TECHNICAL ADVANCE

Unmarked Gene Editing in *Clavibacter michiganensis* Using CRISPR/Cas9 and 5-Fluorocytosine Counterselection

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Plant-pathogenic bacteria in the genus *Clavibacter* are important quarantine species that cause considerable economic loss worldwide. The development of effective gene editing techniques and additional selectable markers is essential to expedite gene functional analysis in this important Gram-positive genus. The current study details a highly efficient unmarked CRISPR/Cas9-mediated gene editing system in *Clavibacter michiganensis*, which couples the expression of *cas9* and single-guide RNA with homology-directed repair templates and the negative selectable marker *codA::upp* within a single plasmid. Initial experiments indicated that CRISPR/Cas9-mediated transformation could be utilized for both site-directed mutagenesis, in which an A to G point mutation was introduced at the 128th nucleotide of the *C. michiganensis rpsL* gene to generate a streptomycin-resistant mutant, and complete gene knockout, in which the deletion of the *C. michiganensis celA* or *katA* genes resulted in transformants that lacked cellulase and catalase activity, respectively. In subsequent experiments, the introduction of the *codA::upp* cassette into the transformation vector facilitated the counterselection of unmarked transformants by incubation in the absence of the selective antibiotic, followed by plating on M9 agar containing 5-fluorocytosine at 100 µg/ml, in which an unmarked *katA* mutant lacking the transformation vector was recovered. Compared with conventional homologous recombination, the unmarked CRISPR/Cas9-mediated system was more useful and convenient because it allowed the template plasmid to be reused repeatedly to facilitate the editing of multiple genes, which constitutes a major advancement that could revolutionize research into *C. michiganensis* and other *Clavibacter* spp.

Keywords: 5-fluorocytosine, bacterial pathogenesis, *Clavibacter michiganensis*, CRISPR/Cas9, homologous recombination

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Clavibacter is a genus of Gram-positive plant-pathogenic bacteria that are noted for their high (65 to 75%) GC content (Davis et al. 1984; Whitman et al. 2012). The damage caused by *Clavibacter* pathogens results in significant losses to crop production throughout the world, and *Clavibacter michiganensis*, the causal agent of bacterial canker of tomato, has long been considered a quarantine bacterium, the spread of which must be controlled to prevent dramatic economic losses to global tomato production. However, research regarding the pathogenicity of *C. michiganensis* has been somewhat limited, and has mainly focused on the cell-wall-degrading enzymes and serine proteases associated with the development of wilt and canker symptoms (Chalupowicz et al. 2017; Dreier et al. 1997; Jahr et al. 2000) and, although several virulence factors have been identified based on single-gene exploration or whole-genome sequence analyses, molecular investigation of the host–pathogen interaction has been hampered by the absence of convenient techniques for the genetic manipulation of *C. michiganensis*.

A major breakthrough in the transformation of *C. michiganensis* came in 1991 with development of the pDM100 cloning vector (Meletzus and Eichenlaub 1991). The subsequent development of a mutagenesis system based on antibiotic resistance transposons in combination with an improved electroporation protocol (Kirchner et al. 2001) provided greater opportunities for gene functional analysis in *C. michiganensis*. For example, knockout and complementation studies of *celA*, *chpC*, and *chpG* were conducted using homologous recombination via suicide plasmids, which confirmed that cell-wall-degrading enzymes and serine proteases play an important role in the development of wilt and canker symptoms (Hwang et al. 2019; Jahr et al. 2000; Stork et al. 2008), while *pbpC*, an enzyme involved in peptidoglycan assembly, was found to be associated with stress resistance (Chen et al. 2021). However, the limited availability of selectable markers has proven to be an obstacle in the construction of multigene knockout mutants, resulting in a stagnation of research focused on the interaction of different pathogenesis-related genes. Therefore, the development of a novel molecular technique to facilitate the routine production of multigene mutants is essential to further understanding of the *C. michiganensis* infection process.

The discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) bacterial immune system, which provides protection against exogenous nucleic acids and bacteriophage invasion (Garneau et al. 2010; Horvath and Barrangou 2010; Mohanraju et al. 2016), was quickly recognized as an opportunity to greatly improve strategies for genetic manipulation in all manner of circumstances. The

CRISPR/Cas-mediated defense system involves three stages: adaption, in which foreign DNA (protospacers) are acquired and incorporated into the CRISPR locus, a portion of the bacterial genome that acts as a repository to recognize the foreign DNA; expression, which involves transcription of the CRISPR RNA (crRNA) that acts as a guide to identify invading nucleic acids; and interference, in which the Cas9-crRNA complex interacts with foreign DNA, resulting in cleavage of the target DNA by the Cas9 endonuclease (Gameau et al. 2010; Marraffini 2015; Mohanraju et al. 2016; van der Oost et al. 2009). The key elements in the CRISPR/Cas system that allows it to be exploited for genetic manipulation are the protospacer adjacent motifs (PAMs) that initially select the protospacer sequences, because they allow artificial or guide RNA (gRNA) to be created that can target Cas9 to any site in the genome (Jinek et al. 2012; Mojica et al. 2009). The CRISPR/Cas system is extremely versatile and can be applied in both prokaryotes such as *Escherichia coli* (Jiang et al. 2015; Zhao et al. 2016), *Streptomyces* spp. (Chen et al. 2017; Huang et al. 2015), and *Bacillus* spp. (Li et al. 2018; K. Zhang et al. 2016) and in eukaryotes such as the fungus *Phytophthora infestans* (Ah-Fong et al. 2021), as well as higher plants (Zong et al. 2017) and animal cells (Shalem et al. 2014). However, to date, there have been no reports of CRISPR/Cas being used in *Clavibacter* spp., which likely results from the combination of the high GC content of the *Clavibacter* genome as well as the lack of suitable selectable markers and expression vectors for the Cas9 and gRNA components.

