Title: RecT recombinase expression enables efficient gene editing in *Enterococcus*

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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 the expression of *E. faecium* RecT recombinase significantly improves the efficiency of recombineering technologies in commensal strains of *E. faecium* and other *Enterococcus* species such as *E. durans* and *E. hirae*. Notably, we demonstrate that *E. faecium* RecT expression facilitated the chromosomal insertion of both single-stranded and double-stranded DNA templates encoding antibiotic selectable markers. Moreover, the expression of RecT in combination with clustered regularly interspaced palindromic repeat (CRISPR)-Cas9 and guide RNAs (gRNAs) enabled highly efficient scar-less ssDNA recombineering to generate specific gene editing mutants in *E. faecium*. The 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 underlining its diverse activities. However, genetic engineering in *E. faecium* still requires passive homologous recombination, which often requires cloning of multiple homologous fragments and screening. To alleviate these challenges, we discovered that RecT-recombinase enables more efficient integration of mutagenic DNA templates to generate insertions, deletions and substitutions of genomic DNA in *E. faecium*. These improved recombineering methods should facilitate functional and mechanistic studies of *Enterococcus*.

Introduction

The Gram-positive bacterial genus of *Enterococcus* can be found in diverse origins including the environment, food, diverse animals and humans[1–3]. *E. 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 antibiotic resistant genes and cause nosocomial infections[1–3]. Yet, commensal *E. faecium* has been discovered to confer immune modulation to promote protection against other enteric pathogens, decrease diarrheal severity and has been associated with enhanced immunotherapy efficacy[4–10]. To better understand both the mechanisms of pathogenesis and commensal functions of *Enterococcus*, it is imperative to perform genetic-based studies on *Enterococcus*. Although there are some existing tools to genetically manipulate and analyze enterococci[11,12], the study of *E. faecium* in the laboratory to elucidate these mechanisms is largely limited by inefficient reverse genetics.

To date, the generation of scar-less genetic mutants in *Enterococcus* relies heavily on homologous recombination[11–14]. This process involves cloning homologous templates hundreds to thousands of bases long flanking the DNA edit of interest into an *Enterococcus* compatible vector that is then transformed into *Enterococcus*. Exconjugants are then screened for the double crossover insert using a multistep selection and counterselection process[13]. While homologous recombination are proven ways to acquire genetic *Enterococcus* mutants, this process is laborious and takes weeks to perform. In this regard, the development of CRISPR-Cas9 gene editing methods offers new opportunities to improve scar-less mutagenesis in bacteria[15]. CRISPR-Cas9 is an endonuclease system that site-specifically cleaves genomic DNA using a homologous RNA guide[15]. Cas9 is a nuclease that uses CRISPR RNA (crRNA) and the antisense trans-activiating crRNA (tracrRNA) to produce a guide RNA (gRNA) for targeted DNA cleavage[16]. In bacteria, efficient Cas9 guided genomic cleavage usually leads to bacterial cell death, since bacteria lack inherent DNA repair mechanisms to repair and escape Cas9 double-stranded DNA breaks. Thus, CRISPR-Cas9 can be used alongside complementary gene editing methods to select for mutants by targeting Cas9 to the wild-type, unedited genomic sequence, thereby enriching for bacteria that mutated and escaped Cas9 chromosomal cleavage. Indeed, a recent study sought to ameliorate homologous recombination selection process using CRISPR-Cas9 counterselection with in *E. faecium*[17]. 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 to generate a targeted mutation.

Recombineering is a gene editing method in bacteria utilizing phage derived recombinases to mediate the incorporation of single-stranded (ss) or double-stranded (ds) DNA templates into bacterial genomes[18]. 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 scar-less bacterial mutants in select species[16,19]. 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. Additionally, recombineering can also be used with dsDNA template. In this study, we report a recombineering system using RecT that is compatible with *E. faecium* and other related species (**Fig. 1**). By combining recombineering with CRISPR-Cas9 counterselection, we were able to produce scarless mutants via point mutations and deletions using this method. Additionally, we report the unexpected result of our system to have gene editing activity with dsDNA templates. Overall, we show the versatility of our RecT-mediated recombineering to produce mutants in *E. faecium* with and without CRISPR-Cas9 counterselection.

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 *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*[19]. The vector pVPL3004 encodes *S. pyogenes cas9*, 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 for successful amplification and cloning in *E. coli*. Additionally, we found that erythromycin selection for pVPL3004 to be unreliable, as spontaneously resistant colonies formed over a few days after transformation of this plasmid. Thus, we exchanged the erythromycin resistance cassette for the chloramphenicol resistance gene, chloramphenicol acetyltransferase (*cat*). The resulting plasmid pCas9 encodes for *S. pyogenes Cas9*, *tracrRNA*, *crRNA*, *cat*, and the pUC19 origin or replication (**Fig. 2A**). pCas9 was transformed into *E. faecium* via electroporation, and exconjugants were assayed for Cas9 expression. Western blot analysis was performed to confirm 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 is a tool for CRISPR-Cas9 delivery across other *Enterococcus* species (**Fig. S1**).

