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CRISPR-Cas9-mediated genome editing in vancomycin-resistant *Enterococcus faecium*

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One sentence summary: An approach based on CRISPR-Cas9 was developed to efficiently generate targeted mutations in the multi-drug resistant opportunistic pathogen *Enterococcus faecium*.

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ABSTRACT

The Gram-positive bacterium *Enterococcus faecium* is becoming increasingly prevalent as a cause of hospital-acquired, antibiotic-resistant infections. A fundamental part of research into *E. faecium* biology relies on the ability to generate targeted mutants but this process is currently labour-intensive and time-consuming, taking 4 to 5 weeks per mutant. In this report, we describe a method relying on the high recombination rates of *E. faecium* and the application of the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Cas9 genome editing tool to more efficiently generate targeted mutants in the *E. faecium* chromosome. Using this tool and the multi-drug resistant clinical *E. faecium* strain E745, we generated a deletion mutant in the *lacL* gene, which encodes the large subunit of the *E. faecium* β -galactosidase. Blue/white screening using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) could be used to distinguish between the wild-type and *lacL* deletion mutant. We also inserted two copies of *gfp* into the intrinsic *E. faecium* macrolide resistance gene *msrC* to generate stable green fluorescent cells. We conclude that CRISPR-Cas9 can be used to generate targeted genome modifications in *E. faecium* in 3 weeks, with limited hands-on time. This method can potentially be implemented in other Gram-positive bacteria with high intrinsic recombination rates.

Keywords: *Enterococcus faecium*; genome editing; CRISPR-Cas9; molecular biology; mutants; counterselection

INTRODUCTION

Antibiotic resistance is currently recognised as a global threat to human health (Ferri *et al.* 2017). Enterococci are among the most problematic multi-drug resistant bacteria causing infections among hospitalised patients, contributing to

10 000–25 000 deaths per year in the USA alone (McKinnell *et al.* 2012). Clinically, the two most important enterococcal species are *Enterococcus faecalis* and *Enterococcus faecium*. While historically *E. faecalis* has been the most prominent enterococcal pathogen, since the 1990s *E. faecium* has rapidly emerged as a nosocomial pathogen of major importance. Infections caused

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by *E. faecium* are generally more difficult to treat as vancomycin resistance is more widespread in *E. faecium* than in *E. faecalis* (Gilmore, Lebreton and Schaik 2013; García-Solache and Rice 2019). Until we understand the molecular underpinnings that contribute to the transfer of antibiotic-resistant genes and pathogenicity, we will be hampered in our ability to develop treatment strategies. To drive functional studies, efficient genome editing tools are essential, which are currently lacking. Current methods to generate targeted mutations in *E. faecium* mostly rely on allelic exchange between the chromosome and a temperature-sensitive vector which contains an antibiotic resistance cassette and sequences that flank the target site on the *E. faecium* genome (Maguin et al. 1996; Nallapareddy, Singh and Murray 2006; Zhang et al. 2012). The antibiotic cassette can be removed using the Cre-lox system, but a single lox site remains as a scar (Zhang et al. 2012). These protocols are time-consuming, taking upwards of 4 to 5 weeks. The process involves several days of sub-culturing and selection of colonies on media with different antibiotics, to screen for a double cross-over event and then removal of the resistance marker by Cre-lox. In addition, extensive screening by colony PCR is needed to eliminate false positives and retrieve the desired double cross-over mutant. The process to generate targeted mutants in *E. faecium* was improved by the use of counter-selection system against single cross-over mutants by the use of *pheS**, a mutated allele of the *E. faecalis* phenylalanyl tRNA synthetase α -subunit that confers susceptibility to p-chloro-phenylalanine in enterococci (Kristich, Chandler and Dunny 2007; Thurlow, Thomas and Hancock 2009; Somarajan et al. 2014; Bhardwaj, Ziegler and Palmer 2016).