Although antibiotic resistance genes provide a very convenient method for selecting successful transformation events, they can limit the number of times a particular cell line can be altered, because each new transformation requires a different selectable marker. However, the use of negative selectable markers for counterselection presents the opportunity to sequentially knockout multiple genes using an unmarked gene strategy. The *codA::upp* cassette has been developed for counterselection in *Rhodococcus equi* (van der Geize et al. 2008), which contains the *E. coli* cytosine deaminase and uracil phosphoribosyltransferase genes (*codA* and *upp*, respectively) that convert the innocuous cytosine analog, 5-fluorocytosine (5-FC), first into 5-fluorouracil and then into 5-fluorouridine monophosphate, an extremely toxic compound that irreversibly inhibits thymidylate synthase, resulting in the inhibition of DNA and RNA synthesis and, ultimately, cell death (Andersen et al. 1989; Daher et al. 1990; Danielsen et al. 1992; Greco and Dachs 2001; Hughes et al. 2005; Loyse et al. 2013; Rezzoagli et al. 2018; van der Geize et al. 2008; Xing et al. 2009).

To date, the transformation of *Clavibacter* spp. has depended on techniques that utilize homologous recombination, which has a low transformation efficiency and results in genetic “scars” resulting from the marker genes that confer resistance to the antibiotics gentamycin (Gm) or chloramphenicol (Jahr et al. 2000; Stork et al. 2008). Therefore, the objective of the current study was to develop a novel gene-editing strategy for *Clavibacter* based on CRISPR/Cas9-mediated transformation and counterselection using the *codA::upp* cassette. It was hoped that such a system would greatly increase the efficiency of *Clavibacter* transformation as well establish a system for unmarked gene editing that would facilitate the silencing of multiple genes in combination. Such a breakthrough would undoubtedly result in rapid progress in *Clavibacter* research and lead to a much greater understanding of the host-pathogen interaction of these important plant-pathogenic bacteria.

RESULTS

Cas9 and single-guide RNA adaption for use in *C. michiganensis*.

Preliminary investigation of the CRISPR database indicated that the *C. michiganensis* genome possessed no endogenous

CRISPR system (Grissa et al. 2007a and b, 2008) and, therefore, that a foreign CRISPR/Cas9 system could be introduced to perform gene editing. Further analysis using the Codon Usage Database revealed that the GC content of the *C. michiganensis* genome and its codon usage was similar to that of *Streptomyces coelicolor*, which was a strong indicator that the chimeric single-guide RNA (sgRNA) and codon-optimized *cas9* system developed for gene editing in *S. coelicolor* may also be effective in *C. michiganensis*, although further investigation was required to confirm whether the *tipA* and J23119 promoters used would be capable of driving expression in *Clavibacter* spp. Experiments interchanging the *tipA* and J23119 promoters for the native *pcmx* promoter of the *Corynebacterium striatum* antibiotic resistance gene *cmx*, which was known to confer chloramphenicol resistance in *Clavibacter michiganensis*, revealed that only the *pcmx* and J23119 promoters were functional in *C. michiganensis*, because transformants carrying the pHN216*pcmx-cmx* and pHN216J23119-*cmx* could be recovered on Luria-Bertani (LB) agar containing chloramphenicol at 10 µg/ml, while none could be recovered with pHN216*tipA-cmx* (Fig. 1). Consequently, the J23119 promoter from the *S. coelicolor* CRISPR/Cas9 system was retained for the transcription of target-specific sgRNA in *C. michiganensis*, while the *pcmx* promoter was selected for the expression of *cas9*.

CRISPR/Cas9-mediated site-directed mutagenesis of *rpsL* in *C. michiganensis*.

Having established suitable promoters for CRISPR/Cas9-mediated transformation in *C. michiganensis*, a gene-editing plasmid was developed to test whether the system could be used for site-directed mutagenesis. The *C. michiganensis* ribosomal protein S12 was selected as the target for the transformation because a conserved mutation (A to G at the 128th nucleotide) in the *rpsL* gene was known to confer streptomycin (Str) resistance in a range of plant-pathogenic bacteria, including *C. michiganensis* (Lyu et al. 2019; McManus et al. 2002; Zhang et al. 2011). The transformation vector pHN216-*rpsL*128 (Fig. 2A), which utilized the pHN216 *E. coli*-*C. michiganensis* shuttle vector as a backbone, included four components: a neomycin (Neo)-selectable marker, Cas9 under the control of the *pcmx* promoter, 500 bp upstream and downstream regions of the *C. michiganensis* *rpsL* gene (which included the A to G point mutation), and a modified sgRNA scaffold designed to be a sequence in close proximity to the 128th nucleotide but which had an altered PAM sequence and a nonsense altered sequence to eliminate the *BlnI* restriction site, all under the control of the J23119 promoter (Fig. 2B and C). The modified PAM and sgRNA served two functions. First, the nucleotide nonsense alteration allowed the transformation to avoid the cleavage of the Cas9/sgRNA complex again to recover untransformed cells; and, second, the elimination of the *BlnI* restriction site provided a means to confirm that the site-directed mutation had been successful because the transformed cells would have a different restriction profile compared with the wild type. The pHN216-*rpsL*128 plasmid was successfully introduced to *C. michiganensis* cells by electroporation and the resulting Neo-resistant transformants were selected for further analysis. Amplification of the *rpsL* flanking region from both the wild type and selected transformants using the *rpsLF-F/rpsLF-R* primer set (Supplementary Table S1) coupled with *BlnI* digestion confirmed the success of the mutagenesis, because the transformant produced a single band of 2,486 bp, whereas the wild type yielded a smaller band corresponding to two restriction fragments 1,281 and 1,205 bp in length (Fig. 2D). Subsequent DNA sequencing of the transformants revealed that they did, indeed, contain the A to G point mutation associated with Str resistance (Fig. 2E), which was confirmed by phenotype analysis because the $\Delta rpsL^{A128G}$ mutant was found to have a minimal inhibitory concentration (MIC) value in excess of 3,000 µg/ml, which far exceeded the

8 µg/ml obtained with the wild-type isolate BT0505 (data not shown). Taken together, these results not only confirmed the findings of previous research regarding the link between the site-specific mutation (A to G) at the 128th nucleotide acid of the *rpsL* gene and Str resistance but also demonstrated that CRISPR/Cas9-mediated transformation could be an extremely useful technique for genetic manipulation in *Clavibacter* spp.