Previous studies have indicated that Cas9 targeting of genomic DNA is lethal in bacteria[15]. Additionally, Cas9 nuclease activity is highly sensitive to mismatches between the gRNA and the 3' region of the targeted sequence[16]. To confirm that Cas9 is active in *E. faecium*, we performed transformation assays of pCas9 into *E. faecium* with a gRNA 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, transformation with 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 Cas9 is active in *E. faecium* and kills the bacteria post-transformation (**Fig. 2C**). We also examined whether catalytically dead Cas9 (dCas9) could also be used for gene knockdown by CRISPR interference (CRISPRi)[20]. Mutation of the *Cas9* active sites, D10A and H840A, yielded and incorporation of the *thyA* targeting gRNA yielded pdCas9-thyA, which was then transformed into *E. faecium*. Resulting exconjugants were then grown in thymidine-rich liquid BHI medium and plated onto MM9YEG agar medium with or without thymidine. Colonies from the resulting plates show *E. faecium* was able to grow on medium containing thymidine but not minimal medium alone, indicating dCas9 actively repressed the expression of thymidylate synthase to cause thymidine auxotrophy (**Fig. 2D**).

RecT expression enables efficient recombineering in *E. faecium*. Recombineering methods in various bacteria utilize RecT proteins to incorporate introduced DNA templates[21]. In nature, RecT proteins are ssDNA annealing proteins that help integrate bacteriophage DNA into bacterial genomes as prophage[18]. Because RecT proteins can exhibit species-specific activity, 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* to be under the control of an 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* using CRISPR-Cas9 mediated recombineering. We first transformed *E. faecium* with pRecT and selected for exconjugants using erythromycin on BHI agar. *E. faecium* containing pRecT were then grown in liquid culture and induced with IPTG for ectopic RecT expression until mid-log phase, before being harvested and co-transformed with 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 that introduced two base-pair substitutions into *thyA* that would produce an early stop codon and ablate the protospacer-adjacent motif (PAM) sequence to prevent Cas9 cleavage[16]. 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 MM9YEG agar with or without thymidine. We found that in the wild-type condition, none of the colonies selected were thymidine auxotrophs. Conversely, RecT-expressing *E. faecium* successfully produced thymidine auxotrophs, with the non-induced and IPTG induced conditions yielding 53.8% and 93% editing efficiencies, respectively (**Fig. 3B**). This data suggests that the ectopic expression of RecT greatly improves recombineering activity in *E. faecium*. Consistently, we found that the number of colony formation units as a result of the recombineering transformation for *E. faecium* was higher in the IPTG induced condition as opposed to the uninduced or wild-type conditions (**Fig. 3C**). We confirmed that the mutant genotype sequences contained the desired two base-pair 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 that would generate short deletions (64 and 100 bp) in *E. faecium thyA* that were discernable by agarose gel analysis after PCR amplification. Using these new templates with pCas9-thyA and pRecT, we found that colony formation units were significantly higher in RecT-expressing cells compared to 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-bp and 100-bp deletions, respectively (**Fig. S2A**). Surprisingly, we found that WT *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 resulting thymidine auxotrophs show deletions in *thyA* of expected size in both 64 bp and 100 bp deletions, indicating 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 *E. durans*, *E. hirae* and *E. mundtii*. We were able to generate small deletions in their respective *thyA* genes for *E. durans* and *E. hirae* using CRISPR-Cas9 mediated recombineering (**Fig. S2B-G**). This indicates our developed technology can be applied to other *Enterococcus* species as well.