To further expand the genetic toolbox for multi-drug resistant *E. faecium*, we explored the use of clustered regularly interspaced palindromic repeats (CRISPR) and its associated Cas9 protein to generate mutants in *E. faecium*. The Cas9 nuclease introduces double-strand breaks in DNA that is targeted by a CRISPR and, together with other CRISPR-associated proteins, serves as a defence against invading bacteriophages and plasmids in prokaryotes (Brouns et al. 2008; Marraffini and Sontheimer 2008). The combination of CRISPR and Cas9 has been successfully used for genome editing in eukaryotes where CRISPR-Cas9 drives the generation of mutants by inducing double-strand DNA breaks, which are then repaired by non-homologous end-joining (NHEJ) (Cong et al. 2013). While some bacteria have NHEJ systems, there is no evidence for their presence in *Enterococcus* and other *Lactobacillales* and thus *E. faecium* can only escape the lethal effect of CRISPR-Cas9 targeting a chromosomal site by utilising homologous recombination (HR) (Bowater and Doherty 2006). One approach to use CRISPR-Cas to identify recombinant genotypes is to introduce a vector that contains DNA identical to the flanking sequence of the target region while the cell produces Cas9 and a CRISPR-array homologous to the target sequence. Most surviving cells will have undergone a HR event thereby escaping CRISPR-Cas-mediated killing (Jiang et al. 2013; Wang et al. 2015, 2018). Genome editing approaches using HR and CRISPR-Cas9 have been used for numerous bacterial species, including Gram-positive lactic acid bacteria (Mougiakos et al. 2016; Leenay et al. 2019). *Enterococcus faecium* has a high intrinsic recombination rate and readily integrates novel exogenous DNA in its genome (de Been et al. 2013), making it particularly suited for the implementation of CRISPR-Cas9 as a counterselection strategy during the generation of targeted mutants.

In this study, we aimed to develop a CRISPR-Cas9 based genome editing approach for *Enterococcus faecium*. We adapted

a CRISPR-Cas9-based genome editing approach previously developed for the lactic acid bacterium *Lactobacillus reuteri* (Oh and Van Pijkeren 2014), relying on the high intrinsic recombination rate of *E. faecium* for allelic exchange combined with CRISPR-Cas9 to counterselect against wild-type cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions and oligonucleotides

The vancomycin-resistant *E. faecium* strain E745 (Zhang et al. 2017) was used throughout this study. This strain was isolated from a rectal swab of a hospitalized patient, during routine surveillance of a VRE outbreak in a Dutch hospital. Unless otherwise mentioned, *E. faecium* was grown in brain heart infusion broth (BHI; Oxoid) at 37°C. The *E. coli* strain EC1000 (Leenhouts et al. 1996) was grown in Luria-Bertani (LB) medium at 37°C with shaking at 200 rpm. *Lactobacillus lactis* MG1363 was grown in M17 broth supplemented with 0.5% w/v lactose. When required, antibiotics were used at the following concentrations: erythromycin 50 $\mu\text{g ml}^{-1}$ for *E. faecium* and 5 $\mu\text{g ml}^{-1}$ for *L. lactis* and spectinomycin 200 $\mu\text{g ml}^{-1}$ for *E. faecium*, 100 $\mu\text{g ml}^{-1}$ for *E. coli*, and tetracycline 10 $\mu\text{g ml}^{-1}$ for *L. lactis*. Where indicated, plates were supplemented with 20 $\mu\text{g ml}^{-1}$ 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). The vectors pREG696 (Grady and Hayes 2003), pWS3 (Zhang et al. 2011) and pET-3 α (Novagen) were obtained from our laboratory's culture collection. pREG696-*gfp* was derived from pREG696 by inserting the *gfp* gene under the control of the promoter of the *bacA* gene (P_{bac}) of *E. faecalis* (Heikens, Bonten and Willems 2007) in the NotI and XhoI restriction sites of pREG696 (J. Top, personal communication). Plasmids pVPL3004 and pVPL3115 were described in previous work (Oh and van Pijkeren 2014). The sequences of the oligonucleotides used in this study are listed in Table 1.

Isolation and transformation of plasmids

Plasmid isolation from *E. coli* was performed using the GeneJET plasmid miniprep kit (Thermo Fischer Scientific, Bleiswijk, the Netherlands) according to the manufacturer's instructions. Isolation of plasmids from *L. lactis* was as described previously (O'Sullivan and Klaenhammer 1993) with slight modifications. In short, 5 ml overnight cultures were centrifuged for 10 min at 3000 g. The cell pellet was resuspended in 250 μl THMS-buffer (30 mM Tris-HCl pH 8, 25% sucrose, 3 mM MgCl₂) supplemented with 2 mg ml⁻¹ lysozyme. The cell suspension was incubated for 10 min at 37°C after which 500 μl 1% SDS in 0.2 M NaOH was added. The tubes were mixed gently and incubated on ice for 5 min. About 375 μl ice-cold 3 M potassium acetate pH 5.5 was added and mixed by inversion, followed by incubation on ice for 5 min. Cell debris was pelleted via centrifugation at 20 000 g for 5 min, after which the supernatant was transferred to a new tube and an equal amount of isopropanol was added. After a 10-min incubation at room temperature the tubes were centrifuged at 20 000 g for 10 min to precipitate the DNA. The pellet was washed with 70% ethanol, air dried and dissolved in sterile dH₂O. Transformation of plasmids into *E. faecium* E745 was performed as previously described (Zhang et al. 2012), typically resulting in 500–1000 transformants/ μg DNA.