CRISPR/Cas9-mediated deletion of *celA* and *kata* in *C. michiganensis*.

Having established that CRISPR/Cas9 could be utilized for site-directed mutagenesis in *C. michiganensis*, the current study went on to investigate its potential to generate gene knockout mutants. In this case, the pHN216-rpsL128 described above was utilized as a template plasmid for the development of two new plasmids, pHN216-celA and pHN216-katA, that were designed to knockout

the *CelA* cellulase (located on pCM1) and *KatA* catalase (CAT) (located on the chromosome), respectively, of *C. michiganensis* (Fig. 3A). This was achieved by simply replacing the *rpsL* sequences and sgRNA with flanking regions from *celA* or *kata* and the sgRNA with gene-specific sequences designed using the Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool via *HindIII/AflIII* digestion.

Initial analysis of the transformants obtained from electroporation with pHN216-celA using PCR and the celAF-F/celAF-R primer set (Supplementary Table S1) identified two transformants, 6 and 10 from 14 transformants, that produced a 1,220-bp product in addition to the 3,461-bp product found in the wild type (Fig. 3B), which indicated that incomplete deletion had occurred as a result of the low efficiency of Cas9 cleavage coupled with the bacteria carrying multiple copies of the pCM1 plasmid that encoded the *celA* gene. However, when these

Fig. 1. Availability of candidate promoters in driving the expression of the chloramphenicol resistance gene (*cmx*) in *Clavibacter michiganensis*. Transformation of *C. michiganensis* with three plasmids (pHN216pcmx-cmx, pHN216J23119-cmx, and pHN216tipA-cmx) containing the *cmx* chloramphenicol resistance gene demonstrated that only the native *pcmx* promoter and the J23119 constitutive promoter could efficiently drive expression in *C. michiganensis*, whereas the *Streptomyces tipA* promoter was nonfunctional and prevented the growth of transformants on Luria-Bertani containing chloramphenicol at 10 µg/ml.

Fig. 2. CRISPR/Cas9-mediated site-directed mutagenesis of the *Clavibacter michiganensis* *rpsL* gene. **A**, pHN216-rpsL128 plasmid, which contained *cas9* under the control of the *pcmx* promoter. **B**, The 500-bp upstream and downstream regions of the *C. michiganensis* *rpsL* gene that included the A to G point mutation at the 128th nucleotide. **C**, A nonsense altered single-guide RNA (sgRNA) sequence that lacked a functional protospacer adjacent motif (PAM) sequence and had an altered sequence to eliminate the *BlpI* restriction site in the *rpsL* gene, all under the control of the J23119 promoter, were used to transform *C. michiganensis* by electroporation. **D**, PCR coupled with restriction digestion analysis confirmed that the transformants ($\Delta rpsL^{A128G}$) lacked the *BlpI* restriction site and, therefore, yielded a single larger fragment (2,486 bp) compared with the wild type (WT), which produced two fragments (1,281 and 1,205 bp) of roughly the same size. Lane M = DNA marker. **E**, DNA sequencing confirmed that the *rpsL* gene contained the desired changes in sequence that resulted in the $\Delta rpsL^{A128G}$ mutant exhibiting a streptomycin-resistant phenotype (data not shown).

transformants were subcultured for more than one round and streaked to single colonies, several individuals (10-1, 10-4, and 10-7) were identified that yielded only the 1,220-bp fragment, which were further confirmed by DNA sequencing (Fig. 3C and D). Furthermore, none of the subcultured transformants produced the characteristic 481-bp fragment using the *celA*-F/*celA*-R primer set, which was designed to amplify an internal region of the *celA* gene (Fig. 3C). In addition, phenotype analysis using Congo Red (Fig. 3E) revealed that none of the three transformants produced the orange halo indicative of cellulase activity, as seen in the *C. michiganensis* reference strain NCPPB382, the wild-type

parental isolated BT0505, and the parental isolate transformed with pHN216 as a negative control for the transformation. Taken together, the PCR and phenotype evidence indicated that the subcultured transformants were, indeed, $\Delta celA$ mutants, and that CRISPR/Cas9-mediated transformation was an effective method for producing gene knockout mutants in *C. michiganensis*.

The success of the CRISPR/Cas9 method was further confirmed in transformants obtained from electroporation with pHN216-katA. In this case, all of the subcultured transformants were found to produce smaller PCR products from the *katA*-F/*katA*-R primer set designed to the *katA* flanking region, which