RecT recombineering enables the generation of insertion mutants in *E. faecium*. To test if our CRISPR-Cas9 mediated recombineering system is able to produce insertion mutants, we designed commercially synthesized ssDNA oligonucleotide templates that contained small DNA insertions of 15 bases into the *E. faecium* genome. However, any attempts to produce insertions with the commercial ssDNA templates proved unsuccessful, likely because ssDNA oligonucleotide templates are too short to effectively make insertions. Thus, we attempted to make insertions using longer DNA templates. We cloned an 800 bp *cat* operon flanked by two 1-kb homology arms homologous to *E. faecium thyA* into the plasmid pET21(**Fig. 4A**). To generate long ssDNA template, we PCR amplified the cloned DNA template and selectively degraded one strand of the PCR products using the Guide-it long ssDNA production system (Takara). To avoid complications due to chloramphenicol resistance from pCas9-thyA, we performed the recombineering experiment without the help of Cas9 selection, and transformed, chloramphenicol-resistant colonies were spot assayed as described above. We found that 100% of the tested colonies showed thymidine auxotrophy, suggesting complete on-target insertion of *cat* into the *thyA* locus (**Fig. 4B**). Notably, no colonies were formed in cells lacking pRecT, indicating that insertional mutations using this method is highly dependent on RecT expression. Additionally, PCR of *thyA* in mutant colonies showed a gel shift of about 800 bp, which corresponded to the desired *cat* insertion size (**Fig. 4C**). Sanger sequencing further shows that the *cat* insert into *thyA* in *E. faecium*

was placed in the expected region at high fidelity (**Fig. 4D**). We further tested whether long dsDNA templates were capable of producing a similar result. Previous studies have indicated that dsDNA templates required the exonuclease RecE in combination with RecT to produce dsDNA recombineering mutants in bacteria[18,22]. To our surprise, we were able to produce insertional mutants with dsDNA template in a similar fashion using a dsDNA template encoding *cat* flanked by 1-kb homology arms targeting *thyA* (**Fig. 4E**). Further analyses show that the mutants were indeed thymidine auxotrophs (**Fig. 4F**), and sequencing results show that the edits were of high fidelity (**Fig. 4G**).

Discussion

In this study we developed recombineering and CRISPR-Cas9 technologies to enhance the efficiency of genomic engineering in *E. faecium*. Using these technologies, we were able to produce methods to generate substitutions, deletions and insertions at high efficiency and fidelity on a relatively short timescale. We were also able to show the activity of dCas9 for gene repression in *E. faecium*, thereby opening another method for investigating genes in *E. faecium*. 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 by allowing for gene knockouts, 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 (data not shown), allowing for sequential gene editing as well as the ability to isolate true isogenic mutants. This technology can be readily transferred to other *Enterococcus* species, allowing for other enterococci to be more genetically tractable. Moreover, the reagents used in this approach are highly modular, allowing for the replacement of *E. faecium* RecT with *recT* genes isolated from other species to further improve recombineering in other *Enterococcus* species and strains.

We discovered that *E. faecium* RecT can enable DNA insertions of both long ssDNA and dsDNA templates (**Fig. 4**). The successful insertion of *cat* allowed for the rapid screening of colonies, yielding multiple clones of high fidelity and on-target gene disruption. 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 of laborious passaging and screening. As long ssDNA and dsDNA synthetic technologies 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 cloning in the years to come.

Although some methods to genetically engineer enterococci have been previously described[11,12,17], our approach allows for mutant generation in *E. faecium* more efficiently and on a much faster time scale. Oligonucleotides can be ordered in advanced 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 correct gRNA insertion (day 3) and grown for plasmid harvesting (day 4). Concurrently, pRecT harboring *E. faecium* can be prepared for recombineering by co-transforming harvested plasmid and ssDNA oligonucleotide template (day 4). Upon *E. faecium* colony outgrowth (days 5-6), 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 8-9). 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*.

Acknowledgments

We thank Andrew Varble and Luciano Marraffini at The Rockefeller University for helpful suggestions and sharing plasmids. We also thank Karthik Hullahalli and Kelli Palmer at University of Texas at Dallas for *Enterococcus* transformation tips. V.C. acknowledges support from The Rockefeller University Graduate Program and the National Institutes of Health (T32 A1070084). M.E.G thanks Hope Funds for Cancer postdoctoral fellowship and Melanoma Research Foundation for additional support. H.C.H.

acknowledges support from the National Institutes of Health (NIGMS R01 GM103593 and NCI R01 CA245292) and Kenneth Rainin Foundation Synergy Award.

Materials and methods

Bacterial strains and growth conditions

E. coli DH5a strains were grown at 37°C in LB broth or agar. For liquid cultures, cells are grown in broth in shaking conditions at 220 rpm. Transformations were done according to manufacturer's (New England Biolabs) instructions. Antibiotics for *E. coli* were used at the following concentrations: chloramphenicol, 10 µg/mL; erythromycin, 150 µg/mL. *Enterococcus* strains were grown at 37°C in Brain-heart Infusion broth or agar. Antibiotics for *Enterococcus* strains were used at the following concentrations: chloramphenicol, 10 µg/mL; erythromycin. For spot assays, *Enterococcus* strains were grown on MM9YEG agar which includes 1x M9 salts, 0.25% yeast extract, 0.5% glucose and 1.5% agar. When appropriate, 40 µg/mL thymidine was supplemented to media. 10 µg/mL erythromycin was used for *Staphylococcus aureus* when propagating pRecT. *Staphylococcus aureus* was grown at 37°C in BHI liquid media or agar.

