Clinical Microbiology and Infection 24 (2018) 1095–1099

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Original article

Genome engineering of *Clostridium difficile* using the CRISPR-Cas9 system

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ARTICLE INFO

Article history:
Received 10 October 2017
Received in revised form
22 December 2017
Accepted 14 January 2018
Available online 29 March 2018

Editor: G. Greub

Keywords:
Clostridium difficile
Clostridium difficile infection (CDI)
CRISPR-Cas9
Genome engineering
Infectious disease
PpFbFPm
spo0A
Sporulation

ABSTRACT

Objectives: *Clostridium difficile* is a notorious pathogenic species that can cause severe gastrointestinal infections in humans and animals. *C. difficile* infection (CDI) results in thousands of deaths worldwide every year. The elucidation of related mechanisms of CDI and exploration of potential therapeutic strategies are largely delayed due to the lack of efficient genetic engineering tools for *C. difficile* strains.
Methods: Plasmids carrying the CRISPR-Cas9 system were constructed and transformed into *C. difficile* through conjugation. Mutants were identified using colony PCR with primers annealing to the regions flanking the target gene deletion/integration locus. Heat-survival assay was used to compare the sporulation frequency between the mutant with *spo0A* deletion and the wild type strain. The fluorescence in the mutant with the insertion of the green fluorescent protein (GFP) gene was inspected under a fluorescent microscope.
Results: An efficient genome editing tool was developed for *C. difficile* based on the CRISPR-Cas9 system. With this tool, *spo0A* was deleted with a 100% mutation efficiency. Conversely, an anaerobic GFP gene was successfully inserted into the *C. difficile* chromosome (with a mutation efficiency of 80%).
Conclusions: The developed CRISPR-Cas9-based genome engineering tool will facilitate functional genomic studies in *C. difficile* as well as the elucidation of mechanisms related to host–bacteria interaction and pathogenesis of CDI. This will be highly beneficial for the development of innovative strategies for CDI diagnostics and therapies. **S. Wang, Clin Microbiol Infect 2018;24:1095**

Introduction

Clostridium difficile (renamed as *Clostridioides difficile* in 2016 [1]), a notorious pathogenic clostridia species which can cause severe gastrointestinal infection, has been designated as an urgent threat to the human health by the US Centers for Disease Control and Prevention [2]. Currently, *C. difficile*-associated diarrhoea has been the most frequently occurring nosocomial diarrhoea worldwide [3].

Despite abundant reports concerning the two principal toxins, toxins A and B, few studies have focused on other virulence factors, and little is known about the interaction of the pathogen with the mammalian gut during CDI, largely due to the lack of efficient genetic engineering tools. Compared to other non-pathogenic *Clostridium* strains, fewer genetic engineering tools are available for *C. difficile*. The ClosTron gene knockout system has been implemented in *C. difficile* to generate insertion-based mutagenesis [4]. However, accompanying the gene knockout, the intron (and sometimes the antibiotic marker) is left behind in the genome [5]. Furthermore, the efficiency of ClosTron relies on the precise prediction of insertion sites using a specific algorithm, and it can be very challenging to find an optimal target site when the selected insertion sequence is short. Besides, the intron insertion may be spliced out by the intron-encoding protein, and thus the mutation can be reversed. Although genome engineering in *C. difficile* has been previously achieved with the allelic exchange approach, the implementation of the protocol is time-consuming and laborious and thus remarkably restricts its broad application [6]. Therefore, more efficient and convenient tools are highly desirable for flexible and versatile genome engineering in *C. difficile*.

