-end dsDNA templates for CRISPR-Cas-independent recombineering. In this study, we report a recombineering system using the RecT from a latent bacterial prophage that is compatible with *E. faecium* and other related species (Fig. 1). By combining recombineering with CRISPR-Cas9 counterselection, we were able to produce both scarless mutants via point mutations as well as controllable deletions of various sizes. Additionally, we report our system to have gene-editing activity with PCR-generated dsDNA templates. Overall, we show the versatility of our RecT-mediated

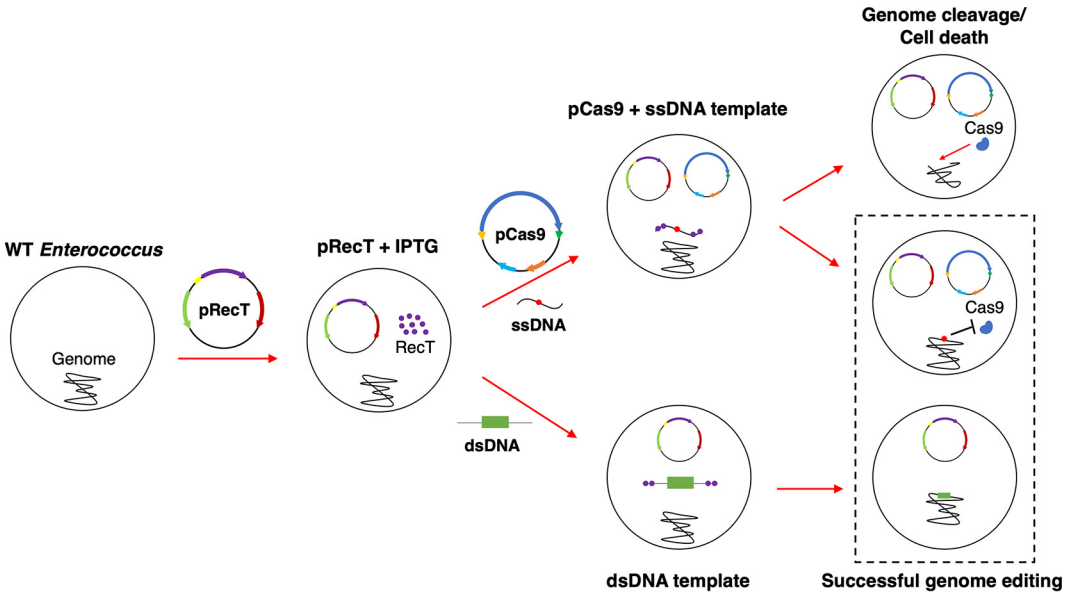

FIG 1 Scheme of recombineering in *Enterococcus* using CRISPR-Cas9 counterselection or genome-inserted antibiotic selection. pRecT- or pRecT_2-harboring cells were induced for RecT production via IPTG. RecT-producing cells were transformed with either an ssDNA template and pCas9 encoding genome-targeting gRNA or a dsDNA template encoding an antibiotic selection marker flanked by homology arms targeting the genome. Cells that successfully recombined with the template escaped Cas9 dsDNA cleavage or were antibiotic resistant mediated by the genomically inserted antibiotic cassette. Cells that did not recombine had their genomes cleaved by Cas9, which led to cell death, or were antibiotic sensitive and did not grow.

recombineering method to produce substitution, deletion, and insertion mutants in *E. faecium* to enable facile genetic-based studies.

RESULTS

***S. pyogenes* Cas9 nuclease is functional in *E. faecium*.** To use CRISPR-Cas as a selection tool for *Enterococcus* genetic engineering, we first tested whether the Cas9 nuclease protein could be functionally expressed in the commensal *E. faecium* strain Com15. We based our expression system on the pVPL3004 vector, which has previously been used for CRISPR-Cas9-mediated recombineering in *Lactobacillus reuteri* (20). The vector pVPL3004 harbors a constitutively expressed *Streptococcus pyogenes cas9* gene, the erythromycin resistance cassette *ermC*, tracrRNA, and a crRNA cloning site. To facilitate cloning, we added a high-copy-number origin of replication from pUC19 into pVPL3004, which allowed successful amplification and cloning in *Escherichia coli*. Additionally, we found that erythromycin selection for pVPL3004 in *E. faecium* was unreliable over long periods, as spontaneously resistant colonies formed over a few days after the transformation of this plasmid. Thus, we exchanged the erythromycin resistance cassette for the chloramphenicol resistance gene, chloramphenicol acetyltransferase (*cat*). The resulting plasmid, pCas9, carries *S. pyogenes cas9*, tracrRNA, crRNA, *cat*, and the pUC19 origin of replication (Fig. 2A). pCas9 was transformed into *E. faecium* via electroporation of lysozyme-treated cells, and transformants were assayed for Cas9 expression. Western blot analysis confirmed that *E. faecium* harboring pCas9 expresses Cas9 protein as opposed to the wild-type control (Fig. 2B). Furthermore, other *Enterococcus* species transformed with pCas9 also successfully expressed Cas9, indicating that this vector can be used to deliver CRISPR-Cas9 machinery across other *Enterococcus* species (see Fig. S1 in the supplemental material).

Previous studies have indicated that Cas9 targeting of genomic DNA is lethal in bacteria (16). Additionally, Cas9 nuclease activity is highly sensitive to mismatches between the gRNA and the 3′ region of the targeted sequence (17). To confirm that Cas9 is active in *E. faecium*, we performed transformation assays of pCas9 in *E. faecium* with a gRNA

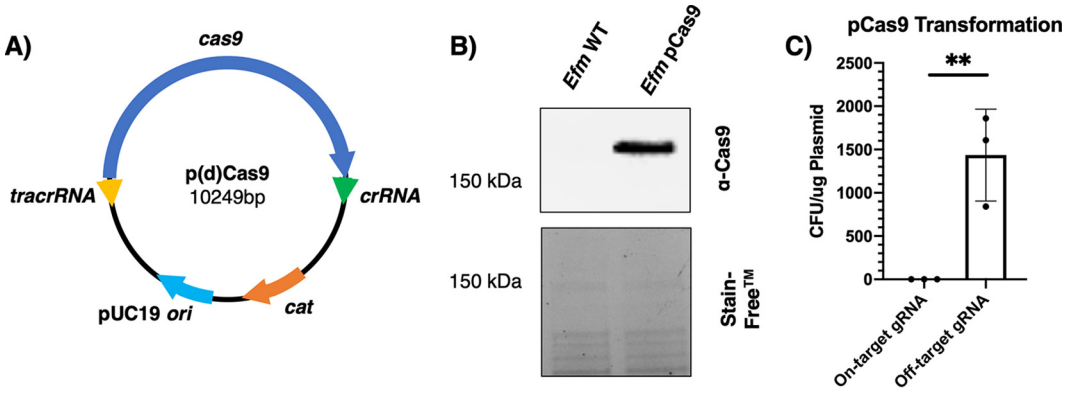

FIG 2 Cas9 expression and activity characterization in *E. faecium*. (A) pCas9 plasmid map. Plasmids carry *S. pyogenes cas9*, tracrRNA, crRNA, the pUC19 origin of replication, and the chloramphenicol acetyltransferase gene (*cat*). (B) Western blot analysis was performed on *E. faecium* (*Efm*) Com15 harboring pCas9, using the wild type (WT) as a control. HRP-conjugated anti-Cas9 was used (Cell Signaling Technologies). The corresponding stain-free gel is shown. A single band appears above 150 kDa. (C) pCas9 encoding on-target or off-target gRNA was transformed into *E. faecium* Com15. CFU were counted and normalized to the amount of plasmid added. Each point represents a transformation replicate. A parametric unpaired *t* test was performed to analyze significance ($P \leq 0.001$). Error bars represent the standard deviations. On-target gRNA targets *thyA*, while off-target gRNA is nearly identical to that of the on-target but with 2 bases mutated at the 3′ end.

that targeted thymidylate synthase (*thyA*) (pCas9-*thyA*). As a control, we created a separate pCas9 vector encoding an off-target gRNA by mutating two nucleotides at the 3′ end of the on-target gRNA. As expected, the transformation of pCas9-*thyA* into *E. faecium* yielded very few colonies (Fig. 2C). In contrast, transforming pCas9 encoding the off-target gRNA yielded significantly more colonies, indicating that Cas9 is active in *E. faecium* and can be used for CRISPR-Cas9-mediated counterselection (Fig. 2C).

RecT expression enables efficient CRISPR-Cas9-mediated recombineering in *E. faecium* Com15. Recombineering methods for various bacteria utilize RecT proteins to incorporate introduced DNA templates (21). In nature, RecT proteins are ssDNA-annealing proteins that function in bacteriophage recombination (22). Because RecT proteins can exhibit species-specific activity (23), we reasoned that prophage operons in *E. faecium* may be a useful source for finding RecT proteins that are active and compatible with enterococci. Within the *E. faecium* Com15 genome, we found one *recT* gene (EFWG_RS08525) in a prophage operon. To ectopically express RecT for recombineering, we cloned *recT* under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter in a plasmid (Fig. 3A). The plasmid pRecT also contains the genes *lacI* and *ermC* for IPTG transcriptional control and erythromycin selection, respectively (Fig. 3A). With both a CRISPR-Cas9 delivery vector and a RecT expression vector in hand, we next investigated whether we could genetically manipulate *E. faecium* Com15 using CRISPR-Cas9-mediated recombineering. We first transformed *E. faecium* Com15 with pRecT and selected for transformants using erythromycin on brain heart infusion (BHI) agar. *E. faecium* cells containing pRecT were then grown in liquid culture and induced with IPTG for ectopic RecT expression until mid-log phase, before being harvested and cotransformed with the ssDNA template and pCas9-*thyA*. Once transformed, cells were selected using chloramphenicol on BHI agar as a proxy for Cas9 expression and screened for recombination events (Fig. 1).

To test for site-specific mutagenesis, we first designed ssDNA oligonucleotides with 49-bp homology arms that introduced 2-bp substitutions into *thyA* that would produce an early stop codon and ablate the protospacer-adjacent motif (PAM) sequence to prevent Cas9 cleavage (17). To determine whether RecT improved recombineering, we performed the experiment using wild-type *E. faecium* and *E. faecium* harboring pRecT grown with or without IPTG induction. Colonies that grew after transformation with pCas9-*thyA* were screened by spotting on modified M9 medium with yeast extract and glucose (45) MM9YEG agar with or without thymidine. We found that under the