Fig. 3. CRISPR/Cas9-mediated deletion of the *celA* and *katA* genes of *Clavibacter michiganensis*. **A**, The pHN216-rpsL128 vector was used as a template plasmid for gene deletion, in which the *rpsL* donor DNA and single-guide RNA (sgRNA) were substituted for gene-specific flanking regions and sgRNA corresponding to either *celA* or *katA*, resulting in plasmids pHN216-*celA* and pHN216-*katA*. **B**, PCR analysis of the transformants resulting from electroporation with pHN216-*celA* indicated that only two (6 and 10) had altered profiles in comparison with the wild type (WT). Lane M = DNA marker. **C**, After streaking to purity, PCR targeting the *celA* flanking region and an internal *celA* sequence identified several deletion mutants (10-1, 10-4, and 10-7) that **D**, were later confirmed by DNA sequencing. **E**, Lack of cellulase activity in the Congo Red assay failed to produce the orange halos observed in the WT (BT0505), type *C. michiganensis* isolate (NCPPB382), and transformant carrying an empty vector (BT0505-pHN216). **F**, Similar PCR analysis of the pHN216-*katA* transformants, all of which produced smaller fragments during amplification of the *katA* flanking region and none corresponding to the internal *katA* sequence, indicated a higher efficiency of gene deletion. **G**, DNA sequencing indicated the deletion of gene *katA*. **H**, Phenotype analysis confirmed that all of the transformant colonies ($\Delta katA$) lacked catalase activity, failing to produce the bubbles of oxygen observed in the WT upon the application of H_2O_2 .

were 1,351 bp in length compared with 2,881 bp for the wild type, and completely failed to produce a product from the *katA*-F/*katA*-R primer set designed to target an internal region of the *katA* gene (Fig. 3F). DNA sequencing confirmed successful deletion of the *katA* gene (Fig. 3G). Phenotype analysis revealed that none of the transformants produced the characteristic bubbles of oxygen when H₂O₂ was applied to colonies growing on LB agar, as seen in the wild-type parental isolate (Fig. 3H), and the CAT activity was undetectable in mutants (Supplementary Fig. S1). These results further validated the potential of CRISPR/Cas9-mediated transformation as a method of gene knockout in *C. michiganensis*, not only the deletion of genes located on the plasmid but also genome-encoded genes.

Verification of aminoglycoside antibiotics and 5-FC for use as selectable markers in *C. michiganensis*.

The first step toward adapting the pSelAct plasmid, which has previously been used for unmarked gene editing in *R. equi*, for use in *Clavibacter* was to verify the functionality of the selectable markers in *C. michiganensis*. Plate assays conducted using LB agar amended with the following aminoglycoside antibiotics, including Gen at 50 µg/ml, kanamycin (Km) at 50 µg/ml, Neo at 50 µg/ml, Str at 50 µg/ml, or apramycin (Apr) at 100 µg/ml, showed that *E. coli* cells with pSelAct-*katA* plasmid, in which the *ccdB* suicide gene and the chloramphenicol resistance gene had been replaced by the flanking region of the *katA* gene, could generate colonies (except for Str). The counts of *E. coli* colonies on LB containing Km or Neo were lower than without antibiotic (Fig. 4A). Moreover, competent cells of *C. michiganensis* were not capable of regenerating on media containing Neo, Km, or Gen (Fig. 4B), which indicated that wild-type *C. michiganensis* was sensitive to these aminoglycoside antibiotics. The combined results confirmed that the *aac(3)IV* gene in the pSelAct vector responsible for conferring resistance to aminoglycoside antibiotics (except for Str) was functional and, consequently, Gen at 50 µg/ml was chosen for positive selection of transformants in the subsequent experiments.

Another experiment confirmed that 5-FC could be used for negative selection in *C. michiganensis*, because the wild-type *C. michiganensis* isolate was found to exhibit no growth inhibition on M9 agar containing 5-FC, whereas transformants carrying the pSelAct-*katA* vector (5-FC^S/Gen^R), which contained the *codA::upp* cassette, were completely inhibited by 5-FC at 100 µg/ml, which indicated that *codA::upp* could endow 5-FC sensitivity to *C. michiganensis* (Fig. 5A and B). However, it

was interesting to note that, when the experiment was repeated substituting LB for M9 agar, all of the bacterial samples, including the *C. michiganensis* transformants carrying the pSelAct-*katA* vector, were capable of growth on LB agar containing 5-FC, which indicated that the use of M9 agar was critical for effective 5-FC selection (Fig. 5C and D).

Unmarked gene deletion via homologous recombination and CRISPR/Cas9-mediated transformation.

Although the Δ *katA* mutant generated using the pHN216-*katA* was adequate for further phenotype analysis, the fact that it contained the transformation vector expressing Neo resistance limited its potential in the development of further mutants with multiple gene deletions. However, the transformants carrying pSelAct-*katA* (Fig. 6A) were also confirmed by PCR analysis because they produced a band of 1,351 bp, except for the 2,881 bp seen in the wild type (Fig. 6B). The 5-FC counterselection on M9 agar supplemented with 5-FC, which was endowed by the *codA::upp* cassette of the pSelAct-*katA* vector, provided a solution to this problem. This was simply achieved by subculture and shaking the candidate transformants in LB broth; then, 100 µl of suspension was inoculated onto another LB broth for second shaking. This process was repeated one more time, and the 10⁻⁵ and 10⁻⁴ dilutions of each round of shaking cultures were plated onto M9 agar with 5-FC at 100 µg/ml. Then, these 5-FC-resistant colonies were picked onto LB agar with or without Gen. Transformants exhibiting the 5-FC^R/Gen^S feature were verified by PCR and DNA sequencing (Fig. 6C and D). It was found that the more rounds of subculture in the absence of Gen the more efficient the process, because the numbers of transformants recovered were found to increase from 0 in the first 24 h to 4.65% in the second and 11.54% in the third (data not shown). The recovery of this 5-FC^R/Gen^S Δ *katA* mutant (Figs. 5 and 6) confirmed that the *codA::upp* cassette and 5-FC selection could be an effective method to achieve unmarked gene deletion in *C. michiganensis* via homologous recombination.