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https://doi.org/10.1016/j.cmi.2018.03.026
1198-743X/

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) system is an RNA-mediated immune system in bacteria and archaea [7]. Recently, the CRISPR system, especially the Type II CRISPR-Cas9 system from *Streptococcus pyogenes*, has been exploited as a cutting-edge genome-engineering tool for both eukaryotic and prokaryotic cells [8]. In the CRISPR-Cas9 system, the Cas9 can be directed to the site-specific DNA sequence by a chimeric guide RNA (gRNA), leading to the double strand breakage (DSB) [9]. To generate the genetic mutation in cells where an efficient non-homologous end-joining (NHEJ) or functional recombineering system is not available (as is the case in most of bacterial strains), CRISPR-Cas9 serves as a selection tool for the mutant cells generated through homologous recombination out of the unedited background cells [8]. Thus, stable and marker-free mutations can be generated on the host chromosome. In the past few years, the CRISPR-Cas9 system has been successfully exploited for genome editing in various bacteria including several non-pathogenic clostridia species [10–18].

In this study, an efficient CRISPR-Cas9-based genome-editing system was developed for *C. difficile*. With this system, both gene deletion and gene integration were achieved with high efficiencies. This developed genome editing system will facilitate functional genomic studies in *C. difficile* such as virulence factor discovery as well as elucidation of mechanisms for example, concerning host–pathogen interaction. This will provide valuable information for the development of effective strategies for CDI diagnostics and therapies.

Methods

Bacteria strains, plasmids, growth conditions and oligonucleotides

All the strains, plasmids and DNA primers used in this study are listed in Supplementary Tables S1 and S2. *E. coli* DH5α was used for DNA cloning. *E. coli* CA434 (HB101 carrying the IncPβ conjugative plasmid, R702) was used as the donor strain for conjugation [19]. Growth conditions for the strains are described in the Supplementary Material.

Plasmid construction

The fragment including the Cas9 open reading frame (ORF) under the control of a lactose inducible promoter along with the chimeric gRNA sequence was amplified from pYW34 using primers YW1237 and YW1223 [8], and then inserted into the *Eco*RI site of pMTL82151 [20], generating pSH12. The plasmid pSH12 was then used as the mother vector to construct plasmids for genome engineering in *C. difficile* [17].

Spo0A, the master transcriptional regulator for sporulation, has been reported to play a key role in initiating sporulation in *Clostridium* species [23]. To construct the plasmid for the deletion of the *spo0A* gene (CD630_12140), two homology arm sequences (1 kb for each) flanking the *spo0A* ORF were amplified from *C. difficile* 630 genomic DNA with primer pairs YW1226 & YW1227 and YW1228 & YW1229, respectively. These two fragments were then fused together with primers YW1230 and YW1231 through splicing by overlapping extension PCR (SOE-PCR) and inserted into the *Not*I site of pSH12 with Gibson Assembly, generating pSH13. Afterwards, the 20-nt guiding sequence (5′-GACATGCAATAGAGGTTGCA-3′) fused with the small RNA (sRNA, sCbei_5830) promoter was amplified from *C. beijerinckii* 8052 genomic DNA using primers YW484 and YW1232, and inserted into the *Btg*ZI site of pSH13, generating pSH14.

For the insertion of *PpFbFPm* [21,22], the sRNA promoter along with the 20-nt guiding sequence (5′-GGATTTCTCACATAAAATAG-3′) was amplified with primers YW484 and YW1930, and then inserted into the *Btg*ZI site of pSH12, generating pSH20. The upstream homology arm sequence (443 bp), the *PpFbFPm* gene under *thiolase* promoter and the downstream homology arm sequence (487 bp) were amplified using primer pairs YW2060 & YW1931, YW1934 & YW1935 and YW1932 & YW2062, respectively. These three fragments with *PpFbFPm* in the middle were fused together using primers YW2060 and YW2062 through SOE-PCR, and then inserted into the *Not*I site of pSH20, generating pSH21.

Conjugation of C. difficile

The desirable plasmid (pSH14 or pSH21) was first transformed into the conjugation donor strain *Escherichia coli* CA434 with electroporation, and then into *C. difficile* through conjugation (see Supplementary Material).