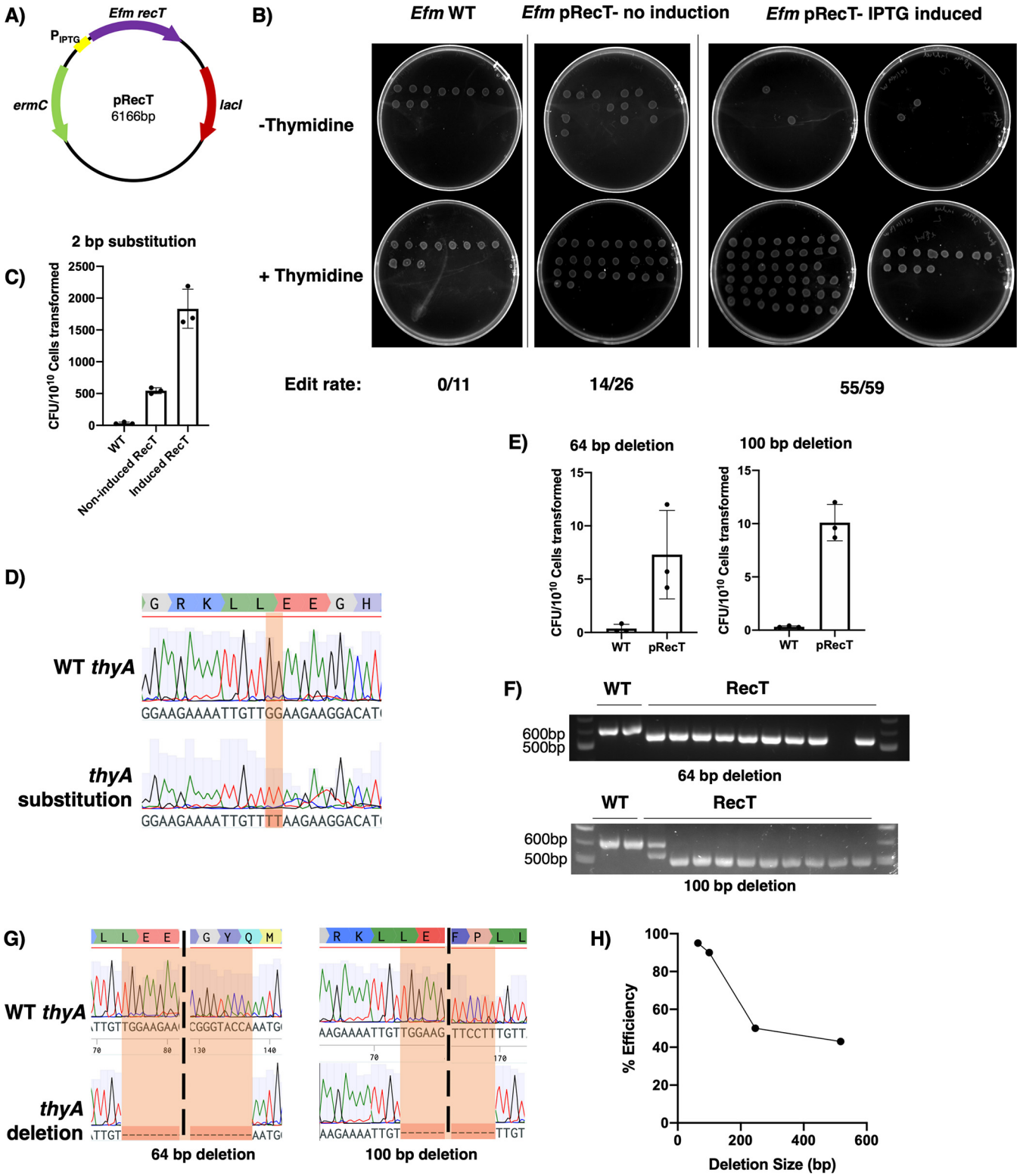

FIG 3 *E. faecium* Com15 CRISPR-Cas9-mediated recombineering. (A) Plasmid map of pRecT. pRecT carries *recT* derived from the genome of *E. faecium* under the control of an IPTG-inducible promoter, *ermC*, and *lacI*. (B) Spot assay of the resulting CRISPR-Cas9-mediated recombineering experiment to induce a 2-base substitution in *E. faecium* Com15 (*Efm*) *thyA*. *E. faecium* Δ*thyA* mutants are unable to grow on MM9YEG medium lacking thymidine. (C) Relative CFU for the 2-bp substitution experiment. CFU are normalized to 10^{10} *E. faecium* cells transformed in the experiment. Each point represents a transformation replicate. Error bars represent the standard deviations. (D) The sequence identity of the 2-bp substitution matches the proposed induced mutation. (E) Relative CFU per 10^{10} *E. faecium* cells transformed for *thyA* 64-bp and 100-bp deletion mutants made via CRISPR-Cas9-mediated recombineering. Each point represents a transformation replicate. Error bars represent the standard deviations. (F) DNA gel of colony PCR of the resulting mutants from panel E. Mutants show a modest gel shift corresponding to

(Continued on next page)

wild-type conditions, none of the colonies selected were thymidine auxotrophs. Conversely, RecT-expressing *E. faecium* successfully produced thymidine auxotrophs, with the noninduced and IPTG-induced conditions yielding 53.8% and 93% editing efficiencies, respectively (Fig. 3B). Background colonies that exhibited wild-type *thyA* were likely caused by spontaneous deletions of the targeting spacer in pCas9, similar to what has been previously described (17). These data suggest that the ectopic expression of RecT greatly improves recombineering activity in *E. faecium*. Consistently, we found that the number of CFU as a result of the recombineering transformation for *E. faecium* was higher under the IPTG-induced conditions than under the uninduced or wild-type conditions (Fig. 3C). We confirmed that the mutant genotype sequences contained the desired 2-bp substitutions in *thyA*, indicating that this system is both efficient and accurate in producing substitution mutations in *E. faecium* (Fig. 3D).

Although we successfully generated substitutions via recombineering, our experiments show a low, basal level of spontaneous CRISPR-Cas9 escape that may make screening for other genes without obvious phenotypes more difficult. To address this concern, we asked whether PCR amplification was a comparable method to screen for recombineering. Here, we designed oligonucleotide templates with 45-bp homology arms that would generate short deletions (64 and 100 bp) in *E. faecium thyA* that were discernible by agarose gel analysis after PCR amplification. Using these new templates with pCas9-*thyA* and pRecT, we found that the numbers of CFU were significantly higher for RecT-expressing cells than for wild-type cells (Fig. 3E). Using spot assays as described above, we found that the rates of on-target recombineering were 95% and 90% for the 64- and 100-bp deletions, respectively (Fig. S2A). We found that wild-type *E. faecium* also produced thymidine mutants without the help of RecT at a markedly lower rate of efficiency, a phenomenon that has been previously described in *Staphylococcus aureus* (21) (Fig. S2A). Colonies were also screened via colony PCR for short deletions. Compared to the wild-type cells, PCR amplification of the recombineered cells showed observable gel shifts corresponding to the proper deletion sizes (Fig. 3F). Sanger sequencing of the resulting thymidine auxotrophs showed deletions in *thyA* of the expected sizes for both the 64- and 100-bp deletions, indicating that the generation of deletions via CRISPR-Cas9-mediated recombineering is of high fidelity (Fig. 3G). We further performed additional gene deletion experiments to approximate the maximum deletion size achievable using 100-base ssDNA oligonucleotide templates in CRISPR-Cas9-mediated recombineering. We found that this technology is able to generate deletions of up to at least 517 bp with reduced editing efficiency (Fig. 3H). To determine if this editing system can be applied to closely related species, we applied our CRISPR-Cas9-mediated recombineering protocol to *Enterococcus faecalis*, *Enterococcus durans*, *Enterococcus hirae*, and *Enterococcus mundtii*. Unfortunately, we were unable to observe any colony formation in *E. mundtii*, possibly due to inefficient transformation or recombineering efficiency. Additionally, we were unable to produce efficient Cas9 counterselection in *E. faecalis*, likely due to insufficient Cas9 expression, as previously described (14). However, we were able to generate small deletions in the respective *thyA* genes for *E. durans* and *E. hirae* using CRISPR-Cas9-mediated recombineering, without significant modification or optimization of our standardized approach (Fig. S2B to G). This indicates that our developed technology can be readily applied to other *Enterococcus* species as well.

RecT recombineering enables the generation of insertion mutants in *E. faecium* Com15. To test if our CRISPR-Cas9-mediated recombineering system is able to produce insertion mutants, we designed commercially synthesized ssDNA oligonucleotide templates with 50-bp homology arms that contained small DNA insertions of 15 bases into the *E. faecium* genome. However, any attempts to produce insertions with the commercial

FIG 3 Legend (Continued)
the deletion size on the DNA gel. (G) The sequence identity of the deletion mutants is shown to be of high fidelity, with the exact number of proposed nucleotides deleted for both the 64-bp and 100-bp deletions. (H) Editing efficiency was measured against the size of the deletion. The percent efficiency was calculated as correctly recombineered colonies/total number of colonies × 100%. The sizes of deletions depicted are 64 bp, 100 bp, 256 bp, and 517 bp.

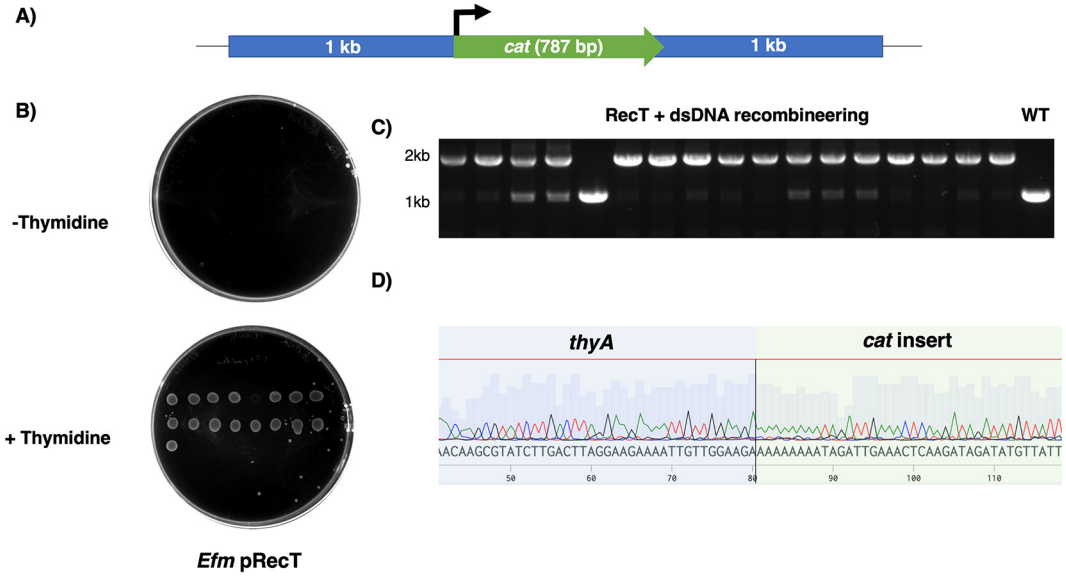

FIG 4 Recombineering of a long DNA template enables the insertion mutations. (A) Scheme of the template depicting chloramphenicol acetyltransferase under the control of the constitutive *bacA* promoter, flanked by 1-kb homology arms targeting *thyA* in *E. faecium*. The fragment was cloned into pET21 for PCR amplification. The *bacA* promoter was used for the constitutive expression of *cat*. (B) Spot assay of colonies resulting from long dsDNA recombineering from *E. faecium* Com15 harboring pRecT. (C) DNA gel of colony PCR of the resulting colonies produced by dsDNA recombineering. WT *E. faecium* is included as a control. Notably, one colony picked exhibited a band corresponding to the WT but was unable to grow on screening plates because of the lack of an inserted *cat* gene. (D) Sequence identity of mutants showing the *cat* insertion in *thyA* at the correct location for dsDNA recombineering.

ssDNA templates proved unsuccessful, likely because ssDNA oligonucleotide templates are too short to effectively make insertions. Thus, we attempted to make insertions using longer dsDNA templates (Fig. 1). We cloned the 787-bp chloramphenicol acetyltransferase gene (*cat*) under the control of a constitutive *bacA* promoter (14) flanked by two 1-kb homology arms homologous to *E. faecium thyA* into the plasmid pET21 (Fig. 4A). The homology arms upstream and downstream of *cat* carry parts of the *thyA* gene such that the successful incorporation of the template would produce a clean insertion of *cat* inside *thyA* without deleting any preexisting genomic DNA. To generate a long DNA template, we PCR amplified the cloned DNA template and purified the DNA products for transformation into *E. faecium* Com15. To avoid complications due to chloramphenicol resistance from pCas9-*thyA*, we performed the recombineering experiment without the help of Cas9 selection, relying solely on the *cat* gene insertion for chloramphenicol-based selection. Transformed, chloramphenicol-resistant colonies were spot assayed as described above. We found that 94% of the tested colonies showed thymidine auxotrophy, with only one false chloramphenicol-resistant colony that was unable to grow on chloramphenicol-containing MM9YEG plates and exhibited a wild-type *thyA* genotype, suggesting the nearly complete on-target insertion of *cat* into the *thyA* locus (Fig. 4B and C). Although previous studies have indicated that dsDNA templates required the exonuclease RecE in combination with RecT to produce dsDNA recombineering mutants in bacteria (19, 24), our results suggest that insertional mutants could be produced in our system with only a dsDNA template and RecT. Notably, no colonies were formed in cells lacking pRecT, indicating that insertional mutations using this method are highly dependent on RecT expression. Additionally, PCR of *thyA* in mutant colonies showed a gel shift of about 787 bp, which corresponded to the desired *cat* insertion size (Fig. 4C). However, some mutant colony PCRs showed a dual banding pattern reflecting both the insertional and WT genotypes (Fig. 4C). Since the phenotype of these colonies still reflected that of the *thyA* knockouts (Fig. 4B), we suspect that the dual bands are a result of residual nonedited *E. faecium* that contaminated the PCR mixture due to the fact that mutant selection relied on the bacteriostatic