The study then went on to investigate whether a similar system could be applied to CRISPR/Cas9-mediated transformation by integrating the *codA::upp* cassette into the pHN216-*katA* plasmid. As expected, the derivative vector pHN216-*katA*-*codAupp* (Fig. 6E) successfully produced transformants which, when verified by PCR and DNA sequencing, produced profiles that indicated that gene deletion had occurred (data not shown). After three cycles of subculture in liquid media lacking Neo,

Fig. 4. Sensitivity of *Escherichia coli* and *Clavibacter michiganensis* to aminoglycoside antibiotics. **A**, *E. coli* cells harboring pSelAct-*katA* plasmid and **B**, wild-type *C. michiganensis* competent cells (Cm) were plated on Luria-Bertani (LB) agar supplement with gentamicin (Gen) at 50 µg/ml, kanamycin (Km) at 50 µg/ml, neomycin (Neo) at 50 µg/ml, streptomycin (Str) at 50 µg/ml, and apramycin (Apr) at 100 µg/ml. LB agar without any antibiotic was used as control (CK).

followed by plating on M9 agar supplemented with 5-FC for counterselection, 5-FC^R $\Delta katA$ mutants were recovered that had evidently lost the pHN216-*katA*-*codA*^{upp} vector because they were no longer able to grow on LB amended with Neo (Fig. 6F). Phenotype analysis confirmed that both the 5-FC^R/Gen^S $\Delta katA$ mutants obtained by homologous recombination using pSelAct-*katA*, and the 5-FC^R/Neo^S $\Delta katA$ mutants generated via CRISPR/Cas9-mediated transformation using pHN216-*katA*-*codA*^{upp}, completely lacked the CAT activity observed in the wild-type BT0505 (Fig. 6G; Supplementary Fig. S1). Taken together, these results confirmed that CRISPR/Cas9-mediated transformation with 5-FC counterselection was an efficient method for creating unmarked gene knockout mutants that could be further transformed to produce *C. michiganensis* mutants with multiple gene knockouts.

DISCUSSION

The current study developed a CRISPR/Cas9-mediated genome editing strategy that was successfully used for site-directed mutagenesis of the *rpsL* gene to generate Str-resistant transformants, as well as to produce knockout mutants of the *C. michiganensis* *celA* and *katA* genes that lacked cellulase and CAT activity, respectively. In addition, the study also integrated an efficient method of counterselection that exploited the cytotoxic effect of 5-FC associated with the *codA::upp* cassette to

facilitate the production of an unmarked $\Delta katA$ mutant without the need for large-scale colony validation. The combination of the two approaches resulted in an improved CRISPR/Cas9 system that could be implemented numerous times within a single *C. michiganensis* cell line to establish mutants with alterations to multiple genes.

Traditionally, the use of CRISPR/Cas-mediated transformation in bacteria has relied on a two-plasmid approach (especially CRISPR/Cas9), in which the *cas9* gene and sgRNA are located separately on two compatible plasmids: a pCas9 plasmid carrying the transactivating crRNA and *cas9* gene, and a second gRNA plasmid carrying the array of CRISPR spacers (Jiang et al. 2013; Liu et al. 2017). However, the current study adapted a single-plasmid system recently developed for the transformation of *Streptomyces* (Huang et al. 2015), in which the *cas9* gene and an sgRNA, as well as a flanking region of the target gene, which acts as a donor for homology directed repair, were carried on one plasmid. The analogous plasmid used in the current study placed the *cas9* gene under the control of the *pcmx* promoter, while retaining the J23119 promoter to drive the expression of the sgRNA. Although the J23119 promoter has previously been used for the expression of sgRNA in *E. coli* and *S. coelicolor* (Huang et al. 2015; Larson et al. 2013), to the best of our knowledge this represents the first report of the J23119 promoter being used to drive expression in *C. michiganensis*. Indeed, research regarding appropriate promoters for the genetic

Fig. 5. Confirmation of 5-fluorocytosine (5-FC) counterselection in *Clavibacter michiganensis*. Panels show the growth of the wild-type (WT), recombinant strain transformed with pSelAct-*katA* vector, which contained the *codA::upp* cassette (*C. michiganensis* *codA::upp*), and transformants lacking the plasmid as a result of counterselection (5-FC^R/Gen^S, *C. michiganensis* $\Delta katA$), as well as the growth of the WT *C. michiganensis* on four different media: **A**, M9 agar; **B**, M9 agar containing 5-FC at 100 $\mu\text{g/ml}$; **C**, Luria-Bertani (LB) agar; and **D**, LB agar containing 5-FC at 100 $\mu\text{g/ml}$.

modification of *C. michiganensis* is currently very limited, and the identification of more efficient *C. michiganensis*-specific promoters could be one avenue to increase the editing efficiency of the CRISPR/Cas9 system and decrease the rounds of subculture required to eliminate incomplete deletion mutants, especially when there may be multiple copies of a target gene carried on a plasmid rather than within the main bacterial genome. Another strategy might be to develop a novel plasmid based on the CRISPR/Cas12a (Cpf1) system. Previous research has shown that Cas12a recognizes a different PAM sequence and exhibits

more precise cleavage of target sequences compared with Cas9 (Leenay et al. 2016). Gene editing strategies based on CRISPR/Cas12a have already been developed in *E. coli*, *Corynebacterium glutamicum*, *Yersinia pestis*, and *Mycobacterium smegmatis* (Jiang et al. 2017; Yan et al. 2017), and have been found to provide a highly efficient method of genetic manipulation in these bacteria.