Mutant screening

Single colonies were picked randomly from the selection plate for colony PCR (cPCR) using primers YW1066 and YW1067 to confirm the presence of plasmid. Further, cPCR with primers YW1345 and YW1346 was performed to detect mutants with the deletion of the *spo0A* gene. Similarly, cPCR with primers YW2231 and YW2233 was carried out to detect the insertion of *PpFbFPm*. If no mutant was detected in the original transformants, colonies were picked and inoculated into BHIST liquid medium (BHIS with thiamphenicol, Tm) [14]. The culture was incubated overnight to reinforce homologous recombination. It was then diluted in series and spread onto BHISLT (BHIS with Tm and lactose) agar plates for the induction of Cas9 expression. Once colonies were observed, screening for mutants was further carried out using cPCR as described above. The detailed procedures for generating the mutants using the CRISPR-Cas9 system are illustrated in Figs S1 and S2.

Plasmid curing

To cure the plasmid, the mutant was subcultured in BHIS medium without antibiotics for about 7–10 cycles, and then the culture was spread onto BHIS plates to obtain colonies [14]. The colonies were afterwards replica plated onto BHIS and BHIST agar plates. The culture that could grow well on the BHIS plate but failed to grow on the BHIST plate was identified as putative clean mutant with the plasmid cured. The loss of the plasmid was then further confirmed by cPCR using primers YW1066 and YW1067.

Heat-survival assay for sporulation evaluation

Spores were prepared as described by Heap et al. [4]. The Δ*spo0A* mutant as well as the wild type *C. difficile* 630 strain were grown on brain heart infusion (BHI) agar plates for 14 days. Cells were scraped off the plate and resuspended in phosphate-buffered saline (PBS) buffer. Cultures before and after heat-treatment (80°C, 10 min) were spread onto BHIS plates supplemented with 0.1% sodium taurocholate. Colonies (cfu/mL) were enumerated after 24–48 h incubation. The sporulation frequency was determined as the ratio of heat-resistant cfu (grown from the culture with heat treatment) out of the total cfu (grown from the culture without heat treatment). Reported results were based on the average of three biological replicates.

Fluorescence microscopy

In vivo fluorescence in *C. difficile*::*PpFbFPm* was detected with an Olympus BX51 fluorescent microscope. Cells in the logarithmic

growth phase were harvested, washed twice with PBS and then resuspended in 1/10 volume of PBS. Two microlitres of culture were loaded to make the microscope slide for the intracellular fluorescence detection (Ex = 490 nm, Em = 520 nm). The fluorescence intensity was measured semi-quantitatively using the ImageJ software (http://rsb.info.nih.gov/ij/index.html).

Results

spo0A deletion in C. difficile 630

The *spo0A* gene has been successfully disrupted in *C. difficile* 630 with ClosTron previously [4]; thus, we selected it to delete as a starting point for the development of CRISPR-Cas9 system for genome editing in *C. difficile*. With the transformation of pSH14 through conjugation, an efficiency of 4.9×10^{-5} per donor cell was obtained. To detect possible mutants on the BHIST agar plates (with transformants from the original transformation without induction for Cas9 expression using lactose), eight colonies were picked randomly for cPCR. All of the eight colonies were confirmed as positive mutants with *spo0A* deleted, indicating a 100% mutation rate (Supplementary Fig. S3). Then the plasmid within the mutant was cured through subculturing and replica plating. The deletion in the clean mutant was further verified through cPCR (Fig. 1(a)). The resultant mutant was named as *C. difficile Δspo0A*.

PpFbFPm insertion into the genome of C. difficile 630

To demonstrate the capability for gene integration into the genome of *C. difficile* using CRISPR-Cas9, a green fluorescent protein (GFP) gene *PpFbFPm* which could be actively expressed under anaerobic conditions was selected to be inserted [21,22]. The insertion site was selected between the 101st and 102nd nucleotide downstream of the stop codon of *slpA*, exactly between the terminator of *SlpA* (CD630_27930) and promoter of *secA2* gene (CD630_27920) (Supplementary Material). Therefore, technically, the insertion of *PpFbFPm* would not influence the expression of either *slpA* or *secA2*.