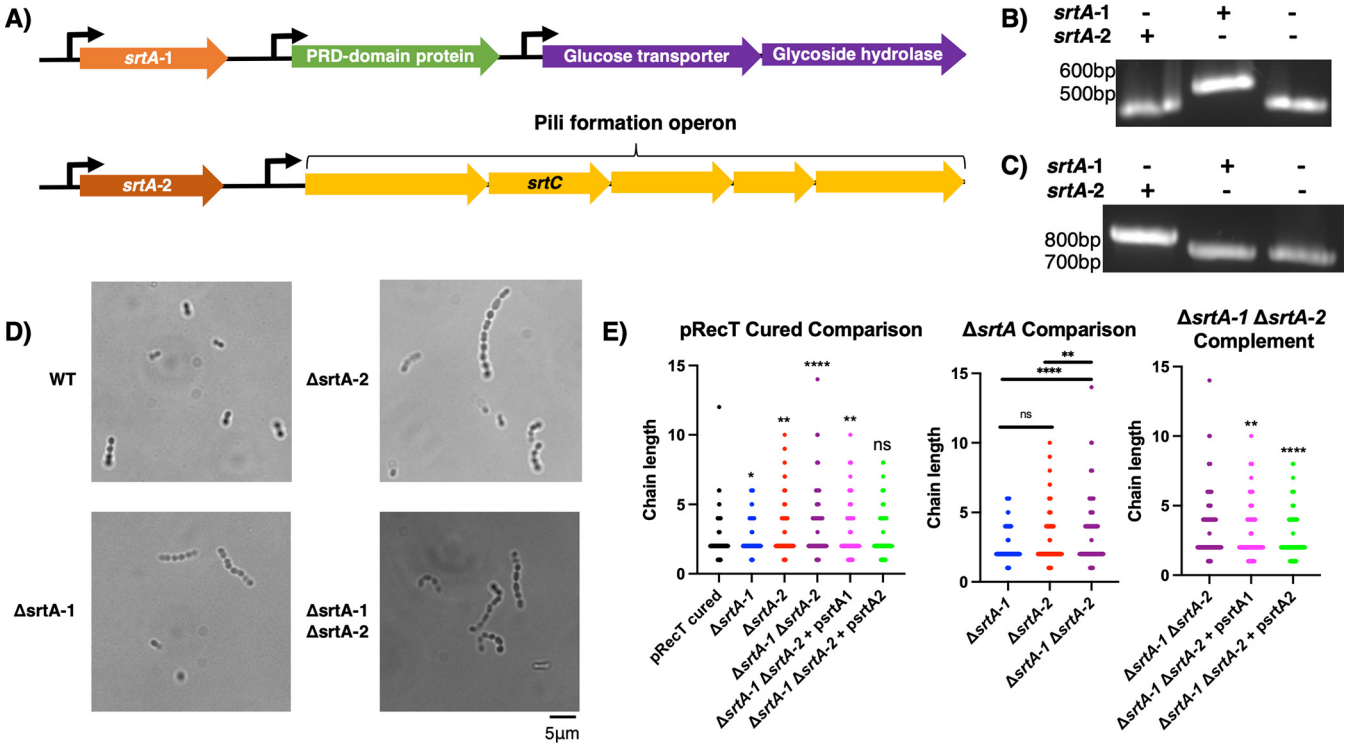

FIG 5 CRISPR-Cas9-mediated recombineering enables the creation of double knockouts in *E. faecium*. (A) *E. faecium* Com15 encodes two copies of sortase A in separate parts of the genome. *srtA-1* localizes around genes that are seemingly unrelated to cell surface proteins, including a phosphotransferase system regulation domain (PRD)-containing protein and glucose utilization genes. *srtA-2* localizes next to a pilin formation operon that harbors a copy of pilin-forming *srtC*. (B) DNA gel showing PCR of *srtA-1* in *E. faecium* Δ*srtA-1*, Δ*srtA-2*, and Δ*srtA-1* Δ*srtA-2* mutants. Gel shifts corresponding to a 76-bp deletion can be observed in the Δ*srtA-1* and Δ*srtA-1* Δ*srtA-2* mutants compared to the Δ*srtA-2* mutant that has the WT form of *srtA-1*. (C) DNA gel showing PCR of *srtA-2* in *E. faecium* Δ*srtA-1*, Δ*srtA-2*, and Δ*srtA-1* Δ*srtA-2* mutants. Gel shifts corresponding to a 62-bp deletion can be observed in the Δ*srtA-2* and Δ*srtA-1* Δ*srtA-2* mutants compared to the Δ*srtA-1* mutant that has the WT form of *srtA-2*. (D) Light microscopy representing *E. faecium* Com15 WT, Δ*srtA-1*, Δ*srtA-2*, and Δ*srtA-1* Δ*srtA-2* mutant strains. Increased chaining effects can be seen in the mutant strains compared to the WT. (E) Quantification of chain length for control, mutant, and complemented strains. pRecT-cured *E. faecium* Com15 was used for the control conditions. Approximately 300 particles were picked under each condition. Kruskal-Wallis analysis of variance (ANOVA) with Dunn's correction was used to statistically compare multiple conditions. The statistical comparisons were done together and are represented in the three graphs shown. Graphs showing chaining comparisons for pRecT versus all other conditions, *E. faecium* Δ*srtA*, and *E. faecium* Δ*srtA-1* Δ*srtA-2* versus the complemented strain are shown. Asterisks in each graph represent significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; ns, not significant) in chaining. In the pRecT-cured comparison graph, asterisks represent significant chaining effects compared to the pRecT-cured control strain. In the Δ*srtA-1* Δ*srtA-2* complement graph, asterisks represent significant chaining effects compared to the Δ*srtA-1* Δ*srtA-2* strain. Further comparisons are graphed in Fig. S3C in the supplemental material.

antibiotic chloramphenicol (25). Sanger sequencing further shows that the *cat* insertion into *thyA* in *E. faecium* was placed in the expected region with high fidelity (Fig. 4D).

CRISPR-Cas9-mediated recombineering can be used to efficiently generate double knockouts. To demonstrate the utility of our CRISPR-Cas9-mediated recombineering method in efficiently generating multigene knockouts, we chose the sortase A gene (*srtA*) as our gene target of interest. Importantly, sortase A is a protein that is responsible for attaching many cell surface proteins to the cell walls of Gram-positive bacteria (26). In enterococci, sortase-dependent proteins have been found to be responsible for adhesion, biofilm formation, and host colonization (27–29). In our genome analyses, we found that *E. faecium* Com15 carries two copies of *srtA*, which we named *srtA-1* (EFWG_RS17355) and *srtA-2* (EFWG_RS05700) for this study (Fig. 5A). The two sortase A genes were verified by standard protein Basic Local Alignment Search Tool (BLAST) analysis to both contain sortase A domains and exist in multiple *E. faecium* strains beyond Com15. Within the genome, *srtA-1* is localized near genes that encode proteins that do not appear to be cell wall associated (Fig. 5A). However, a pilin formation operon can be found next to *srtA-2* (Fig. 5A). Pili have been previously shown to be important in Gram-positive bacteria for pilus formation and are dependent on *srtA* for attachment to the cell wall (30, 31). To test the effects of knocking out *srtA* in *E. faecium*, we generated single and double knockouts of *srtA-1* and *srtA-2* in *E. faecium* Com15 using our CRISPR-Cas9-mediated recombineering method as described

above by using distinct gRNAs for each sortase A gene. (Fig. 5B and C). To generate knockouts, we produced a 76-bp deletion in *srtA-1* (Fig. 5B) and a 62-bp deletion in *srtA-2* (Fig. 5C), with the double-knockout strain harboring both mutations (Fig. 5B and C). In order to generate the Δ*srtA-1* Δ*srtA-2* double-knockout strain, we took the Δ*srtA-1* mutant and cured its CRISPR and recombineering plasmids. In brief, cells were passaged, streaked for single colonies, and screened for erythromycin and chloramphenicol sensitivity over the course of 3 days. Cured *E. faecium* Δ*srtA-1* cells were then retransformed with pRecT before knocking out *srtA-2* using CRISPR-Cas9-mediated recombineering. To assess the phenotypic effects of these knockouts, we performed light microscopy and observed that the knockouts had modest cell chaining effects compared to the wild-type conditions (Fig. 5D). To investigate whether the cell chaining effects were due to *srtA* knockouts, we performed quantitative analyses of the cell chains as previously described (32), using isogenic knockout strains and a control. To produce true isogenic mutants, we cured all the plasmids (pRecT and pCas9) from our knockout strains using a pRecT-cured *E. faecium* Com15 strain as our wild-type background control. Notably, we found that all of the mutants produced some level of increased chaining compared to the control (Fig. 5E and Fig. S3A). However, the Δ*srtA-1* Δ*srtA-2* double-knockout strain yielded an increased chaining effect compared to both the Δ*srtA-1* and Δ*srtA-2* strains (Fig. 5E). To reinforce these results, we created complementation plasmids by cloning either *srtA-1* or *srtA-2* constitutively expressed under the control of the *bacA* promoter into pKH12 (14) to generate the resulting plasmids psrtA1 and psrtA2 (Fig. S3B). psrtA1 or psrtA2 was then transformed into *E. faecium* Δ*srtA-1* Δ*srtA-2* and assayed for chaining. Overall, we found that the complementation strains formed shorter chains than the *E. faecium* Δ*srtA-1* Δ*srtA-2* double-knockout strain (Fig. 5E), with chaining levels comparable to those of *E. faecium* Δ*srtA-1* and *E. faecium* Δ*srtA-2* (Fig. S3C). These proof-of-principle experiments highlight the versatility of our approach to rapidly assay gene function in cells.

Recombineering can be applied to clinically relevant vancomycin-resistant enterococci. Previous literature has suggested that human-associated *E. faecium* strains are phylogenetically separated into two distinct clades, clade A1 and clade B, representing pathogenic and commensal *E. faecium* strains, respectively (33, 34). To determine whether our recombineering technology is compatible with clinically relevant, multidrug-resistant, clade A1 *E. faecium* strains, we chose two vancomycin-resistant *E. faecium* strains, *E. faecium* TX0082 and *E. faecium* ERV165. Unfortunately, we found that vancomycin-resistant *E. faecium* strains are commonly naturally resistant to erythromycin (35), rendering them incompatible with our original pRecT plasmid. To alleviate this, we transferred the IPTG-inducible *recT* and *lacI* genes from pRecT to the vector pIZ12, which harbors a spectinomycin resistance gene, to produce pRecT_2 (Fig. S4A). Given our previous observations of highly variable transformation efficiencies across different *Enterococcus* species and strains, we also chose to further optimize our transformation protocol by applying either our original protocol of lysozyme-mediated cell wall degradation or growth overnight in high concentrations of glycine (36).