TABLE 1. List of oligonucleotides used in this study.

Name	Sequence 5'- 3' (restriction sites are underlined)
oVDM1001	AAA <u>ACTCGAGCCACTCACC</u> ATGGGTACTGCAG
oVDM1002	AAA <u>AGAATTCAACGTTGGCG</u> ATTCGTTGGCGATTGA
oVDM1003	/5Phos/ <u>GGCGAGTCCTTTTGA</u> AGAAAATATTGCC
oVDM1004	/5Phos/ <u>AGCCATCTTTTCCG</u> TTTTATTGAGCG
oVDM1005	TCAT <u>TGTTCGCAACAG</u> ATAGC
oVDM1006	GGA <u>ACATCTGTGG</u> TATGGCG
oVDM1007	GGCCGA <u>ATTGATGACAG</u> TTG
oVDM1008	CTCTCCAGCGATTTGGTAG
oVDM1009	GTAGGCAATCTGTACC <u>ACTC</u>
oVDM1011	TGCGTCCTTTGATCCGTTTC
oVDM1012	CACGATGGTACCTGCGTCCTTTGATCCGTTTC
oVDM1013	CATGATGGGCCC <u>CATG</u> TAAAAACAACAATTATCG
oVDM1014	CATGAT <u>ACTAGT</u> ATCCGCAAACAAGGAGAAGG
oVDM1015	CTAGATGCGGCGCGTAGGCAATCTGTACC <u>ACTC</u>
oVDM1016	CATGATGAATT <u>CAGGAGG</u> ATTAACATATGAGCAAAGGAGAAG
oVDM1018	CATGATGCATGCATGAGCAAAGGAGAAG
oVDM1020	CATGATGGGCGCGCTTGCATCAAAAATAAAC
oVDM1021	CACGATGAATT <u>CGTAGAAA</u> ATATTTTTGAAATGCATTTTC
oVDM1022	AAACGATCTTCAGAGATGTCTTCTTAGTTGCTCGG
oVDM1023	AAAACCGAGCAACTAAGAAGACATCTCTGAAGATC
oVDM1024	AAACTCCCGCTCTGAAGTTTCTTCCAGTCTTAACG
oVDM1025	AAAACGTTAAGACTGGAAGAACTTCAGAGCGGAA
oVDM1026	CACTATGCATGCTTAGTGGTGGTGGTGGTGGGATC
oVDM1027	CATGATCCCGGGTTAGTGGTGGTGGTGGTGGGATC
oVDM1028	CTAGATCCCGGGGCTGAGCAATAACTAGCATAAC
oVDM1029	CACGAT <u>ACTAGT</u> CAAAAAACCCCTCAAGACC
oVDM1052	/5Phos/TGCGTCCTTTGATCCGTTTC
oVDM1053	/5Phos/GTAGGCAATCTGTACC <u>ACTC</u>
oVDM1054	GGGCGGTGATCACTGATGAATATA
oVDM1055	ACCAATAATTCCTCAGTACCATCCAT
oVDM1056	ATGACCAATTTGATTAACGG
oVDM1057	CTAATTGAGAGAAGTTCTATA

Construction of the pVDM1001 CRISPR delivery vector and generation of *lacI*-deletion and *gfp*-insertion mutants

We first aimed to construct a vector that could be used for genome editing in *E. faecium* E745. This vector, termed pVDM1001, was constructed by cloning a 0.7-kbp fragment, which contains the CRISPR sequences from pVPL3115 in the *Xho*I and *Eco*RI sites of pWS3. The fragment was amplified from pVPL3115 using the primers oVDM1001–oVDM1002. The pVDM1001 vector was then implemented for the generation of a *lacI* deletion and *gfp* insertion mutant by modifying the CRISPR sequence via digestion with *Bsa*I and annealing two oligos, oVDM1022–oVDM1023 and oVDM1024–oVDM1025, which contain a protospacer targeting *lacI* or *msrC*, respectively. This created pVDM-*xlacI* and pVDM-*xmsrC*. CRISPRs were designed by identifying 30 bp sequences, that were directly followed by the protospacer adjacent motif (PAM) NGG (Jiang and Doudna 2017). The CRISPR was only used if the last 6 bp, corresponding to the seed sequence, which is crucially important for target site recognition (Jiang and Doudna 2017), did not align to another site on the *E. faecium* E745 genome with an immediately adjacent PAM site. Finally, additional nucleotides were added to create the necessary overhang for ligation into the *Bsa*I site in pVDM1001. The nucleotide sequence of pVDM1001 has been made available on NCBI Genbank with accession number MN580666.