A major limitation of conventional homologous recombination based approaches for producing gene knockout mutants is that they leave genetic “scars” because the antibiotic resistance

Fig. 6. Unmarked deletion of the *Clavibacter michiganensis* *katA* gene via homologous recombination and CRISPR/Cas9-mediated transformation. **A**, Electroporation with the pSelAct-*katA* plasmid, which contained the aminoglycoside antibiotics resistance cassette (*aac(3)/IV*), and the 5-fluorocytosine (5-FC) sensitivity cassette (*codA::upp*), as well as 500-bp upstream and downstream flanking regions of the *katA* gene, resulted in **B**, the regeneration of several *C. michiganensis* transformants (1, 2, and 3) that had altered PCR profiles compared with the wild-type (WT). Lane M = DNA marker. **C**, After further subculture, two transformants were recovered that produced smaller PCR products when the flanking region was amplified and none corresponding to an internal *katA* sequence. **D**, DNA sequencing also indicated that complete deletion had occurred. **E**, Similar PCR results were obtained after transformation with the pHN216-*katA*-*codAupp* plasmid, which contained the *codA::upp* cassette in addition to *cas9* and the *katA*-specific single-guide RNA (sgRNA) and flanking region (data not shown). **F**, Subculture of the pHN216-*katA*-*codAupp* transformants in broth lacking antibiotic selection followed by counterselection on M9 media containing 5-FC resulted in transformants no longer capable of growth in the presence of antibiotic selection, indicating the loss of the transformation vector. **G**, Phenotype analysis confirmed that both the pSelAct-*katA*- and pHN216-*katA*-*codAupp*-derived transformants (5-FC^R/Gen^S Δ *katA* and 5-FC^R/Neo^S Δ *katA*, respectively) lacked catalase activity, failing to produce the bubbles of oxygen observed in the WT upon the application of H₂O₂.

markers (Gen or chloramphenicol) remain in the genome, limiting the potential for further genetic manipulation (Hwang et al. 2019; Jahr et al. 2000). Thus, the confirmation that the 5-FC counterselection developed for unmarked gene deletion in *R. equi* (van der Geize et al. 2008) can also be applied in *Clavibacter michiganensis* represents a significant advancement for the study of this important plant-pathogenic species. The use of oligotrophic M9 medium rather than nutrient-rich LB medium seemed to be a critical factor in the effectiveness of the counterselection system, which was probably a consequence of the pyrimidine salvage pathway that can affect 5-FC uptake under nutrient-rich conditions (van der Geize et al. 2008). It is also important to note that although natural 5-FC resistance has been documented in many species of actinobacteria (van der Geize et al. 2008), further research is required to determine whether the 5-FC resistance observed in *C. michiganensis* occurs in other *Clavibacter* spp., and whether this counterselection can be universally applied across the genera.

Although the CRISPR/Cas9 coupled with 5-FC counterselection system developed in the current study could be improved further—for example by the use of multiple sgRNA sequences (Ren et al. 2021; Z. Zhang et al. 2016)—it still represents a major breakthrough for genome editing in *C. michiganensis*, by providing a convenient and reliable method for both site-directed mutagenesis and the production of unmarked deletion mutants. The application of such technology could lead to rapid advances in our understanding of the host–pathogen interaction of *C. michiganensis*, as well as providing the groundwork for the genetic manipulation of other *Clavibacter* spp.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions.

The parental wild-type *C. michiganensis* isolate BT0505 (24944; China Center of Industrial Culture Collection) used in the current study was originally isolated from infected tomato plants growing in the Inner Mongolia province of China in 2005 (Jiang et al. 2016). All *C. michiganensis* strains were routinely maintained on LB agar (tryptone at 10 g/liter, yeast extract at 5 g/liter, and NaCl at 5 g/liter) at 28°C for 3 days, while a modified M9 medium (Na₂HPO₄·12H₂O at 6 g/liter, KH₂PO₄ at 3 g/liter, NH₄Cl at 1 g/liter, NaCl at 0.5 g/liter, 1 mM MgSO₄·7H₂O, 0.01 mM CaCl₂·2H₂O, glucose at 2 g/liter, methionine at 200 mg/liter, thiamine at 200 mg/liter, nicotinic acid at 20 mg/liter, and agar

at 16 g/liter) (Chalupowicz et al. 2010) and an extended period of incubation (7 days) was used during the 5-FC counterselection procedure. Similarly, LB broth was used to multiply cells prior to transformation, while sorbitol broth (SB) (tryptone at 10 g/liter, yeast extract at 5 g/liter, NaCl at 4 g/liter, D-sorbitol at 91 g/liter, 20 mM MgCl₂·6H₂O, 20 mM CaCl₂·2H₂O, and agar at 16 g/liter if required) was used to recover *C. michiganensis* transformants after electroporation. When selection was required, antibiotics, including Neo, Gm, and chloramphenicol, were added to a final concentration of 50, 50, and 10 µg/ml, respectively, while, during the negative selection, 5-FC was added at 100 µg/ml. The 5-FC (Sigma) stock solution itself was prepared by dissolution in water (10 mg/ml), a process that was accelerated by heating in a 50°C water bath. The optical density of bacterial suspensions was measured at 580 nm (OD₅₈₀) using an ultraviolet spectrophotometer U-T6A (Yipu, Shanghai, China).

The initial plasmid construction was performed using *E. coli* DH5α competent cells using conventional cloning procedures, with the plasmids used in the *C. michiganensis* transformation being prepared using the TIANprep Mini Plasmid Kit (TIAN-GEN Biotech, Beijing, China). All plasmids used in the study have been listed in Table 1.

Electroporation of *C. michiganensis* cells.

The electroporation protocol used to routinely transform *C. michiganensis* cells was adapted from a method detailed in a previous study (Kirchner et al. 2001). Briefly, cells of *C. michiganensis* (250 ml) at the early log phase of growth (OD₅₈₀ = 0.6) were mixed with 20% (wt/vol) glycine stock solution to a final concentration of 2.5% (wt/vol), and incubated at 28°C with shaking (120 rpm) for 1 h. The cells were then harvested by centrifugation and washed in 25 ml of ice-cold distilled water three times, before being washed in ice-cold 10% (vol/vol) glycerol twice. The competent cells were then resuspended in 500 µl of 15% glycerol and divided into five tubes, which were stored at –80°C until required. The electroporation itself was performed at 12.5 kV/cm with a time constant of 12 to 16 ms. The transformed cells were then mixed with 400 µl of SB liquid medium and incubated at 28°C with shaking (200 rpm) for 3 h before being spread on SB agar plates containing the appropriate antibiotic for selection. Individual colonies were picked after incubation at 28°C for 4 to 5 days, and streaked to purity on LB amended with the selective antibiotic.