Recombineering of the two strains of vancomycin-resistant enterococci (VRE) was performed using a purified PCR product containing a *cat* gene under the control of a constitutive promoter flanked by 1-kb homology arms targeting *thyA*. The resulting colonies from both transformation methods were counted and screened for the *cat* insertion into their *thyA* genes using PCR. In both the *E. faecium* TX0082 and ERV165 strains, we found that cells harboring pRecT_2 produced more transformants per cell than did their respective wild-type controls (Fig. 6A and B). In general, the recombineering efficiency of the glycine transformation method was higher than that of the lysozyme transformation method in both strains (Fig. 6A and B). Surprisingly, we observed that several colonies formed under the wild-type conditions without pRecT_2 using the glycine transformation method but not the lysozyme transformation method in both VRE strains (Fig. 6A and B). To check if the correct insertion was made, we picked colonies formed under all conditions and performed PCR analysis on the *thyA* region of the genome. We found that for *E. faecium* TX0082, every colony possessed an insertional mutation corresponding to the size of the *cat* gene (Fig. 6C and

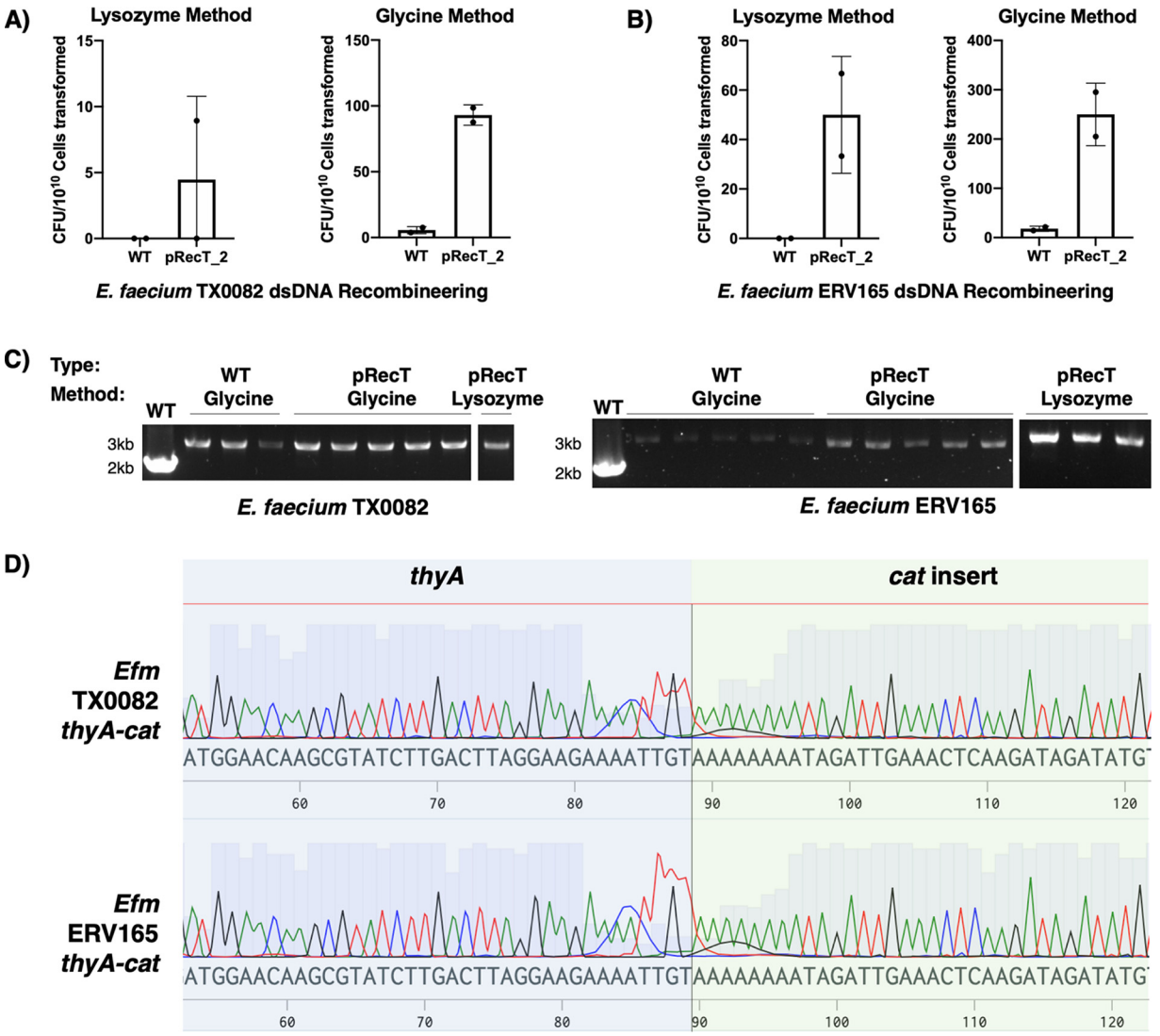

FIG 6 Recombineering technologies can be applied to pathogenic, multidrug-resistant strains of *E. faecium*. (A) Relative CFU resulting from dsDNA recombineering to insert *cat* into *thyA* for vancomycin-resistant *E. faecium* TX0082 using both the lysozyme and glycine transformation methods. WT cells were used as a control for pRecT_2-harboring cells. Each point represents a transformation replicate. Error bars represent the standard deviations. (B) Relative CFU resulting from dsDNA recombineering to insert *cat* into *thyA* for vancomycin-resistant *E. faecium* ERV165 using both the lysozyme and glycine transformation methods. WT cells were used as a control for pRecT_2-harboring cells. Each point represents a transformation replicate. Error bars represent the standard deviations. (C) Representative DNA gel of colony PCR from the resulting *E. faecium* TX0082 and *E. faecium* ERV165 colonies produced via dsDNA recombineering using the two transformation methods. PCRs of WT *E. faecium thyA* of the respective strains were included as controls. (D) Sequence identity of mutants showing the *cat* insertion in *thyA* at the correct location for dsDNA recombineering in both recombineered *E. faecium* TX0082 and *E. faecium* ERV165.

Fig. S4B). Similarly, nearly all colonies of *E. faecium* ERV165 possessed an insertional mutation corresponding to the size of the *cat* gene (Fig. 6C and Fig. S4C). To validate the insertional mutations, we performed Sanger sequencing on a subset of the PCR products produced under each condition and found that every sampled amplicon possessed the *cat* gene insertion in the expected region of the *thyA* gene (Fig. 6D). We found that the VRE strains were unable to grow effectively on MM9YEG screening plates, which precluded functional validation of *thyA* auxotrophy by spot assays.

We then asked whether CRISPR-Cas9 can be used to produce scarless mutations in VRE as described above for *E. faecium* Com15, *E. durans*, and *E. hirae* (Fig. 2 and Fig. S2). Therefore, we transformed the VRE strains with pCas9-*thyA* and an ssDNA template designed to produce a 61-bp deletion using both the lysozyme and glycine

transformation methods. We were able to produce transformants using both transformation methods in *E. faecium* ERV165, with the lysozyme transformation method being slightly more efficient than the glycine transformation method (Fig. S5A). To check for the introduced deletions, we performed PCR analysis of the *thyA* gene and found gel shifts corresponding to the 61-bp deletion in 89% of the colonies picked in the lysozyme transformation method and 75% of the colonies picked in the glycine transformation method (Fig. S5B). As expected, all PCR products with apparent deletions had the exact 61-bp deletion (Fig. S5C). Taken together, these data suggest that our developed recombineering technologies can be applied to pathogenic clade A1 *E. faecium* strains.

DISCUSSION

In this study, we developed recombineering and CRISPR-Cas9 technologies to enhance the efficiency of genomic engineering in both commensal and pathogenic *E. faecium* strains. Using these approaches, we were able to generate substitutions, deletions, and insertions with high efficiency and fidelity on a relatively short timescale. By utilizing Cas9 selection, we generated scarless substitutions and deletions using ssDNA oligonucleotides that can be readily purchased from commercial vendors (Fig. 1). Thus, our method enables the functional assessment of any gene or protein in the *E. faecium* genome via genetic knockout, site-specific mutations, or whole-protein-domain deletions. The pRecT plasmid is readily lost from modified bacteria without erythromycin selection, and pCas9 can be cured upon cell passaging, allowing sequential gene editing as well as the ability to isolate true isogenic mutants. This technology can be readily transferred to other *Enterococcus faecium* strains and *Enterococcus* species, allowing other enterococci to be more genetically tractable. Moreover, the reagents used in this approach are highly modular, allowing the replacement of *E. faecium* RecT with *recT* genes isolated from other species or the replacement of the plasmid backbone to further improve recombineering in other *Enterococcus* species and strains.

We discovered that *E. faecium* RecT can enable DNA insertions using dsDNA templates (Fig. 4 and Fig. 6). The successful insertion of *cat* allowed the rapid screening of colonies, yielding multiple clones of high fidelity and on-target gene disruption. Unfortunately, dsDNA recombineering currently requires the insertion of an antibiotic selection marker, which may lead to polar mutational effects. Studies are ongoing to further improve the capabilities of insertional mutation by optimizing CRISPR-Cas9 selection for shorter sequences that do not include antibiotic resistance. Nevertheless, the insertion mutation method provides a straightforward, facile means to produce gene knock-ins without the need for laborious passaging and screening. As technologies for custom long DNA synthesis improve and become more affordable, this technique will be powerful in providing a method to generate mutants in enterococci in a quick and easy fashion without the need for extensive cloning.

As a proof of concept, we were able to show the generation of single and double knockouts of sortase A in *E. faecium* Com15. Although we retransformed the single sortase A mutant with pRecT in order to generate the double mutant, we envision the possibility of retaining pRecT during the first mutant generation step by selecting recombineered colonies for pRecT as well. Notably, we observed chaining effects in the sortase A mutants compared to the WT background control (Fig. 5E). We suspect that the chaining effects are due to sortase-dependent substrates, through either their inability to function properly or their accumulation in the cell without sortase A to attach them to the cell wall. Indeed, previous literature suggested that sortase A accumulates at distinct cell foci associated with cell division (30, 37). In the case of *E. faecalis*, it was found that a sortase substrate, aggregation substance, accumulated when sortase A is knocked out. In *E. faecium*, it may be possible that the accumulation of sortase substrates at cell foci may delay daughter cell separation, causing a chaining effect. The precise mechanism responsible for this effect in *E. faecium* will need to be further investigated.

Although some methods to genetically engineer enterococci have been previously described (11–13, 15, 18), our approach allows mutant generation in *E. faecium* more efficiently and on a shorter timescale. Oligonucleotides can be ordered in advance for both gRNA cloning and eventual ssDNA template delivery. Once available, gRNA can be constructed and cloned into pCas9 (day 1) and transformed into *E. coli* for propagation (day 2). Clones can then be analyzed for the correct gRNA insertion (day 3) and grown for plasmid harvesting (day 4). Concurrently, pRecT-harboring *E. faecium* can be prepared for recombineering by cotransforming the harvested plasmid and the ssDNA oligonucleotide template (day 4). Upon *E. faecium* colony outgrowth (days 5 to 6), clones can be screened by colony PCR and sequencing for mutant identification (day 7). True isogenic strains cured of CRISPR-Cas9/recombineering plasmids can then be generated by plasmid curing (days 8 to 9). As this process can be performed in parallel to analyze multiple targets, our recombineering method provides an efficient and potentially high-throughput means to characterize genes in *Enterococcus*. Given the difficulty in genetically manipulating certain isolates of enterococci, this study provides a promising method to expand the genetic tractability of *Enterococcus*.

MATERIALS AND METHODS

Individual bacterial strains, oligonucleotides, and plasmids used or created in this study can be found in Tables 1 to 4. Unless stated otherwise, all reagents were sourced from MilliporeSigma.