Bacterial transformations

E. coli transformations were performed using New England Biolabs 5-alpha chemically competent cells. *E. coli* transformations were performed according to manufacturer's instructions. Transformations and plasmid extraction for *Staphylococcus aureus* was performed using a protocol described from a previous study[23]. *Enterococcus* transformations were performed via electroporation modified from a previously published method[24]. Overnight cultures of *Enterococcus* were diluted in 25-50 mL of fresh media at 1:100. Diluted cultures were grown until OD_{600} 0.6-0.8 before harvesting. Harvested cells were centrifuged at 5000 RPM in a falcon tube. Supernatant was removed, and the cell pellet was then resuspended in 1mL of ice cold 10% glycerol and transferred to an Eppendorf tube. Cells were centrifuged at 7000 RPM for 8 minutes. Supernatant was aspirated, and the cell pellet was resuspended in 500 µL lysozyme mix containing 10 mM Tris pH 8.0, 20% sucrose, 10 mM EDTA. 50 mM NaCl and 30 µg/mL lysozyme and incubated at 37°C for 20-30 minutes. Cells were pelleted again at 7000RPM for 8 minutes before aspirating the supernatant. Cell pellet was then resuspended in 1mL electroporation solution containing 0.5M sucrose and 10% glycerol. This step was repeated 3-4 times and resuspended in electroporation solution to the amount desired enough to complete the transformation. 100 µL aliquots of cells in electroporation solution was taken for each transformation. Cells were transformed in 0.2 cm gap electroporation cuvettes at 25 µF, 400 ohm and 2.5 kV. After electroporation, 400 µL SBHI, containing 0.5 M sucrose in BHI medium, was immediately added to transformed cells and left to recover for 3 hours at 37°C without shaking. Recovered cells were then plated on selective BHI agar plates.

Recombineering

Recombineering experiments were performed *Enterococcus* transformed with pRecT. pRecT's plasmid backbone originates from a Gram-positive bacterial vector that is propagated from *Staphylococcus aureus* RN4220. pRecT harboring cells were used to perform recombineering experiments using the electroporation protocol similar to what is described above, with a couple distinct changes. Notably, during the growth phase before cells are harvested, *Enterococcus* cultures were induced with IPTG for RecT production at OD_{600} 0.3-0.4 using a final culture concentration of 1 µM, before proceeding to make competent cells. pRecT competent cells were transformed with either ssDNA template and accompanying pCas9 containing gRNA targeting the desired mutation site, or dsDNA template. For ssDNA substitution recombineering, 100 base ssDNA template was designed to flank upstream and downstream of the desired mutation location, with the desired substitutional changes located in the middle of the ssDNA template. For ssDNA deletion recombineering, 90 or 100 base ssDNA was designed to flank upstream and downstream of the desired deletion. All oligonucleotides used in this study were ordered from Integrated DNA Technologies and diluted to a final concentration of 1 mM. ssDNA and dsDNA templates were generated by cloning the desired sequence into pET21 and

amplified using PCR. For ssDNA templates, PCR products were generated with an appropriate phosphorylated primer and treated with Guide-It Long ssDNA Production System according to manufacturer's instructions (Takara Bio). Recombineering electroporations were performed by electroporating either dsDNA template, or ssDNA template accompanied with corresponding pCas9 containing appropriate gRNA. Prior to transformation, DNA templates were dilated on MilliQ water using 0.025 μM membrane filter from Millipore (VSWP01300) for 15 minutes. Approximately 10-20 μL of DNA template was used for each recombineering experiment. After cell recovery, transformed cells were plated on BHI agar containing chloramphenicol. Resulting colonies were picked, grown again before PCR screening. PCR screening was performed by adding 10 μL of culture from grown colonies to 90 μL of TE buffer and heated at 95°C for 10 minutes. After heating, solution was spun at 20,000 RPM at 4°C for 5 minutes. 1 μL of supernatant was added to appropriate PCR reaction.