To create a *lacI* deletion mutant, a DNA template consisting of a 365 bp upstream region of *lacI* fused together with a 225 bp

downstream region of *lacI* (Table S1) was ordered from Integrated DNA Technologies (Leuven, Belgium) and amplified using oVDM1003–oVDM1004. The amplified template was cloned into pVDM-*xlacI* after digestion with *Sma*I and a blunt end ligation creating pVDM- Δ *lacI*.

To create a *gfp* knock-in construct we amplified 773 bp upstream region of *msrC* and a 507 bp fragment overlapping with the 3' region of *msrC* using primers oVDM1012–oVDM1013 and oVDM1014–oVDM1015, respectively. Each fragment was separately cloned into pWS3 using *Kpn*I-*Apa*I for the upstream fragment and *Sma*I-*Not*I for the downstream fragment, creating pWS3-*msrCup* and pWS3-*msrCdown*, respectively. Downstream of the *msrCup* fragment a *Pbac* promoter was inserted. The promoter site was amplified from pREG696-*gfp* using primers oVDM1020–oVDM1021 and inserted after *Apa*I-*Eco*RI digestion creating pWS3-*msrCup-Pbac*. To pWS3-*msrCdown* a T7 terminator was added which was amplified from pET3 α using primers oVDM1028–oVDM1029 and digested with *Sma*I-*Spe*I to create pWS3- T7-*msrCdown*. pWS3-*msrCup-Pbac* was then digested with *Kpn*I-*Eco*RI and the *msrCup-Pbac* fragment was transferred to pWS3-*msrCdown-T7* to create pWS3-*msrC-Pbac-T7*. To compensate for the low copy number of the *gfp* integration in the chromosome, we amplified two copies of *gfp* from pREG696-*gfp* (laboratory collection) using primers with different restriction sites, oVDM1016–oVDM1026 (*Eco*RI-*Sph*I) and oVDM1018–oVDM1027 (*Sph*I-*Sma*I), and consequently ligated together after digestion with *Sph*I. This construct with two *gfp* genes in tandem was inserted into pWS3-*msrC-Pbac-T7* via *Eco*RI-*Sma*I digestion

creating the complete *msrC::gfp* template. This template was amplified using oVDM1052- oVDM1053 and transferred to pVDM1001 by digestion with SmaI creating pVDM-*msrC::gfp*.

To perform the chromosomal modifications we first transformed E745 with pVPL3004, with selection for transformants by plating on BHI with 50 $\mu\text{g ml}^{-1}$ erythromycin and 24 h incubation at 37°C. Presence of pVPL3004 in E745 was confirmed via PCR using primers oVDM1005-oVDM1006. A colony positive for pVPL3004 was grown in the presence of 50 $\mu\text{g ml}^{-1}$ erythromycin and made competent to receive pVDM1001 or one its derivatives described above. After transformation with these vectors the transformants were selected on BHI agar with 200 $\mu\text{g ml}^{-1}$ spectinomycin and 70 $\mu\text{g ml}^{-1}$ erythromycin and incubated 48–72 h at 30°C. Successful deletion of *lacI* was confirmed by PCR with primers oVDM1007-oVDM1008. Insertion of *gfp* was confirmed by PCR with primers oVDM1009-oVDM1011.

Curing of CRISPR and Cas9 plasmids

A colony that was positive for the desired mutation was transferred to 200 ml BHI without antibiotics and incubated overnight at 37°C at 250 rpm after which 200 μl was transferred to 200 ml pre-warmed BHI and incubated overnight at 37°C. This process was repeated a third time after which a 100 μl sample was taken and diluted 1000 times of which 25 μl was transferred and spread on a BHI agar plate. After 24 h incubation at 37°C, 50 colonies were transferred to BHI agar, BHI agar with 200 $\mu\text{g ml}^{-1}$ spectinomycin or BHI agar with 50 $\mu\text{g ml}^{-1}$ erythromycin. After incubation overnight at 37°C the plates were examined for colonies that were susceptible to both spectinomycin and erythromycin. Curing of the Cas9 delivery vector pVPL3004 and the CRISPR-containing vectors derived from pVDM1001 was confirmed via colony PCR using the primer sets oVDM1054- oVDM1055 and oVDM1056- oVDM1057, respectively

Flow cytometric analysis of GFP fluorescence in E745

To confirm the phenotype of the *gfp* integration mutant, cultures of E745, E745:*msrC::gfp*, and E745 + pREG696-*gfp* in 3 ml BHI, supplemented with 250 $\mu\text{g ml}^{-1}$ spectinomycin if required, were started and incubated overnight at 37°C. The fluorescence of the cultures was then determined by flowcytometric analysis after adjusting the cultures to an OD₆₀₀ of 0.2. These were then diluted 25-fold in a 2-ml volume of PBS of which 200 μl was transferred to a round bottom 96-well plate, which was placed into a MACSQuant (Miltenyi Biotech) machine. Flow cytometric analysis was performed by measuring fluorescence at 488 nm excitation and 525 nm emission at 35.000 events in total. Bacteria were gated on single cells based on forward and side scatter. Data was further processed in FlowJo (FlowJo LLC).