Bacterial strains and growth conditions. *E. coli* DH5α strains were grown at 37°C in LB (BD Difco) broth or LB agar. For liquid cultures, cells were grown in broth under shaking conditions at 220 rpm. Antibiotics for *E. coli* were used at the following concentrations: chloramphenicol at 10 μg/ml, erythromycin at 150 μg/ml, and spectinomycin at 50 μg/ml. *Enterococcus* strains were grown at 37°C in BD Bacto brain heart infusion (BHI) broth or BHI agar. Antibiotics for *Enterococcus* strains were used at the following concentrations: chloramphenicol at 10 μg/ml, erythromycin at 50 μg/ml, and spectinomycin at 250 μg/ml. For thymidine auxotrophy assays, *Enterococcus* strains were grown on MM9YEG agar (1$\times$ M9 salts [BD Difco], 0.25% yeast extract [Fisher Bioreagents], 0.5% glucose, and 1.5% agar [BD Bacto]) supplemented with chloramphenicol and 40 μg/ml thymidine (Abcam) when appropriate. A total of 10 μg/ml erythromycin was used for *Staphylococcus aureus* when propagating pRecT. *Staphylococcus aureus* was grown at 37°C in BHI broth or BHI agar.

Bacterial transformations. *E. coli* transformations were performed using New England BioLabs (NEB) 5-alpha chemically competent cells. *E. coli* transformations were performed according to the manufacturer's instructions. Transformations and plasmid extraction for *Staphylococcus aureus* were performed using a protocol described in a previous study (38). *Enterococcus* transformations were performed via electroporation modified from previously published methods (36, 39, 40).

For the lysozyme transformation method, cultures of *Enterococcus* grown overnight were subcultured 1:100 in 25 to 50 ml of fresh BHI medium. Subcultures were grown until the optical density at 600 nm (OD_{600}) reached 0.6 to 0.8 and then harvested by centrifugation at 5,000 relative centrifugal force (RCF) for 10 min at 4°C in a Falcon tube. The supernatant was decanted, and the cell pellet was then resuspended in 1 ml of ice-cold 10% glycerol and transferred to an Eppendorf tube. Cells were pelleted by centrifugation at 7,000 RCF for 8 min at 4°C. The supernatant was then aspirated, and the cell pellet was resuspended in a 500-μl lysozyme mixture (10 mM Tris [pH 8.0], 20% sucrose, 10 mM EDTA, 50 mM NaCl, and 30 μg/ml lysozyme from chicken egg white [Amresco]) and incubated at 37°C for 20 to 30 min. Cells were pelleted again at 7,000 RCF for 8 min at 4°C before aspirating the supernatant. The cell pellet was then resuspended in a 1-ml electroporation solution (0.5 M sucrose and 10% glycerol). This step was repeated 3 to 4 times, and the cells were resuspended in electroporation solution to the amount desired to complete the transformation. One-hundred-microliter aliquots of cells in electroporation solution were taken for each transformation. The remaining cells were stocked at −80°C to be used at a later date. Cells were transformed using a Gene Pulser II instrument with Pulse Controller Plus (Bio-Rad) in 0.2-cm-gap electroporation cuvettes (Bio-Rad) at 25 μF, 400 Ω, and 2.5 kV. After electroporation, 400 μl SBHI medium, containing 0.5 M sucrose in BHI medium, was immediately added to transformed cells, and the cells were left to recover for 3 h at 37°C without shaking. The recovered cells were then plated on selective BHI agar plates.

For the glycine transformation method, cultures of *E. faecium* grown overnight were subcultured 1:50 in 50 ml fresh BHI broth containing 2% glycine and 0.5 M sucrose and again grown overnight. The next day, the cultures were pelleted again by centrifugation at 5,000 RCF for 10 min, resuspended with 25 ml of prewarmed (37°C) BHI medium supplemented with 2% glycine and 0.5 M sucrose, and incubated with shaking at 220 rpm for 1 to 1.5 h at 37°C. Cells were then pelleted again by centrifugation at 5,000 RCF for 10 min, resuspended in 1 ml of electroporation solution, and transferred to an Eppendorf tube. Cells were then pelleted again at 7,000 RCF for 8 min at 4°C. The supernatant was aspirated, and the pellet was resuspended with 1 ml of electroporation solution. This step was repeated once more before resuspending the cells in 1.2 ml of electroporation solution for transformation. One-hundred-microliter aliquots of cells in electroporation solution were taken for each transformation and kept on ice prior to electroporation. The remaining cells were stocked at −80°C to be used at a later date.

TABLE 1 Oligonucleotides used in this study for creating and detecting mutants in enterococci[a]

Oligonucleotide	Sequence[b]	Description
oVC12	TCACTGGTCTTTATGAAACACG	F primer for gRNA sequencing in pCas9
oVC41	AGAAAAAATGGCACGTTGGG	F primer for sequencing/PCR of *thyA* from *E. faecium* Com15; used for screening *thyA* recombineered mutants
oVC42	AATCCGTTTTCGTCCTGTGC	R primer for sequencing/PCR of *thyA* from *E. faecium* Com15
oVC43	AAACAAGCGTATCTTGACTTAGGAAGAAAATTGTG	gRNA; targets *E. faecium thyA*; anneals with oVC44
oVC44	AAAACACAATTTTCTTCCTAAGTCAAGATACGCTT	gRNA; targets *E. faecium thyA*; anneals with oVC43
oVC45	AAACAAGCGTATCTTGACTTAGGAAGAAAATTACG	Mutated gRNA; does not target *E. faecium* Com15 *thyA*; anneals with oVC46
oVC46	AAAACGTAATTTTCTTCCTAAGTCAAGATACGCTT	Mutated gRNA; does not target *E. faecium* Com15 *thyA*; anneals with oVC45
oVC61	AAACCAATATCCTCTTGTTCCTCTTGTTATTAGTG	gRNA; targets *E. faecium* Com15 sortase A; anneals to oVC62
oVC62	AAAACACTAATAACAAGAGGAACAAGAGGATATTG	gRNA; targets *E. faecium* com15 sortase A; anneals to oVC61
oVC79	AAACGGAAACCCTTTTTGCAGATCAAACCGCATTG	gRNA for *E. faecium* Com15 *thyA* dCas9 knockdown; anneals to oVC80
oVC80	AAAACAATGCGGTTTGATCTGCAAAAAGGGTTTCC	gRNA for *E. faecium* Com15 *thyA* dCas9 knockdown; anneals to oVC79
oVC110	*AAACTTAACGTACCCGTGCCCGTTCTGTCTTCTTTACGATGTCCTTCTT*AA **AACAATTTTCTTCCTAAGTCAAGATACGCTTGTTCCATCCCTTTTCCTC**	100-base ssDNA for *E. faecium* Com15 *thyA* 2-bp substitution for early stop codon
oVC113	*TTCGTTGTTAACAAAGGAAACCCTTTTTGCAGATCAAACCGCATT* **ACAATTTTCTTCCTAAGTCAAGATACGCTTGTTCCATCCCTTTTC**	90-base ssDNA template for 64-bp deletion in *E. faecium* Com15 *thyA*
oVC114	*AGCAGTTCGCTTTTGATCAAGCCAAAAGGTACTCGTTTCGTTGTT* **ACAATTTTCTTCCTAAGTCAAGATACGCTTGTTCCATCCCTTTTC**	90-base ssDNA template for 100-bp deletion in *E. faecium* Com15 *thyA*
oVC116	*CAGTCATATCTGGTCCGTTATAATCTGTACTTTTGATATAGCGTT* **ACGCTTGTTCCATCCCTTTTCCTCCTAATCTTCTGCCCATTCACT**	90-base ssDNA template for 246-bp deletion *E. faecium* Com15 *thyA*
oVC117	*ATGCTAGGAACATCTTCTGGATTCCATGCAGAGACAATCAATCTT* **ACGCTTGTTCCATCCCTTTTCCTCCTAATCTTCTGCCCATTCACT**	90-base ssDNA template for 517-bp deletion in *E. faecium* Com15 *thyA*
oVC118	*ACATTCGCTATAGCATATTTATCTCCTGTCTCTCTGACAAGATAA* **TTTCTGCTTTTCCTTTTTTTTTCATTTTCTCTCCCTCCAGACTCA**	90-base ssDNA template for 76-bp deletion in *E. faecium* Com15 *srtA-1*
oVC119	AAACAGTAAAATGAGTCTGG	F primer for *E. faecium* Com15 *srtA-1* PCR/ screening
oVC120	CTAAATCTGTCAAATAGATCTTG	R primer for *E. faecium* Com15 *srtA-1* PCR
oVC121	CTAGAAGAAAAGATGGCACG	F primer for *E. durans thyA*
oVC122	AAACCATTCTCATCTTGTGC	R primer for *E. durans thyA*
oVC125	TTAGATGAGAAAATGGCTCG	F primer for *E. hirae thyA*
oVC126	GTCTTCTTTACCGATCAACC	R primer for *E. hirae thyA*
oVC131	AAACTCTCGATTTAGGAAGAAAACTACTCGAAGAG	F crRNA targeting *E. durans thyA*
oVC132	AAAACTCTTCGAGTAGTTTTCTTCCTAAATCGAGA	R crRNA targeting *E. durans thyA*
oVC135	AAACTCTTGATTTAGGCAGGAAACTACTAGAAGAG	F crRNA targeting *E. hirae thyA*
oVC136	AAAACTCTTCTAGTAGTTTCCTGCCTAAATCAAGA	R crRNA targeting *E. hirae thyA*
oVC138	*CAGATCAAACCGCATTTGGTACCCAAATATGCTCCGTGTACCTGT* **TATGCTTGCTCCATGTTACTCCTCCTATTCTTCTGCCCATTCACT**	*E. durans* 58-base *thyA* deletion template
oVC139	AAACGTTTGGCTATGTTAGCTACCAAAAACATGAG	F crRNA targeting *E. faecium* Com15 *srtA-2*
oVC140	AAAACTCATGTTTTTGGTAGCTAACATAGCCAAAC	R crRNA targeting *E. faecium* Com15 *srtA-2*
oVC141	GTTTTTTGTTTTGTCTGAAAGG	F primer for sequencing/PCR of *E. faecium* Com15 *srtA-2*
oVC142	ATTGCCATTTTTTTATCTCCTC	R primer for PCR of *E. faecium* Com15 *srtA-2*
oVC146	TGATTTGATCAATGAAGCCC	R primer for *E. durans thyA* for edit screening
oVC148	CTGGTACCCATAGATGTGAC	R primer for *E. hirae thyA* for edit screening
oVC157	*AACCGCATTTGATAGCCAAAAATGCTGCGTGTACCGGTGCCTGTT* **AATCAAGATATGCTTGCTCCATTTTATTCCTCCTAGTCTTCTACC**	*E. hirae thyA* recombineering template
oVC173	P-TATATCAAGTAAACCAAGAAGATGACTTAG	Phosphorylated F oligonucleotide for PCR *cat* insertion into *thyA*
oVC179	GGCAAACGCCAAGGAAGG	F primer for PCR of *cat* insertion into *thyA*
oVC181	*GCAGATGGATGAAAAAATGGTTATTTGGTTTTTTAGGAGTCGCTT* **AAGCAAAATATAGAAAAAAAGATGCCCGTAGATCAAATCAATGCA**	F template for 62-bp deletion in *srtA-2* in *E. faecium* Com15
oVC182	*TGCATTGATTTGATCTACGGGCATCTTTTTTTCTATATTTTGCTT* **AAGCGACTCCTAAAAAACCAAATAACCATTTTTTCATCCATCTGC**	R template for 62-bp deletion in *srtA-2* in *E. faecium* Com15

(Continued on next page)

TABLE 1 (Continued)

Oligonucleotide	Sequence[b]	Description
oVC203	CCTAGCGACACAACGTTTAGGG	F primer for vancomycin-resistant *E. faecium thyA* homologous insert
oVC204	AACGCCAAGGAAGACGGTCT	R primer for vancomycin-resistant *E. faecium thyA* homologous insert
oVC221	*AACCCTTTTTGCAGATCAAACCGCATTTGGTATCCGAATAAGCTT* **AGTCAAGATACGCTTGTTCCATCCTTTTTCCTCCTAATCTTCTAC**	Template for *thyA* knockout in vancomycin-resistant *E. faecium*; 61-bp deletion
oVC222	AAGCACAACTTGAAGAAAAAATGG	F primer to sequence vancomycin-resistant *E. faecium thyA-cat* insertion
oVC223	AGTAAACCAAGAAGATGATTTAGAGCTG	F primer for vancomycin-resistant *E. faecium thyA* outside *cat* integration site
oVC224	GTTTGAAGAATTTCAGATCATTTGGC	R primer for vancomycin-resistant *E. faecium thyA* outside *cat* integration site
oVC225	CATGCAGAAACGATCAATCTTC	R primer for vancomycin-resistant *E. faecium thyA* screen for short ssDNA-induced deletion

[a]Oligonucleotides include PCR primers, gRNAs, ssDNA templates, and sequencing primers. F, forward; R, reverse.
[b]Homology arms are in boldface and italic type, and mismatched nucleotides are underlined.