gRNA cloning

gRNA cloning into pCas9 was performed using the restriction enzyme BsaI and T4 ligase. BsaI-HFv2 and T4 ligase was purchased from New England Biolabs. Generally, 4-5 μg of pCas9 was digested with 100 units of BsaI-HFv2 overnight in a 50 μL reaction mix. gRNA oligonucleotide pairs of 30 bases 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 diluted to 100 μM and annealed in a polynucleotide kinase (PNK) mix consisting of 1.5 uL of each oligonucleotide, 41 μL of MilliQ water, 5 μL of PNK buffer and 1 μL of T4 PNK. Reaction mix was then incubated at 37°C for 30 minutes. 0.5 μL of 5 M $CaCl_2$ was added to the reaction before being transferred to a 95°C for 5 minutes. The heat block was then removed from the heat source to be cooled at room temperature over the course of 3 hours. For ligation, about 500 ng of digested pCas9 was used, with the addition of 2 μL of annealed oligonucleotides, 2 μL DNA ligase buffer, 1 μL ATP and 2 μL of T4 DNA Ligase in a 20 μL reaction mix. Ligation reaction was incubated at 16°C overnight and transferred to 37°C for an hour before being heated to 80°C for 20 minutes. Ligated pCas9 was transformed into *E. coli* for plasmid propagation.

Figures

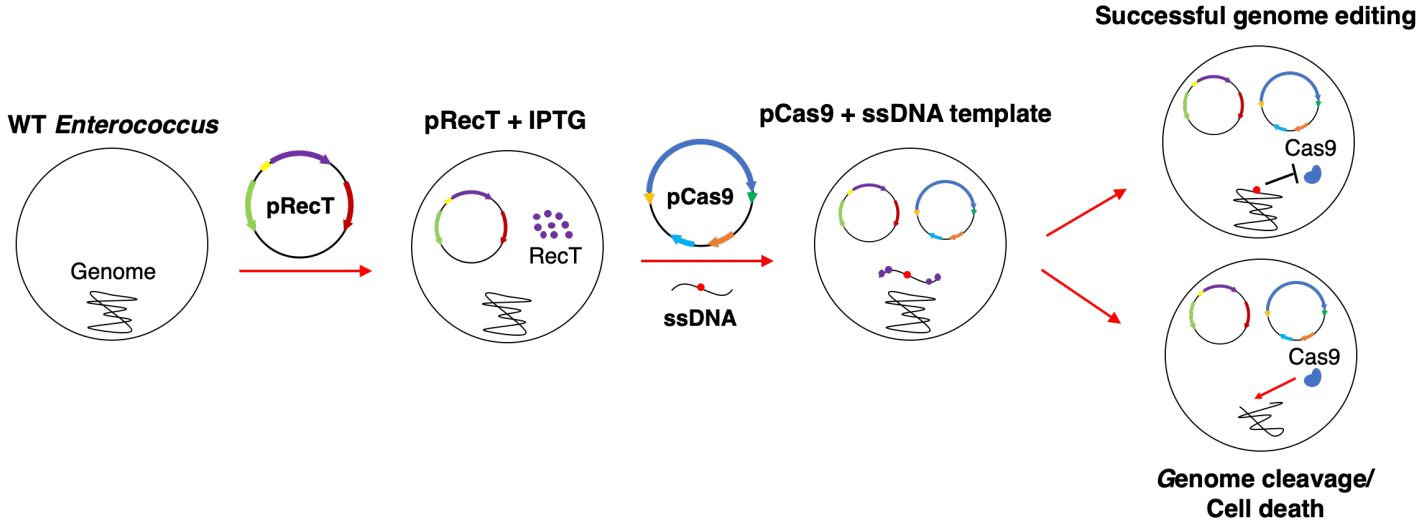

Figure 1. Scheme of CRISPR-Cas9 mediated recombineering in *Enterococcus*. RecT is induced for RecT production. RecT producing cells are transformed with ssDNA template and pCas9 encoding genome targeting gRNA. Cells that successfully recombine with template escape Cas9 dsDNA cleavage. Cells that do not recombine has their genomes cleaved by Cas9, which leads to cell death.