RESULTS AND DISCUSSION

Implementation of CRISPR-Cas9-mediated genome editing in *E. faecium*

We initially attempted to combine single-stranded DNA recombineering and CRISPR-Cas genome editing in *E. faecium*, as was previously demonstrated in the lactic acid bacterium *Lactobacillus reuteri* (Oh and Van Pijkeren 2014). We were, however, unsuccessful in generating mutants in *E. faecium* using this methodology. Either not enough oligonucleotides were transformed into the cells due to the inherent low transformation efficiency in *E.*

faecium, or the activity of the single-stranded DNA binding protein RecT was too low to support incorporation of the oligonucleotide into the chromosome. We then decided to adapt the *L. reuteri* system by relying on the high intrinsic recombination rate of *E. faecium* for allelic exchange and by using CRISPR-Cas9 to counter select against wild-type cells. For this we used the vectors pVPL3004, which encodes Cas9 and pVPL3115, encoding the CRISPR array to which the protospacer target sequence can be added. To facilitate further adaptations needed for genomic modifications we transferred the CRISPR guide RNA section from pVPL3115 to the vector pWS3 to create pVDM1001. This plasmid has the benefit of having a temperature-sensitive replicon for Gram-positive bacteria and can replicate in *E. coli* EC1000, facilitating further cloning procedures.

The *E. faecium* CRISPR-mediated genome engineering plasmid thus relies on pVPL3004 and the novel vector pVDM1001 being present in the strain of interest (Fig. 1A). The general workflow is depicted in Fig. 1B. The plasmid pVPL3004 was first transformed into *E. faecium* E745 to allow for CRISPR-based genome modifications. We then exchanged the control protospacer in pVDM1001 for one that targets the region on the *E. faecium* chromosome that we intended to manipulate. Third, we added a HR template that contained the desired mutation. Lastly, the resulting pVDM1001-derived plasmid was transformed into E745 containing pVPL3004. Transformants were selected on BHI agar plates containing both erythromycin and spectinomycin, and were subjected to PCR to determine the recombinant genotype. As a proof-of-principle in this study, we generated a deletion mutant in *lacI* (locus tag: EfmE745.01561), the gene encoding the large sub-unit of the *E. faecium* β -galactosidase, and we integrated *gfp* in the chromosomal *msrC* gene (Singh, Malathum and Murray 2001)(locus tag: EfmE745.02638) to generate a fluorescently tagged *E. faecium* strain.

Generation of a deletion mutant and a chromosomal integration mutant

To delete *lacI* we adapted pVDM1001 to contain a CRISPR targeting the wild type locus of *lacI* (pVDM- Δ *lacI*). The vector pVDM- Δ *lacI* contained, in addition to the CRISPR targeting *lacI*, a HR template consisting of two regions flanking *lacI*, which allowed the generation of a targeted deletion mutant. To insert *gfp* in the chromosome, we created a HR template containing flanking regions of *msrC* and two copies of *gfp* in tandem as a transcriptional fusion under control of the constitutively expressed Pbac promoter. We cloned the *gfp* HR template and a specific CRISPR targeting *msrC* into pVDM1001 to create pVDM-*msrC::gfp*.

In a representative experiment to generate the *lacI* deletion mutant, we transformed E745 + pVPL3004 with various constructs to quantify the emergence of spontaneously resistant or CRISPR escape mutants and the selective efficiency of the CRISPR. We transformed *E. faecium* E745 with dH₂O (background), pVDM1001 (empty vector), pVDM- Δ *lacI* (carrying a CRISPR that targets *lacI*) and pVDM- Δ *lacI* (carrying both the *lacI*-targeting CRISPR and the HR template for the generation of the *lacI* deletion mutant). This resulted in 70, 250, 68 and 80 colonies, respectively, after selection on BHI agar plates containing erythromycin and spectinomycin to select for both pVPL3004 and pVDM1001 and its derivatives. The relatively high background in the water control revealed the appearance of spontaneously spectinomycin-resistant colonies. Our data also indicated that we could successfully transform pVDM1001, which lacks an *E. faecium* CRISPR-array or HR template, into *E. faecium*. The