Electroporations were performed in 0.2-cm-gap electroporation cuvettes (Bio-Rad) at 25 μF, 400 Ω, and 2.5 kV. After electroporation, 1 ml SBHI medium, containing 0.5 M sucrose in BHI medium, was immediately added to transformed cells, and the cells were left to recover for at least 2 h at 37°C without shaking. The recovered cells were then plated on selective BHI agar plates.

Recombineering. Recombineering experiments were performed with the desired *Enterococcus* strains transformed with pRecT or pRecT_2 plasmids, each containing an IPTG-inducible RecT recombinase. The backbone for pRecT originates from a Gram-positive bacterial vector, pPM145 (Table 3), that is propagated from *Staphylococcus aureus* RN4220. The backbone for pRecT_2 originates from plZ12 propagated from *E. coli*. pRecT- or pRecT_2-harboring cells were used to perform recombineering experiments using the electroporation protocols described above, with certain modifications. Specifically, for cells transformed by the lysozyme method, RecT was induced with 1 mM IPTG when freshly diluted cultures reached an OD_{600} of 0.3 to 0.4. The cultures were then returned to the incubator until they reached an OD_{600} of 0.6 to 0.8, and the protocol was continued as described above. For cells transformed by the glycine transformation method, cultures grown overnight in BHI medium with glycine and sucrose were pelleted and resuspended in fresh BHI medium containing 2% glycine, 0.5 M sucrose, and 1 mM IPTG. Cells were then incubated at 37°C for 1.5 h for RecT induction before continuing with the wash steps in electroporation solution. Recombineering-competent cells were transformed with either an ssDNA template and ~1 μg of the accompanying pCas9 containing an appropriate gRNA targeting the desired mutation site or a dsDNA template. Leftover RecT-induced competent cells were stocked at −80°C to be used in future recombineering experiments.

For ssDNA substitution recombineering, a 100-base ssDNA template was designed with 49-base homology arms flanking a 2-base substitution. For ssDNA deletion recombineering, 90- or 100-base ssDNA was designed with 45- to 50-base homology arms flanking the sequence to be deleted. Prior to the transformation of ssDNA templates, templates were dialyzed in MilliQ water using a 0.025-μm membrane filter from Millipore (catalog number VSWP01300) for 15 min. Typically, the resulting dialyzed ssDNA templates contain a concentration of 2 to 10 μg/μl. Approximately 10 to 20 μl of the dialyzed DNA template was used for each transformation. All oligonucleotides in this study were ordered from Integrated DNA Technologies with standard desalting purification and resuspended to a final concentration of 1 mM in MilliQ water.

For dsDNA recombineering, dsDNA templates were assembled by cloning the desired sequence into pET21 and amplified by PCR. PCR was performed using Q5 high-fidelity DNA polymerase (New England BioLabs) or PrimeSTAR Max DNA polymerase (TaKaRa) according to the manufacturer's instructions. Typically, 3 to 4 μg of dsDNA was used for each transformation where applicable. After cell recovery, transformed cells were plated on BHI agar containing chloramphenicol. The resulting colonies were cultured and then screened for the appropriate mutations by PCR. For dsDNA recombineering, colonies that form are grown with chloramphenicol and passaged at least one time in order to cleanse any bacteriostatic WT cells that remain.

Colony PCR and spot assays. Colonies from recombineering experiments perturbing *thyA* were picked and cultured overnight in BHI medium supplemented with thymidine and chloramphenicol. For colony PCR, the resulting cultures were diluted 1:10 in 1× Tris-EDTA (TE) buffer (catalog number 574793; Calbiochem). Diluted cultures were incubated at 95°C for 10 min. Cell debris was then pelleted by centrifugation at 20,000 RCF at 4°C for 5 min. One microliter of the supernatant was added directly to a 24-μl PCR mixture. GoTaq G2 green master mix (Promega) was used for PCRs involving genotyping. For spot assays, cultures grown overnight were diluted 1:10 in 1× Dulbecco's phosphate-buffered saline (PBS) (Corning Cellgro). Two microliters of diluted cultures was spotted onto MM9YEG screening plates containing thymidine and chloramphenicol. Pictures of spotted plates were taken on the Bio-Rad ChemiDoc imaging system with the Cy5 setting.

TABLE 2 Oligonucleotides used to generate plasmids in this study

Oligonucleotide	Sequence	Description
PM581	TAGAGAGTCGCATGTTCATATTTATCAGAGCTCGTGC	Used to construct pPM145
PM582	CGGGCAGTGAATGAGTGATCGTTAAATTTATACTGC	Used to construct pPM145
PM583	TATGAACATGCGACTCTCTAGCTTGAGGC	Used to construct pPM145
PM584	GATCACTCATTCACTGCCCGCTTTCC	Used to construct pPM145
PM626	AAACACAAGCCCCGGGTAACTAGTAACTGC	Used to construct pPM145
PM598	CATAAATAATCATCCTCCTAAGTACAAGCTTAATTGTTATCCGCTCAC	Used to construct pPM145
PM599	ACTTAGGAGGATGATTATTTATGAATGATTTACAAGAGAGAGAATTA GAAACATTCG	Used to construct pPM145
PM625	GTTACCCGGGGCTTGTGTTTTGCTCATCG	Used to construct pPM145
pPM145_F	CCCGGGTAACTAGTAACTG	Used to construct pRecT
pPM145_R	AAATAATCATCCTCCTAAGTACAAG	Used to construct pRecT
Efm_RecT_F	ACTTAGGAGGATGATTATTTATGGCAACAAATGAATCG	Used to construct pRecT
Efm_RecT_R	GCAGTTACTAGTTACCCGGGCTATTTATTTAATGGTGGATTCG	Used to construct pRecT
plZ12_F	GAAGATCTGGATCCTAATGAATTCATCTGC	Used to construct pRecT_2
plZ12_R	ACGCCAAGCTTGCATGCC	Used to construct pRecT_2
Lac_RecT_F	CAGGCATGCAAGCTTGGCGTTATAAAATTAGTATAATTATAGCACGAG	Used to construct pRecT_2
Lac_RecT_R	TCATTAGGATCCAGATCTTCCTACGACCAAAACTATAAAAC	Used to construct pRecT_2
BacA1_F	CTGATGGGCCCCTGCAGATGTAGATTGAAACTCAAGATAGATATG	Used to construct psrtA_1
BacA1_R	TTTTTTTCATGATACTCCTCCTCAAAATATTTTTG	Used to construct psrtA_1
srtA_1_F	GAGGAGTATCATGAAAAAAAAAGGAAAAGCAG	Used to construct psrtA_1
srtA_1_R	GGAAGATCTGGATCCTAATGCTAAAATGTTTTTGTCTCTAGATTG	Used to construct psrtA_1
BacA2_F	CTGATGGGCCCCTGCAGATGTAGATTGAAACTCAAGATAGATATG	Used to construct psrtA_2
BacA2_R	ATTTTTTCATGATACTCCTCCTCAAAATATTTTTG	Used to construct psrtA_2
srtA_2_F	GAGGAGTATCATGAAAAAATGGTTATTTGGTTTTTTAG	Used to construct psrtA_2
srtA_2_R	GGAAGATCTGGATCCTAATGTTATTGTAGCTCTCCCTG	Used to construct psrtA_2
pVC1_F	GCTTTTATAATTCTATGAGTCGCTTTTG	Used to construct pCas9
pVC1_R	TAAAGTTCATGTAATCACTCCTTCTTAATTACAAATTTTTAG	Used to construct pCas9
CmR_F	GAGTGATTACATGAACTTTAATAAAATTGATTTAGACAATTG	Used to construct pCas9
CmR_R	ACTCATAGAATTATAAAAGCCAGTCATTAGG	Used to construct pCas9
pCas9_ori_F	CCTAGCGCTTAGAATCGC	Used to construct pCas9
pCas9_ori_R	CTCTTTAGCTCCTTGGAAG	Used to construct pCas9
pUC19_ori_F	GCTTCCAAGGAGCTAAAGAGTTGAGATCCTTTTTTTCTGCGC	Used to construct pCas9
pUC19_ori_R	AAGCGATTCTAAGCGCTAGGTTTCCATAGGCTCCGCCC	Used to construct pCas9
Com15_hom_1_F	CTTTAAGAAGGAGATATACATATATCAAGTAAACCAAGAAGATG	Used to construct pET-21-Com15_*thyA-cat*
Com15_hom_1_R	TATTTTTTTTTCTTCCAACAATTTTCTTCC	Used to construct pET-21-Com15_*thyA-cat*
BacA_CmR_F	TGTTGGAAGAAAAAAAAATAGATTGAAACTCAAGATAG	Used to construct pET-21-Com15_*thyA-cat*, amplified from a previously generated vector in which the *bacA* promoter drives *cat* expression
BacA_CmR_R	ACGATGTCCTTAAATTTAACGATCACTCAAAAAATTATAAAAG	Used to construct pET-21-Com15_*thyA-cat*, amplified from a previously generated vector in which the *bacA* promoter drives *cat* expression
Com15_hom_2_F	GTTAAATTTAAGGACATCGTAAAGAAGACAGAAC	Used to construct pET-21-Com15_*thyA-cat*
Com15_hom_2_R	AGTGGTGGTGGTGGTGGTGCTTTGGCAAACGCCAAGGAAG	Used to construct pET-21-Com15_*thyA-cat*
VRE_ThyA_Homo1_F	CTTTAAGAAGGAGATATACATACAGGCACGTGGCACCAAG	Used to construct pET-21-VRE_*thyA-cat*
VRE_ThyA_Homo1_R	TATTTTTTTTACAATTTTCTTCCTAAGTCAAGATACGCTTG	Used to construct pET-21-VRE_*thyA-cat*
ThyA_Cm_insert_F	GAAGAAAATTGTAAAAAAAATAGATTGAAACTCAAGATAG	Used to construct pET-21-VRE_*thyA-cat*, amplified from pET-21-Com15_*thyA-cat*
ThyA_Cm_insert_R	CCTTCTTCCATAAATTTAACGATCACTCAAAAAATTATAAAAG	Used to construct pET-21-VRE_*thyA-cat*, amplified from pET-21-Com15_*thyA-cat*
700221_ThyA_Homo_2_F	GTTAAATTTATGGAAGAAGGGCATCGTAAAGAAG	Used to construct pET-21-VRE_*thyA-cat*
700221_ThyA_Homo_2_R	TCCACCAGTCATGCTAGCCATTCGGCAACGGACGGCTAC	Used to construct pET-21-VRE_*thyA-cat*