Table 1. Plasmids used in the current study

Plasmid	Relevant characteristics ^a	Source
pHN216	<i>Escherichia coli</i> – <i>Clavibacter</i> shuttle vector; Neo ^R	Laine et al. 1996
pOKU9cmBα	Donor of the <i>cmx</i> gene and <i>pcmx</i> promoter	Kaup et al. 2005
pKCcas9dO	Donor of the <i>tipA</i> and J23119 promoters as well as the <i>cas9</i> gene	Huang et al. 2015
pHN216tipA-cmx	Derivative of pHN216; Neo ^R	Current study
pHN216J23119-cmx	Derivative of pHN216; Neo ^R , Chl ^R	Current study
pHN216pcmx-cmx	Derivative of pHN216; Neo ^R , Chl ^R	Current study
pHN216-rpsL128	Derivative of pHN216 for site-directed mutation of <i>rpsL</i> by CRISPR/Cas9-mediated transformation; Neo ^R	Current study
pHN216-celA	Derivative of pHN216-rpsL128 for knockout of <i>celA</i> by CRISPR/Cas9-mediated transformation; Neo ^R	Current study
pHN216-katA	Derivative of pHN216-rpsL128 for knockout of <i>katA</i> by CRISPR/Cas9-mediated transformation; Neo ^R	Current study
pSelAct	Suicide plasmid, including the <i>aac(3)IV</i> , <i>ccdB</i> , and <i>codA::upp</i> cassettes; Gen ^R , Chl ^R , 5-FC ^S	Savory et al. 2020; van der Geize et al. 2008
pSelAct-katA	Derivative of pSelAct containing 500-bp flanking regions for unmarked knockout of <i>katA</i> by homologous recombination; Gen ^R , 5-FC ^S	Current study
pHN216-katA-codAupp	Derivative of pHN216-katA including the <i>codA::upp</i> cassette for unmarked knockout of <i>katA</i> by CRISPR/Cas9-mediated transformation. Neo ^R , 5-FC ^S	Current study

^a Abbreviations: Neo = neomycin, Chl = chloramphenicol, Gen = gentamicin, and 5-FC = 5-fluorocytosine; superscripts ^R and ^S indicate resistant and sensitive, respectively.

Screening for functional promoters to facilitate CRISPR/Cas9-mediated transformation of *C. michiganensis*.

The development of the CRISPR/Cas9 system for use in *C. michiganensis* first required the identification of functional promoters that could drive expression in *Clavibacter* spp. Two candidates were initially considered: the promoter from the *tipA* gene and the constitutive promoter J23119, both of which had been used successfully in the pKCCas9dO plasmid to express *cas9* and sgRNA, respectively, in *S. coelicolor* (Huang et al. 2015). The native promoter of *cmx* cassette (referred as *pcmx*) from plasmid pOKU9cmB α was used as a positive control to turn on the expression of the chloramphenicol resistant gene in *C. michiganensis* (Kaup et al. 2005). The *E. coli*-*Clavibacter* shuttle vector pHN216 digested by *HindIII*/*EcoRV* was used as host backbone for all DNA fragments (the promoter and chloramphenicol-resistant gene), and resulted in recombinant plasmids pHN216tipA-cmx, pHN216J23119-cmx, and pHN216pcmx-cmx. Each of the three plasmids were then introduced into competent *C. michiganensis* cells by electroporation, and verified on LB agar containing chloramphenicol at 10 μ g/ml.

Construction of gene-editing plasmids for CRISPR/Cas9-mediated transformation of *C. michiganensis*.

Having established that both the *pcmx* and J23119 promoters could successfully drive expression in *C. michiganensis*, a series of gene-editing plasmids were constructed with *cas9* under the control of the *pcmx* promoter and the sgRNA under the control of the J23119 promoter to investigate whether the CRISPR/Cas9 system could be used for site-directed mutagenesis and the creation of deletion mutants. The first step in the process involved the creation of a *pcmx-cas9* fusion product, which was produced by amplifying the *pcmx* promoter from pOKU9cmB α using the *pcmx*-F/*pcmx*-R primer set (Supplementary Table S1), which introduced an *Afl*III restriction site (CTTAAG) to facilitate cloning. The *cas9* gene was amplified from pKCCas9dO and ligated to the *pcmx* promoter by overlap PCR. The 500-bp upstream, *rpsL* gene with A128G point mutation and downstream regions were amplified from genomic template DNA of wild-type isolate BT0505 using the *rpsLU*-F/*rpsLU*-R and *rpsLD*-F/*rpsLD*-R primer sets (Supplementary Table S1). Meanwhile, the *rpsL*-specific sgRNA, which was designed to a region near the 128th nucleotide of the *rpsL* gene and contained altered sequences to eliminate the PAM recognition sequence and *Blp*I restriction site (Fig. 2C), was amplified from pKCCas9dO template DNA using the sgRNA-F/*rpsL*sg-R primer set (Supplementary Table S1). These functional elements were then ligated into the pHN216 backbone digested by *HindIII*/*EcoRV*.