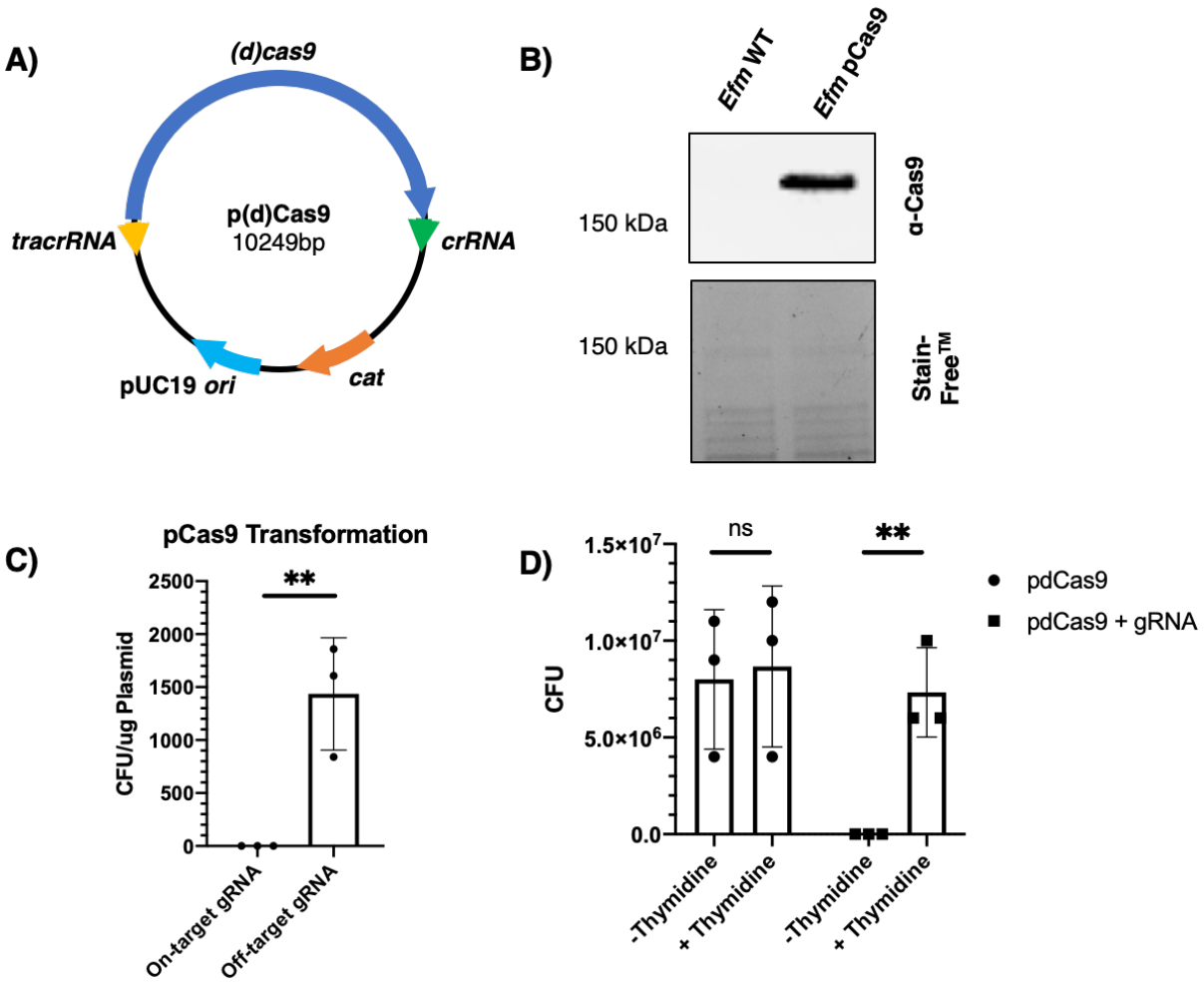

Figure 2. Cas9 expression and activity characterization in *E. faecium*. A) pCas9 and pdCas9 plasmid map is shown. Plasmids encode *S. pyogenes cas9*, *tracrRNA*, *crRNA*, pUC19 origin of

replication and chloramphenicol acetyltransferase (*cat*). B) Western blot analysis was performed on *E. faecium* harboring pCas9, using WT as a control. HRP-conjugated anti-Cas9 was used (Cell Signaling Technologies). Corresponding Stain-Free^{TM} gel is shown. Single band appears above 150 kDa. C) Transformation of pCas9 encoding on-target or off-target gRNA was transformed into *E. faecium*. CFU was counted and normalized for the amount of plasmid added. On-target gRNA targets *thyA*, while off-target gRNA is nearly identical to that of on-target's but with two bases mutated at the 3' end. D) *E. faecium* harboring pdCas9 encoded with or without gRNA targeting *thyA* was grown on MM9YEG agar with or lacking thymidine. CFU counts represent the number cells in 5 μL of culture.