Plasmid construction. Cloning of plasmid constructs was performed using NEBuilder HiFi DNA assembly master mix from New England BioLabs. pCas9 was constructed using pVPL3004 as a backbone, replacing its erythromycin resistance cassette with the chloramphenicol resistance gene *cat* using primer pairs pVC1_F/pVC1_R and CmR_F/CmR_R. Additionally, the pUC19 origin of replication was inserted into the final pCas9 plasmid using primer pairs pCas9_ori_F/pCas9_ori_R to amplify the backbone and pUC19_ori_F/pUC19_ori_R to amplify the origin of replication. pPM145 was constructed by first constructing pPM141. pPM141 was constructed from pE194 (41) and pER65 (42). The pE194 backbone was PCR amplified with primer pair PM581/PM582, and the IPTG-inducible promoter was amplified from pER65 with primer pair PM583/PM584. The two PCR products were assembled into a plasmid by Gibson

TABLE 3 Bacterial strains used or created in this study

Strain	Source
E. faecium Com15	Our laboratory
E. faecium TX0082	BEI Resources
E. faecium ERV165	BEI Resources
E. durans ATCC 6056	ATCC
E. mundtii ATCC 43186	ATCC
E. hirae ATCC 8043	ATCC
E. coli DH5α	NEB
S. aureus RN4220	Gift from Luciano A. Marraffini
E. faecium Com15 Δ*thyA* 2-bp_sub	This study
E. faecium Com15 Δ*thyA* 61 bp	This study
E. faecium Com15 Δ*thyA* 100 bp	This study
E. faecium Com15 Δ*thyA* 256 bp	This study
E. faecium Com15 Δ*thyA* 517 bp	This study
E. faecium Com15 *thyA-cat*	This study
E. faecium Com15 Δ*srtA-1*	This study
E. faecium Com15 Δ*srtA-2*	This study
E. faecium Com15 Δ*srtA-1* Δ*srtA-2*	This study
E. faecium TX0082 *thyA-cat*	This study
E. faecium ERV165 *thyA-cat*	This study

assembly (43), yielding plasmid pPM141. The Gibson assembly product was electroporated into *S. aureus* RN4220 as previously described (38). Plasmid pPM141 was amplified with primer pair PM626/PM598, and phage ϕ11γ2 (38) was amplified with primer pair PM599/PM625. The two PCR products were assembled by Gibson assembly, resulting in plasmid pPM145. The Gibson assembly was electroporated into *S. aureus* RN4220. pRecT was constructed using pPM145 as a backbone, inserting *recT* after its encoded IPTG-inducible promoter. The construction of pRecT utilized primer pairs pPM145_F/pPM145_R to amplify the pPM145 backbone and Efm_RecT_F/Efm_RecT_R to amplify *E. faecium recT*. pRecT_2 was constructed using plZ12 (44) as a backbone, inserting *lacI* and IPTG-inducible *recT* from pRecT. The construction of pRecT_2 utilized primer pairs plZ12_F/plZ12_R to amplify the plZ12 backbone and Lac_RecT_F/Lac_RecT_R to amplify IPTG-inducible *recT* and *lacI* from pRecT. psrtA1 and psrtA2 were constructed using pKH12 as a backbone. The construction of psrtA1 and psrtA2 involved the digestion of pKH12 using EcoRI (New England BioLabs). psrtA1 cloning involved amplifying the *bacA* promoter using primer pair BacA1_F/BacA1_R from *E. faecalis* and amplifying *srtA-1* using primer pair srtA_1_F/srtA_1_R from *E. faecium*. psrtA2 cloning involved amplifying the *bacA* promoter using primer pair BacA2_F/BacA2_R from *E. faecalis* and amplifying *srtA-2* using primer pair srtA_2_F/srtA_2_R from *E. faecium*. The construction of pET-21-Com15_*thyA-cat* and pET-21-VRE_*thyA-cat* was done using XhoI (New England BioLabs) and NdeI (New England BioLabs) double digestion and NdeI single digestion on the pET21a(+) (EMD Biosciences) backbone, respectively. Primers used to amplify each fragment can be found in the supplemental material.

Plasmid curing. To create isogenic mutants of *E. faecium*, pRecT and pCas9 were removed from the cells. This was accomplished by growing cultures of newly generated mutants overnight in antibiotic-free BHI medium, subculturing the cultures grown overnight 1:50 in fresh antibiotic-free BHI medium, and incubating the cultures for several hours until they reached an OD_{600} of at least 0.8. The cultures were streaked on regular BHI plates for single colonies. Single colonies were then picked and screened for antibiotic sensitivity by inoculating the colonies into liquid BHI medium with or without supplemented antibiotic. The resulting cultures containing antibiotic were then checked for a lack of growth, indicating that the plasmid from the original colony has been cured. The corresponding culture grown in regular BHI medium was then stocked for further experiments. Usually, we found that picking three to five colonies for screening was sufficient to acquire plasmid-cured cells.

gRNA cloning. gRNA cloning into pCas9 was accomplished by the ligation of an annealed oligonucleotide pair into BsaI-HFv2-digested pCas9. BsaI-HFv2 and T4 ligase were purchased from New England BioLabs. Generally, 4 to 5 μg of pCas9 was digested with 100 U of BsaI-HFv2 overnight in a 50-μl reaction mix at 37°C. Oligonucleotide pairs containing the 30-base gRNA sequences with appropriate overhangs were ordered from Integrated DNA Technologies. To facilitate gRNA design, a script was written in Python to process DNA sequences (https://github.com/victorrchen/CRISPR). Oligonucleotide pairs were resuspended to 100 μM and annealed in a polynucleotide kinase (PNK) mix (New England BioLabs) consisting of 1.5 μl of each oligonucleotide, 41 μl of MilliQ water, 5 μl of PNK buffer, and 1 μl of T4 PNK. This mixture was incubated at 37°C for 30 min, supplemented with 0.5 μl of 5 M $CaCl_2$, and transferred to a 95°C heat block for 5 min. The heat block was then removed from the heat source and allowed to cool at room temperature over the course of 3 h. The oligonucleotide mixture was then diluted 1:10 to be used for the ligation reaction. For ligation, 20-μl reaction mixtures were prepared with ~500 ng of BsaI-HFv2-digested pCas9, 2 μl of diluted annealed oligonucleotide pairs, 2 μl of DNA ligase buffer, 1 μl of ATP, and 2 μl of T4 DNA ligase in a 20-μl reaction mix. The reaction mixtures were incubated at 16°C

TABLE 4 Plasmids used or created in this study

Plasmid	Source
pCas9	This study
pRecT	This study
pRecT_2	This study
pPM145	This study
pKH12	Gift from Kelli L. Palmer
pCas9-*thyA*	This study
pdCas9-*thyA*	This study
pVPL3004	Gift from Jan-Peter van Pijkeren
psrtA1	This study
psrtA2	This study
pCas9-*srtA-1*	This study
pCas9-*srtA-2*	This study
pIZ12	Gift from Luciano A. Marraffini
pET21	Addgene
pET21-Com15_*thyA-cat*	This study
pET21-VRE_*thyA-cat*	This study

overnight and transferred to 37°C for an hour before being heated to 80°C for 20 min. Ligated pCas9 was transformed into *E. coli* for plasmid propagation.

Cas9 Western blotting. *Enterococcus* strains harboring pCas9 were grown overnight in 5 ml BHI medium. Cells were pelleted at 5,000 RCF and resuspended in lysis buffer (10% sodium dodecyl sulfate, 1× PBS, and 1× cOmplete protease inhibitor cocktail in MilliQ water). Approximately 100 μl of 0.1-mm-diameter zirconium beads and 180 μl of 1× Laemmli sample buffer (Bio-Rad) were added to 2-ml screw-cap microtubes (Sarstedt Inc.). Sixty microliters of resuspended cells was added to the tubes, and bead beating was performed at maximum speed for 20 s in a FastPrep FP120 cell disruptor. Beating was repeated twice. Tubes were then heated to 95°C for 10 min and spun down at 20,000 RCF at 4°C for 5 min. Twenty microliters of the supernatant was run on a stain-free gel (Bio-Rad). The gel was transferred onto a nitrocellulose membrane. The membrane was blocked for an hour in block buffer (5% nonfat milk, 1× Tris-buffered saline, and 0.1% Tween 20). Immunostaining was performed using 1× horseradish peroxidase (HRP)-conjugated anti-Cas9 (clone 7A9-3A3; Cell Signaling Technology) in block buffer overnight at 4°C on a rocker. The membrane was washed 3 times with TBS-T (1× Tris-buffered saline and 0.1% Tween 20). Protein detection was performed using Clarity and Clarity Max ECL Western blotting substrates (Bio-Rad) on a Bio-Rad ChemiDoc imaging system.

Microscopy and analysis. Microscopy and image quantification were performed as previously described (32). Light microscopy was performed at the Rockefeller University Bio-imaging Resource Center using a Zeiss Axioplan 2 upright microscope with a plan apochromat 63× oil 1.4-numerical-aperture (NA) 0.19-mm objective. Images were captured using the equipped Hamamatsu high-resolution digital black-and-white (B/W) charge-coupled-device (CCD) camera and acquired using MetaVue version 7.7.0.0. Images were processed using ImageJ. Bacterial cells to be imaged were grown overnight, pelleted, resuspended in PBS, placed onto Fisher Superfrost Plus microscope slides, and covered with Zeiss cover glasses. Slides were sealed using Sally Hansen Insta-Dri nail color. The chain length was quantified for live cells under the microscope and manually counted. Approximately 300 particles were picked under each condition, where one particle is defined by one continuous chain of bacteria. Statistical analysis was performed with Prism 9 using the Kruskal-Wallis test with Dunn's correction.

Data availability. The following reagents were obtained through BEI Resources, NIAID, NIH, as part of the Human Microbiome Project: *Enterococcus faecium* strain TX0082, HM-460, and *Enterococcus faecium* strain ERV165, HM-970. The following reagents were obtained from the ATCC: *Enterococcus durans* ATCC 6056, *Enterococcus hirae* ATCC 8043, and *Enterococcus mundtii* ATCC 43186. pPM145 and pIZ12 (44) were gifts from Luciano A. Marraffini. pVPL3004 was a gift from Jan-Peter van Pijkeren. pKH12 was a gift from Kelli L. Palmer. Plasmid sequences can be obtained from GenBank under the following accession numbers: MW718993 for pCas9, MW718984 for pRecT, MW718985 for pRecT_2, MW718988 for pCas9-*thyA*, MW718986 for psrtA1, MW718987 for psrtA2, MW718989 for pET-21-Com15_*thyA-cat*, and MW718990 for pET-21-VRE_*thyA-cat*. Additionally, certain plasmids can be obtained through Addgene using the following plasmid numbers: 167547 for pCas9 and 167546 for pRecT_2.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.8 MB.