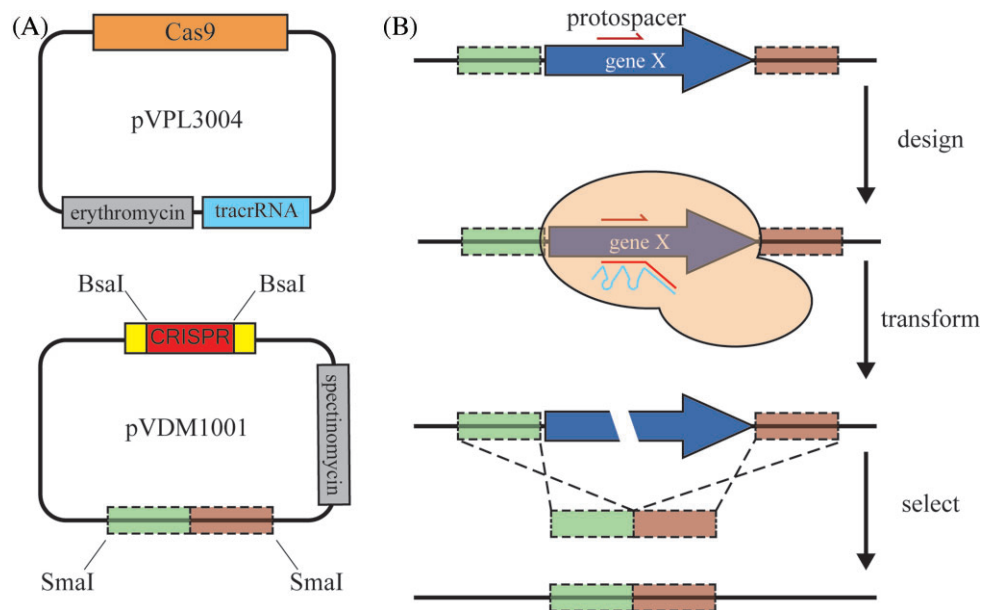


Figure 1. Schematic overview of the CRISPR-Cas9-mediated genome editing. This system consists of two plasmids (panel A), pVPL3004; which contains cas9 from *S. pyogenes*, tracrRNA and an erythromycin selection marker, and pVDM1001; which contains a CRISPR targeting the desired region, the template DNA which carries the desired mutation and a spectinomycin selection marker. The general workflow for generating mutants is shown in panel B, and includes the design of the CRISPR-protospacer and repair template which are incorporated in pVDM1001. The second step is the transformation of the plasmids pVPL3004 and the relevant pVDM1001 derivative into *E. faecium*, followed by direct selection of the mutant.