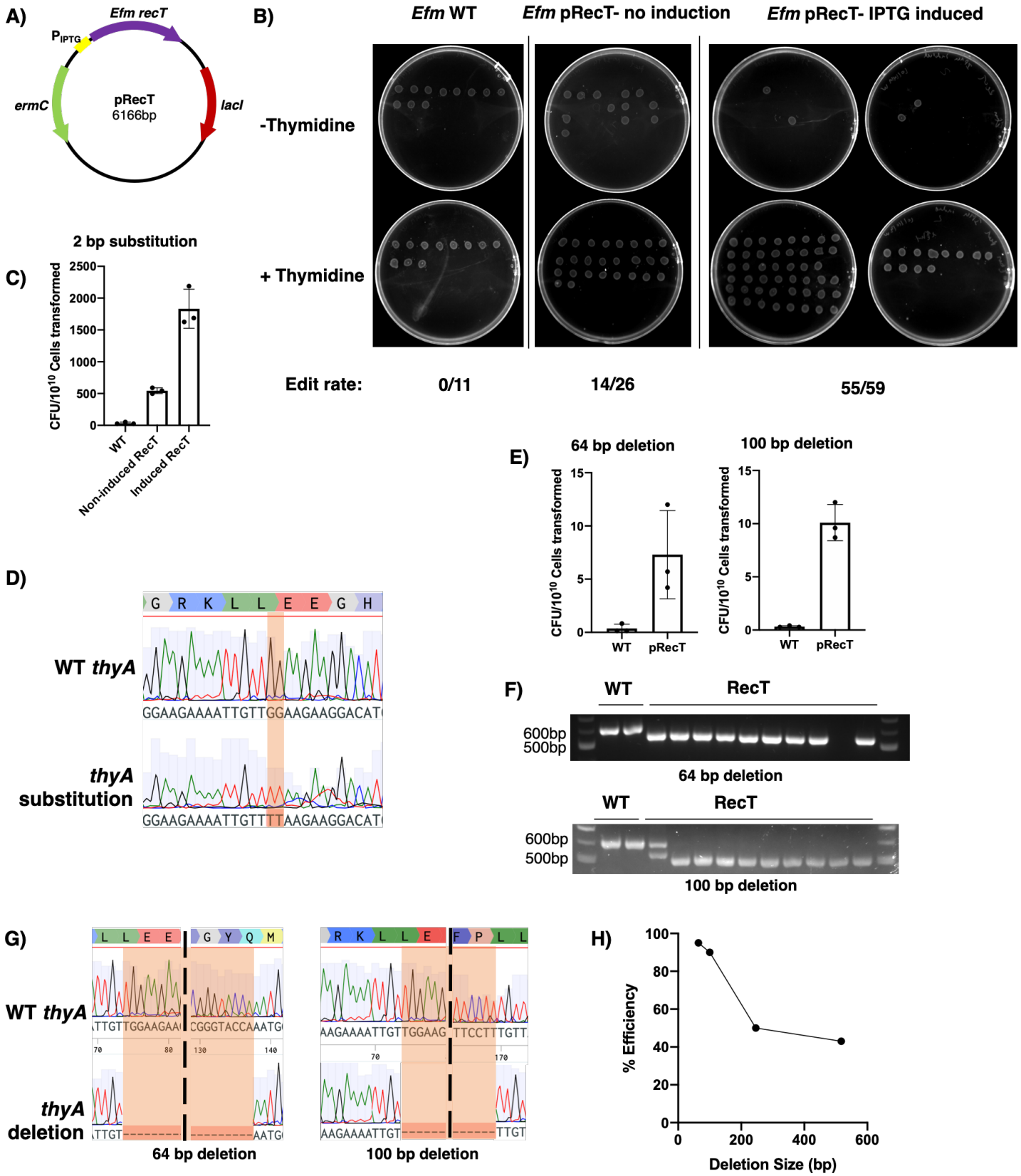

Figure 3. *E. faecium* CRISPR-Cas9 mediated recombineering. A) Plasmid map of pRecT is depicted. pRecT encodes for *recT* derived from the genome of *E. faecium* under an IPTG inducible promoter, *ermC* and *lacI*. B) Spot assay of resulting CRISPR-Cas9 mediated recombineering experiment to induce two base substitution in *E. faecium* (*Efm*) *thyA* is depicted. *E. faecium thyA* mutants are unable to grow on MM9YEG media lacking thymidine. C) Relative CFU for two base pair substitution experiment is shown. CFU is normalized to 10^{10} *E. faecium* transformed in the experiment.

D) Sequence identity of two base pair substitution matches with the proposed induced mutation. E) Relative CFU per 10^{10} *E. faecium* cells transformed is shown for CRISPR-Cas9 mediated recombineering of short deletions of 64bp and 100bp. F) DNA gel of colony PCR of resulting mutants from F) Mutants show a modest gel shift corresponding to the deletion size on DNA gel. G) Sequence identity of deletion mutants is shown to be of high fidelity, with the exact number of proposed nucleotides deleted for both 64 bp and 100 bp deletions. H) Editing efficiency was measured against size of deletion. %Efficiency was calculated via correctly recombineered colonies/total number of colonies x100%. Sizes of deletions depicted are 64bp, 100bp, 256bp and 517bp.