The pHN216-rpsL128 plasmid described above was also used as a template plasmid for the production of the *celA* and *katA* knockout mutants by simply replacing the *rpsL*-specific sgRNA and flanking regions. The sgRNA of *celA* and *katA* was amplified from pKCCas9dO template DNA using the sgRNA-F/*celA*sg-R and sgRNA-F/*katA*sg-R primer sets, respectively (Supplementary Table S1). The *celA* flanking regions were amplified from BT0505 template DNA by *celA*-F1/*celA*-R1 and *celA*-F2/*celA*-R2 primers, and the *katA* flanking regions were amplified by *katA*-F1/*katA*-R1 and *katA*-F2/*katA*-R2 (Supplementary Table S1). The resulting sgRNA elements and flanking regions were cloned into the *HindIII*/*Afl*II-digested pHN216-rpsL128 backbone to produce pHN216-*celA* and pHN216-*katA*, respectively (Fig. 3A).

The pSelAct-*katA* plasmid (Fig. 6A) used for the unmarked *katA* gene deletion experiment was prepared by replacing the *ccdB* suicide cassette and chloramphenicol resistance gene in pSelAct with the *katA* flanking regions. And pHN216-*katA*-codAupp plasmids (Fig. 6E) used for the unmarked CRISPR/

Cas9-mediated gene deletion experiments were constructed by introducing the *codA::upp* cassette from pSelAct to *EcoRV*/*Spl*I-digested pHN216-*katA*.

All of the primers used during the cloning procedure were designed according to the instructions of the In-Fusion cloning system, which produced 15- to 18-bp overlap regions.

Assessing the sensitivity of *C. michiganensis* to aminoglycoside antibiotics.

The first step in determining whether the pSelAct suicide vector could be adapted for use in *C. michiganensis* was to assess the viability of its selectable markers in *Clavibacter* spp. The sensitivity of both *E. coli* and *C. michiganensis* to a range of aminoglycoside antibiotics associated with the *aac(3)IV* resistance gene detailed in the comprehensive antibiotic resistance database, including Km at 50 μ g/ml, Gen at 50 μ g/ml, Neo at 50 μ g/ml, Str at 50 μ g/ml, and Apr at 100 μ g/ml, was tested by plating on antibiotic-amended LB agar (Dubois et al. 2008; Vliegthart et al. 1991). Aliquots (100 μ l each) of 10⁶ CFU/ml bacterial suspensions of either *E. coli* transformed with the pSelAct-*katA* plasmid, in which the chloramphenicol resistance gene and *ccdB* suicide cassette had been replaced by 500 bp upstream and downstream sequences of the *katA* gene, or electrocompetent *C. michiganensis* cells were spread across the plates and assessed after 12 h of incubation at 37°C in the case of *E. coli* and 5 days incubation at 28°C in *C. michiganensis*.

5-FC negative selection in *C. michiganensis*.

The 5-FC negative selection procedure was carried out according to the protocol of a previous study, with a few modifications (van der Geize et al. 2008). Briefly, *C. michiganensis* transformants carrying the pSelAct-*katA* plasmid were first inoculated in 10 ml of LB broth in the absence of antibiotics and incubated at 28°C with shaking (120 rpm) for 24 h, before serial dilutions were prepared and 100- μ l aliquots of the resulting bacterial suspensions (10⁻⁵ and 10⁻⁴) were plated on M9 agar plates supplemented with 5-FC at 100 μ g/ml. Meanwhile, 100- μ l suspensions were added into another 10 ml of LB broth, shaken for 24 h again, and diluted bacterial suspension was spread on M9 agar with 5-FC. This process was repeated four times. After 9 days of incubation at 28°C, individual colonies on M9 agar were selected and inoculated onto LB plates with and without Gen. Colonies that were found to be resistant to 5-FC but susceptible to Gen were further verified by PCR and DNA sequencing. Having established that counterselection was effective with *C. michiganensis* transformants carrying the pSelAct-*katA* plasmid, a similar process was later used for the counterselection of *C. michiganensis* transformants carrying the pHN216-*katA*-codAupp vector, although in this case Neo at 50 μ g/ml was used, replacing Gen for the antibiotic selection step.

Assessing the phenotype of *C. michiganensis* mutants.

Str resistance. The MIC values for Str in the wild-type parental isolate BT0505, and the Δ *rpsL*^{A128G} mutant were determined by measuring their growth in liquid media amended with Str at 0, 1, 2, 4, 8, 16, 32, 64, 120, 240, and 512 μ g/ml for the wild type and 0, 100, 200, 300, 400, 500, 1,000, 2,000, 3,000, 4,000, and 5,000 μ g/ml for the mutant. Each culture was inoculated to a final concentration of 10⁶ CFU/ml and incubated at 28°C with shaking (120 rpm) for 24 h, before OD₅₈₀ was measured using a Tecan infinite F200 microplate reader (Tecan, Grödig, Austria). Each strain was represented by three replicates, and the entire experiment was performed three times.

Cellulase activity. The cellulase activity of the Δ *celA* mutant was investigated using the Congo Red test on M9 plates containing 0.5% carboxyl methyl cellulose (CMC), as described in a previous study (Meletzus et al. 1993). The Δ *celA* mutant and

wild-type isolate were streaked on M9-CMC plates and incubated at 28°C for 4 to 7 days before 10 ml of 0.1% Congo Red was added to each plate. After a further 20 min of incubation, the dye was removed and the plates rinsed five or six times with NaCl at 1 mol/liter. The formation of orange halos around bacterial colonies indicated the presence of cellulase activity in the wild type, while the absence of halos in the $\Delta celA$ mutant indicated a loss of extracellular cellulase activity.

CAT activity. The absence or presence of CAT activity was determined using a very simple assay in which drops of 30% H₂O₂ (w) were applied to colonies of the wild-type *C. michiganensis* isolate and the $\Delta katA$ mutants that had been grown on LB agar for 3 days at 28°C. The rapid formation of oxygen bubbles indicated the presence of CAT activity in the wild-type isolate, while the lack of bubbles in the mutants confirmed the loss of CAT activity.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Codon Usage Database: <http://www.kazusa.or.jp/codon/>

CRISPR database: <https://crispr.i2bc.paris-saclay.fr/>

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