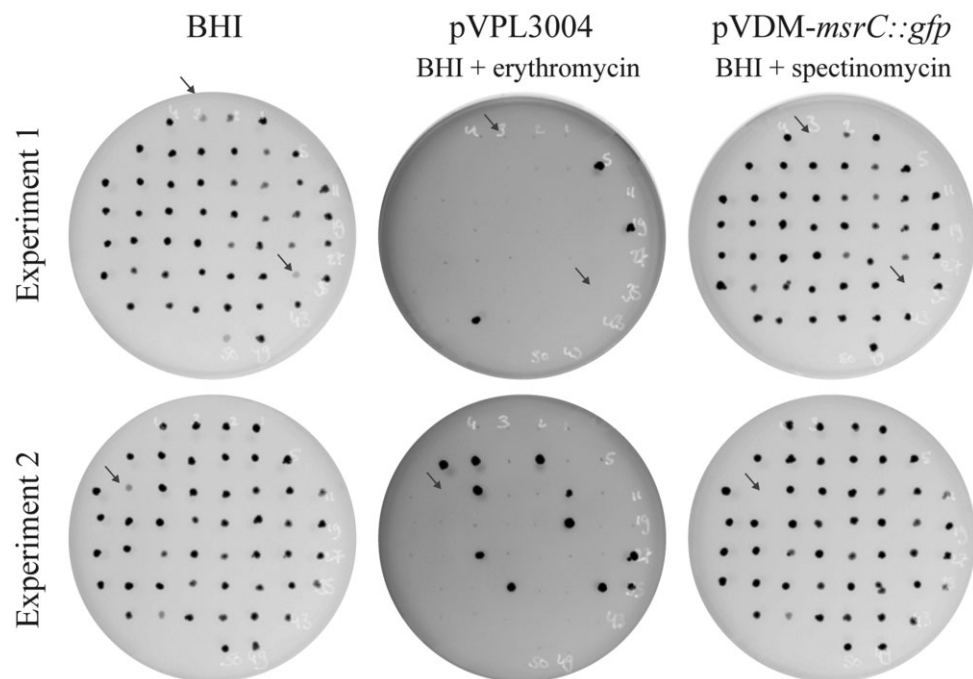


Figure 2. Clearing efficiency of pVPL3004 and pVDM-*msrC*::*gfp*. After three days of sub-culturing to clear the plasmids, 50 colonies per mutant were transferred to BHI, BHI + 50 μ g/ml erythromycin and BHI 200 μ g/ml spectinomycin to screen for clones that have lost both plasmids (indicated by the red arrows). The overall clearance of pVPL3004 is 80%–90% and of pVDM-*msrC*::*gfp* is 2%–5%, resulting in at least one colony that has lost both plasmids. The results show results of two independent experiments to clear pVPL3004 and pVDM-*msrC*::*gfp* from the insertion mutant. Colonies were visualized by the ImageQuant LAS4000 imager through their production of GFP. Note that the fluorescent signal is lower in the *gfp* integration mutants than in the colonies where *gfp* is still present on a multi-copy plasmid.

addition of a CRISPR that targets the *lacl* gene in pVDM-*xlacl* reduced colony numbers down to background levels (68 colonies versus 70 in the water control), suggesting that CRISPR-Cas9 generated lethal double-strand DNA breaks in the *E. faecium* chromosome. Transformation of pVDM- Δ *lacl* resulted in a slight

increase in colony numbers (80 colonies), potentially indicating successful integration of the HR template. This was confirmed by PCR (Fig. 2A) and subsequent Sanger sequencing as we found that approximately 15% of screened colonies were *lacl* deletion mutants. We obtained comparable results in our

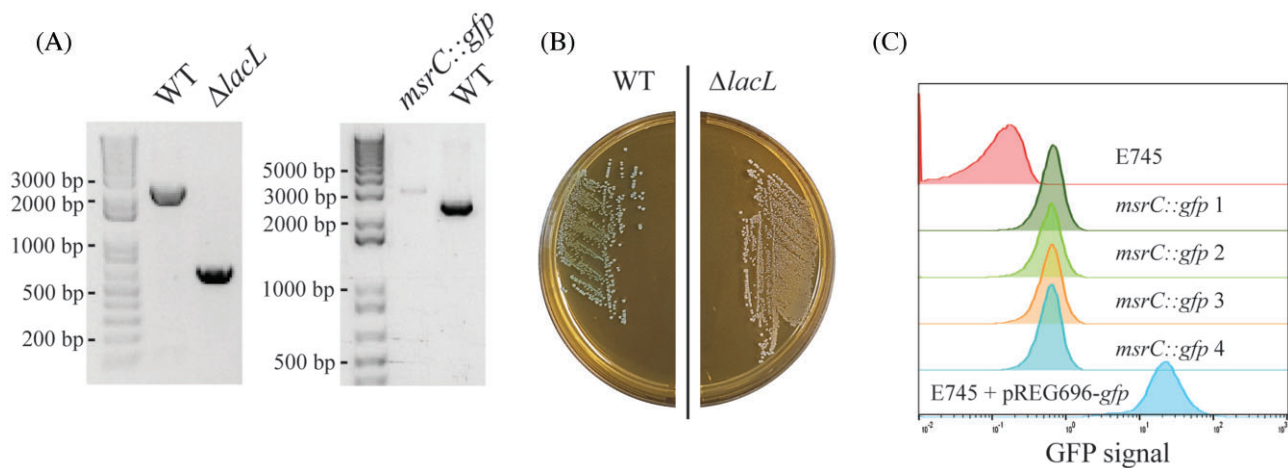


Figure 3. Generation and phenotypes of the $\Delta lacI$ and $msrC::gfp$ mutants. (A), Confirmation of $lacI$ deletion and gfp insertion into $msrC$ via PCR. Deletion of $lacI$ results in a 1800 bp reduction in size of the PCR product from 2.5 kbp to 0.7 kbp, while insertion of the gfp construct into the $msrC$ site results in a shift from 2.8 kbp to 3.2 kbp. (B), Growth of wild-type E745 and $\Delta lacI$ on BHI with 20 μ g/ml X-gal. (C), Flow cytometric analysis of GFP fluorescence levels, from top to bottom, wild-type E745, four different $msrC::gfp$ clones and, as a positive control, E745 containing pREG696- gfp .

attempt to integrate gfp in the $msrC$ gene, with a background of spontaneously spectinomycin-resistant mutants in the control experiments but a higher number of transformants upon electroporation with pVDM- $msrC::gfp$ (data not shown). Our overall success rate in generating mutants was considerable higher in comparison to the homologous recombination-based technique we previously developed (Zhang *et al.* 2012), in which we routinely have to screen 100 or 200 colonies, after several days or even weeks of sub-culturing, before we can isolate the desired mutant that had undergone a double cross-over event.