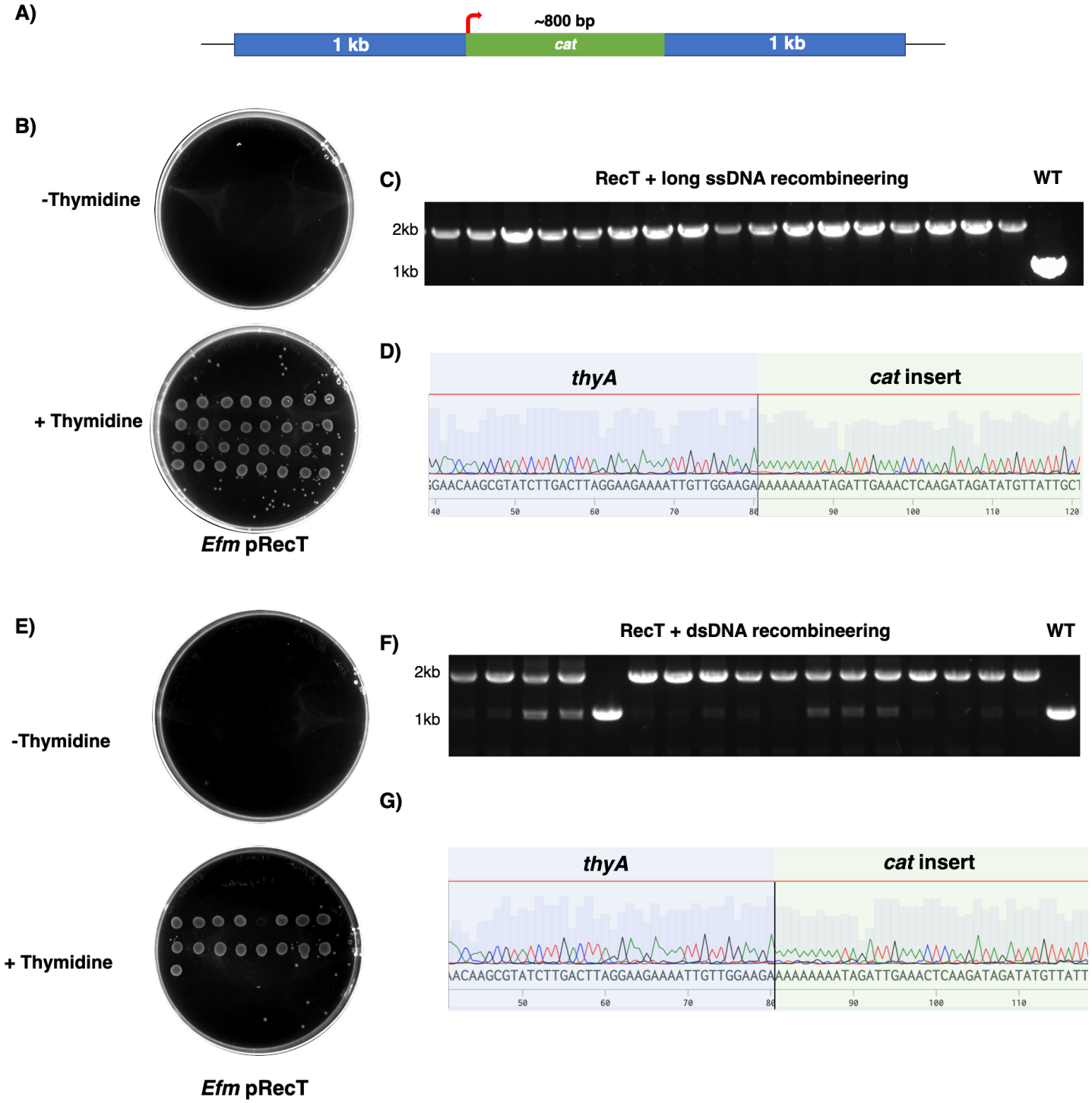

Figure 4. Recombineering of long DNA template enables the insertion mutations. A) Scheme of template depicts chloramphenicol acetyltransferase encoding its own promoter, flanked by 1kb homology arms targeting *thyA* in *E. faecium*. Fragment was cloned into pET21 for PCR amplification.

BacA promoter was used for constitutive expression of *cat*. B) Spot assay of colonies resulting from long ssDNA recombineering is depicted. No colonies were formed from the WT condition. C) DNA gel of colony PCR of resulting colonies produced in ssDNA recombineering. WT *Efm* is included as a control. D) Sequence identity of mutants show *cat* insertion in *thyA* at the correct location for ssDNA recombineering. E) Spot assay of colonies resulting from long dsDNA recombineering is depicted. No colonies were formed from the WT condition. F) DNA gel of colony PCR of resulting colonies produced in dsDNA recombineering. G) Sequence identity of mutants show *cat* insertion in *thyA* at the correct location for dsDNA recombineering.

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