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REFERENCES

1. Lebreton F, Willems RJL, Gilmore MS. 2 February 2014, posting date. Enterococcus diversity, origins in nature, 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. Van Tyne D, Gilmore MS. 2014. Friend turned foe: evolution of enterococcal virulence and antibiotic resistance. Annu Rev Microbiol 68:337–356. https://doi.org/10.1146/annurev-micro-091213-113003.
3. Lebreton F, Manson AL, Saavedra JT, Straub TJ, Earl AM, Gilmore MS. 2017. Tracing the enterococci from Paleozoic origins to the hospital. Cell 169:849–861.e13. https://doi.org/10.1016/j.cell.2017.04.027.
4. Hanchi H, Mottawea W, Sebei K, Hammami R. 2018. The genus Enterococcus: between probiotic potential and safety concerns—an update. Front Microbiol 9:1791. https://doi.org/10.3389/fmicb.2018.01791.
5. Rangan KJ, Pedicord VA, Wang Y-C, Kim B, Lu Y, Shaham S, Mucida D, Hang HC. 2016. A secreted bacterial peptidoglycan hydrolase enhances tolerance to enteric pathogens. Science 353:1434–1437. https://doi.org/10.1126/science.aaf3552.
6. Pedicord VA, Lockhart AAK, Rangan KJ, Craig JW, Loschko J, Rogoz A, Hang HC, Mucida D. 2016. Exploiting a host-commensal interaction to promote intestinal barrier function and enteric pathogen tolerance. Sci Immunol 1:eaai7732. https://doi.org/10.1126/sciimmunol.aai7732.
7. Bybee SN, Scorza AV, Lappin MR. 2011. Effect of the probiotic Enterococcus faecium SF68 on presence of diarrhea in cats and dogs housed in an animal shelter. J Vet Intern Med 25:856–860. https://doi.org/10.1111/j.1939-1676.2011.0738.x.
8. Kim B, Wang Y-C, Hespen CW, Espinosa J, Salje J, Rangan KJ, Oren DA, Kang JY, Pedicord VA, Hang HC. 2019. Enterococcus faecium secreted antigen A generates muropeptides to enhance host immunity and limit bacterial pathogenesis. Elife 8:e45343. https://doi.org/10.7554/eLife.45343.
9. Griffin ME, Espinosa J, Becker JL, Jha JK, Fanger GR, Hang HC. 2020. Enterococcus peptidoglycan remodeling promotes immune checkpoint inhibitor therapy. bioRxiv https://doi.org/10.1101/2020.08.20.256263.
10. Routy B, Chatelier EL, Derosa L, Duong CPM, Alou MT, Daillère R, Fluckiger A, Messaoudene M, Rauber C, Roberti MP, Fidelle M, Flament C, Poirier-Colame V, Opolon P, Klein C, Iribarren K, Mondragón L, Jacquelot N, Qu B, Ferrere G, Clémenson C, Mezquita L, Masip JR, Naltet C, Brosseau S, Kaderbhai C, Richard C, Rizvi H, Levenez F, Galleron N, Quinquis B, Pons N, Ryffel B, Minard-Colin V, Gonin P, Soria J-C, Deutsch E, Loriot Y, Ghiringhelli F, Zalcman G, Goldwasser F, Escudier B, Hellmann MD, Eggermont A, Raoult D, Albiges L, Kroemer G, Zitvogel L. 2018. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science 359:91–97. https://doi.org/10.1126/science.aan3706.
11. Kristich CJ, Chandler JR, Dunny GM. 2007. Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of Enterococcus faecalis. Plasmid 57:131–144. https://doi.org/10.1016/j.plasmid.2006.08.003.
12. Kristich CJ, Djorić 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.
13. Nallapareddy SR, Singh KV, Murray BE. 2006. Construction of improved temperature-sensitive and mobilizable vectors and their use for constructing mutations in the adhesin-encoding acm gene of poorly transformable clinical Enterococcus faecium strains. Appl Environ Microbiol 72:334–345. https://doi.org/10.1128/AEM.72.1.334-345.2006.
14. Hullahalli 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.
15. Panesso D, Montealegre MC, Rincón S, Mojica MF, Rice LB, Singh KV, Murray BE, Arias CA. 2011. The hylEfm gene in pHylEfm of Enterococcus faecium is not required in pathogenesis of murine peritonitis. BMC Microbiol 11:20. https://doi.org/10.1186/1471-2180-11-20.
16. Marraffini LA, Sontheimer EJ. 2008. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322:1843–1845. https://doi.org/10.1126/science.1165771.
17. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 31:233–239. https://doi.org/10.1038/nbt.2508.
18. de Maat V, Stege PB, Dedden M, Hamer M, van Pijkeren J-P, Willems RJL, van Schaik W. 2019. CRISPR-Cas9-mediated genome editing in vancomycin-resistant Enterococcus faecium. FEMS Microbiol Lett 366:fnz256. https://doi.org/10.1093/femsle/fnz256.
19. Pines G, Freed EF, Winkler JD, Gill RT. 2015. Bacterial recombineering: genome engineering via phage-based homologous recombination. ACS Synth Biol 4:1176–1185. https://doi.org/10.1021/acssynbio.5b00009.
20. Oh J-H, van Pijkeren J-P. 2014. CRISPR-Cas9-assisted recombineering in Lactobacillus reuteri. Nucleic Acids Res 42:e131. https://doi.org/10.1093/nar/gku623.
21. Penewit K, Holmes EA, McLean K, Ren M, Waalkes A, Salipante SJ. 2018. Efficient and scalable precision genome editing in Staphylococcus aureus through conditional recombineering and CRISPR/Cas9-mediated counterselection. mBio 9:e01839-18. https://doi.org/10.1128/mBio.01839-18.
22. Lopes A, Amarir-Bouhram J, Faure G, Petit M-A, Guerois R. 2010. Detection of novel recombinases in bacteriophage genomes unveils Rad52, Rad51 and Gp2.5 remote homologs. Nucleic Acids Res 38:3952–3962. https://doi.org/10.1093/nar/gkq096.
23. Filsinger GT, Wannier TM, Pedersen FB, Lutz ID, Zhang J, Stork DA, Debnath A, Gozzi K, Kuchwara H, Volf V, Wang S, Rios X, Gregg CJ, Lajoie MJ, Shipman SL, Aach J, Laub MT, Church GM. 2021. Characterizing the portability of phage-encoded homologous recombination proteins. Nat Chem Biol 17:394–402. https://doi.org/10.1038/s41589-020-00710-5.
24. Muyrers JPP, Zhang Y, Buchholz F, Stewart AF. 2000. RecE/RecT and Redα/Redβ initiate double-stranded break repair by specifically interacting with their respective partners. Genes Dev 14:1971–1982.
25. Das B, Patra S. 2017. Antimicrobials: meeting the challenges of antibiotic resistance through nanotechnology, p 1–22. *In* Ficai A, Grumezescu AM (ed), Nanostructures for antimicrobial therapy. Elsevier, Amsterdam, Netherlands.
26. Spirig T, Weiner EM, Clubb RT. 2011. Sortase enzymes in Gram-positive bacteria. Mol Microbiol 82:1044–1059. https://doi.org/10.1111/j.1365-2958.2011.07887.x.
27. Kemp KD, Singh KV, Nallapareddy SR, Murray BE. 2007. Relative contributions of Enterococcus faecalis OG1RF sortase-encoding genes, srtA and bps (srtC), to biofilm formation and a murine model of urinary tract infection. Infect Immun 75:5399–5404. https://doi.org/10.1128/IAI.00663-07.
28. Gao W, Howden BP, Stinear TP. 2018. Evolution of virulence in Enterococcus faecium, a hospital-adapted opportunistic pathogen. Curr Opin Microbiol 41:76–82. https://doi.org/10.1016/j.mib.2017.11.030.
29. Banla LI, Pickrum AM, Hayward M, Kristich CJ, Salzman NH. 2019. Sortase-dependent proteins promote gastrointestinal colonization by enterococci. Infect Immun 87:e00853-18. https://doi.org/10.1128/IAI.00853-18.

30. Kline KA, Kau AL, Chen SL, Lim A, Pinkner JS, Rosch J, Nallapareddy SR, Murray BE, Henriques-Normark B, Beatty W, Caparon MG, Hultgren SJ. 2009. Mechanism for sortase localization and the role of sortase localization in efficient pilus assembly in Enterococcus faecalis. J Bacteriol 191:3237–3247. https://doi.org/10.1128/JB.01837-08.
31. Khare B, Narayana SVL. 2017. Pilus biogenesis of Gram-positive bacteria: roles of sortases and implications for assembly. Protein Sci 26:1458–1473. https://doi.org/10.1002/pro.3191.
32. McKenney PT, Yan J, Vaubourgeix J, Becattini S, Lampen N, Motzer A, Larson PJ, Dannaoui D, Fujisawa S, Xavier JB, Pamer EG. 2019. Intestinal bile acids induce a morphotype switch in vancomycin-resistant Enterococcus that facilitates intestinal colonization. Cell Host Microbe 25:695–705.e5. https://doi.org/10.1016/j.chom.2019.03.008.
33. Palmer KL, Godfrey P, Griggs A, Kos VN, Zucker J, Desjardins C, Cerqueira G, Gevers D, Walker S, Wortman J, Feldgarden M, Haas B, Birren B, Gilmore MS. 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:e00318-11. https://doi.org/10.1128/mBio.00318-11.
34. Fiore E, Van Tyne D, Gilmore MS. 2019. Pathogenicity of enterococci. Microbiol Spectr 7:GPP3-0053-2018. https://doi.org/10.1128/microbiolspec.GPP3-0053-2018.
35. Qin X, Galloway-Peña JR, Sillanpaa J, Roh JH, Nallapareddy SR, Chowdhury S, Bourgogne A, Choudhury T, Muzny DM, Buhay CJ, Ding Y, Dugan-Rocha S, Liu W, Kovar C, Sodergren E, Highlander S, Petrosino JF, Worley KC, Gibbs RA, Weinstock GM, Murray BE. 2012. Complete genome sequence of Enterococcus faecium strain TX16 and comparative genomic analysis of Enterococcus faecium genomes. BMC Microbiol 12:135. https://doi.org/10.1186/1471-2180-12-135.
36. Shepard BD, Gilmore MS. 1995. Electroporation and efficient transformation of Enterococcus faecalis grown in high concentrations of glycine. Methods Mol Biol 47:217–226. https://doi.org/10.1385/0-89603-310-4:217.
37. Raz A, Fischetti VA. 2008. Sortase A localizes to distinct foci on the Streptococcus pyogenes membrane. Proc Natl Acad Sci U S A 105:18549–18554. https://doi.org/10.1073/pnas.0808301105.
38. Goldberg GW, Jiang W, Bikard D, Marraffini LA. 2014. Conditional tolerance of temperate phages via transcription-dependent CRISPR-Cas targeting. Nature 514:633–637. https://doi.org/10.1038/nature13637.
39. Bhardwaj P, Hans A, Ruikar K, Guan Z, Palmer KL. 2018. Reduced chlorhexidine and daptomycin susceptibility in vancomycin-resistant Enterococcus faecium after serial chlorhexidine exposure. Antimicrob Agents Chemother 62:e01235-17. https://doi.org/10.1128/AAC.01235-17.
40. Zhang X, Paganelli FL, Bierschenk D, Kuipers A, Bonten MJM, Willems RJL, van Schaik W. 2012. Genome-wide identification of ampicillin resistance determinants in Enterococcus faecium. PLoS Genet 8:e1002804. https://doi.org/10.1371/journal.pgen.1002804.
41. Horinouchi S, Weisblum B. 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibodies. J Bacteriol 150:804–814. https://doi.org/10.1128/jb.150.2.804-814.1982.
42. Ramírez-Guadiana FH, Rodrigues CDA, Marquis KA, Campo N, Barajas-Ornelas RDC, Brock K, Marks DS, Kruse AC, Rudner DZ. 2018. Evidence that regulation of intramembrane proteolysis is mediated by substrate gating during sporulation in Bacillus subtilis. PLoS Genet 14:e1007753. https://doi.org/10.1371/journal.pgen.1007753.
43. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343–345. https://doi.org/10.1038/nmeth.1318.
44. 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 proteins of two-component regulatory systems. J Bacteriol 173:2617–2624. https://doi.org/10.1128/jb.173.8.2617-2624.1991.
45. Willett JLE, Ji MM, Dunny GM. 2019. Exploiting biofilm phenotypes for functional characterization of hypothetical genes in *Enterococcus faecalis*. npj Biofilms Microbiomes 5:1–14. https://doi.org/10.1038/s41522-019-0099-0.