Once we confirmed that we had successfully generated the $lacI$ deletion mutant and the $msrC::gfp$ insertion mutant, the CRISPR-related plasmids were cured by sub-culturing in BHI broth without antibiotics for three days, or between 20 and 25 generations. Between 50 and 100 colonies isolated from this culture were then transferred to three different BHI agar plates, i.e. BHI agar without antibiotics, BHI agar with spectinomycin and BHI agar with erythromycin to isolate colonies that had cleared both pVDL3004 and the pVDM1001-derivative. Two representative examples of experiments in which we cured the pVPL3004 and the pVDM1001-derivative are shown in Fig. 3. Curing ratios for pVPL3004 were typically around 60%–90% while pVDM1001-derived vectors was more difficult to cure as 1%–5% of colonies had lost the vector. Typically, we obtained 3 to 5 colonies in which both plasmids had cleared per 100 colonies.

Phenotypic characterization of E745 $\Delta lacI$ and E745 $msrC::gfp$

Wild-type (WT) E745 and E745 $\Delta lacI$, which were cleared of pVDL3004 and pVDM- $\Delta lacI$ as outlined above, were grown on BHI supplemented with the chromogenic substrate X-gal to confirm that the genomic alteration affected β -galactosidase activity. While WT colonies were light blue upon growth on medium containing X-gal, the E745: $\Delta lacI$ colonies were creamy white (Fig. 2B), indicating that they could no longer convert X-gal due to the lack of an active β -galactosidase. We determined production of GFP by flow cytometry (Fig. 2C) and we found that the GFP signal is higher in E745 $msrC::gfp$ compared to WT, but considerably lower than the strain in which gfp is carried on a plasmid. This most likely reflects differences in copy number of the

chromosomally integrated gfp construct versus gfp carried on the multi-copy pREG696 plasmid.

CONCLUSIONS

In this proof-of-principle study we applied CRISPR-Cas9 as a counter-selection strategy to aid in the generation of targeted modifications in the chromosome of a clinical strain of *E. faecium*. Our approach for genome editing in *E. faecium* does not require specialized media and does not leave a scar in the chromosome. Mutants could be efficiently identified by PCR and the plasmids used to generate the mutants were readily cured. In comparison with our previous protocol (Zhang *et al.* 2012), processing time was reduced by up to 2 weeks and the total number of colonies that need to be screened is reduced by approximately 4-fold. It is important to note that the use of CRISPR-Cas9 allowed us to generate deletion mutants but also to insert genes into the genome, which can be useful for a number of applications. The stable insertion of fluorescent or bioluminescent tags into the genome can be of particular use during *in vivo* experiments, e.g. to track colonization and infection by *E. faecium*. We note that the CRISPR/Cas9 system described here can be improved further, e.g. by changing the selection markers to reduce the number of spontaneously resistant colonies or by the addition of phenotypic markers (e.g. genes encoding for bioluminescent or fluorescent proteins) that can facilitate screening for the loss of the plasmid. For unknown reasons, the vector is also not always lost during growth at 37°C, and this could be another target for further improvement. For any in-depth phenotypic characterization of any mutants generated with this method, or any other method involving genome manipulation, we recommend the use of whole-genome sequencing to rule out the introduction of non-target mutations. We also stress the importance of complementation of mutations upon the generation of mutants. Native CRISPR systems are relatively rare in multi-drug resistant clinical *E. faecium* strains (Palmer and Gilmore 2010; Lebreton *et al.* 2013) and there is therefore little risk of interference with the system we implemented here.

Even though *E. faecium* is broadly recognized as an important multi-drug resistant nosocomial pathogen, there is still a limited mechanistic understanding of its basic biology and

the traits that contribute to its transition from gut commensal to opportunistic pathogen. Efficient genome editing tools for *E. faecium* are essential to mechanistically characterise its of resistance to antimicrobials and disinfectants and other adaptations that have contributed to *E. faecium* becoming a globally important nosocomial pathogen. The CRISPR-Cas9-based approach described here improves the current genetic toolbox for *E. faecium* and we anticipate that it will accelerate research into this species. We note that the approach we developed here for *E. faecium* might also be successfully implemented in other enterococci and low-GC Gram-positive bacteria with high recombination rates, including several species in the genera *Lactobacillus*, *Streptococcus* and *Staphylococcus* (González-Torres et al. 2019).

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSLE](#) online.

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Conflicts of interest. None declared.

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