The plasmid pSH21 was transformed into *C. difficile* 630. Unlike the case for *spo0A* deletion, no mutant with the insertion of *PpFbFPm* was detected with cPCR from the original transformant colonies on the BHIST agar plate. Then colonies were picked and cultivated in BHIST liquid medium overnight and then spread onto BHISLT agar plates to induce the Cas9 expression. When colonies were observed, cPCR was carried out, and 80% of the selected colonies (Supplementary Fig. S4) were demonstrated as positive mutants. Plasmid curing was performed and clean mutant was obtained (Fig. 1(b)). The resultant mutant was named as *C. difficile*::*PpFbFPm*.

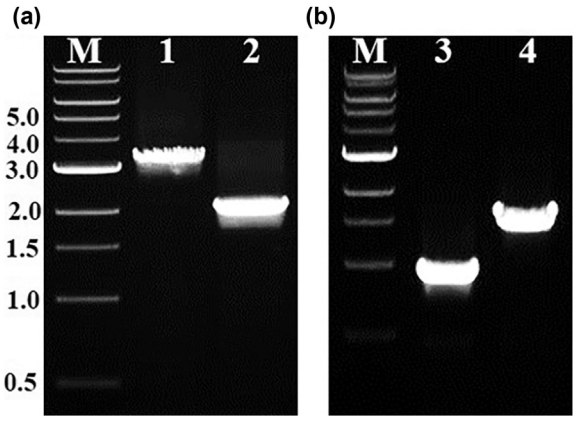

Fig. 1. Confirmation of mutants with colony PCR (cPCR). (a) cPCR results for the detection of *spo0A* deletion (Lane 1, 2984 bp from the wild type; Lane 2, 2159 bp from *C. difficile Δspo0A*). (b) cPCR results for the detection of *PpFbFPm* integration (Lane 3, 1000 bp from the wild type; Lane 4, 2000 bp from *C. difficile*::*PpFbFPm*). Lane M: The 1-kb DNA ladder from New England Biolabs (NEB) with numbers on the left representing the band size in kb.

Sporulation frequency in C. difficile Δspo0A

As shown in Fig. 2, compared with the wild type which demonstrated a sporulation frequency of 23.1 ± 0.6%, *C. difficile Δspo0A* lost the ability to form spores (no colonies were obtained from the *Δspo0A* culture either with or without heat-treatment). This result is similar as that from the previous report [23], demonstrating that *spo0A* is essential for spore formation in *C. difficile*.

Fluorescence in C. difficile::PpFbFPm

The fluorescence generated in *C. difficile*::*PpFbFPm* was detected using fluorescence microscopy and quantified with ImageJ. The wild type *C. difficile* 630 strain was subjected to the same investigation as the control. As shown in Fig. 3, although the wild type strain could generate some intrinsic green autofluorescence as reported previously [24], the fluorescence from *C. difficile*::*PpFbFPm* was much stronger. Based on a semi-quantitative measurement using ImageJ, the fluorescence intensity in *C. difficile*::*PpFbFPm* was about three-times higher than that from the wild type strain (0.31 vs 0.09/pixel), which confirmed the active expression of *PpFbFPm* in the mutant.

Discussion

CDI has become a serious health problem, leading to thousands of deaths worldwide every year [25]. The lack of genome engineering tools for *C. difficile* has delayed the mechanistic understanding of the interaction between this pathogen and its hosts, as well as the pathology. ClosTron was developed in 2007 and has been used for gene disruption in *C. difficile* [4]. However, various drawbacks prevented its broader application. Comparatively, the CRISPR-Cas9 system developed in this study is much more efficient, much easier to implement, and can be used for versatile genome

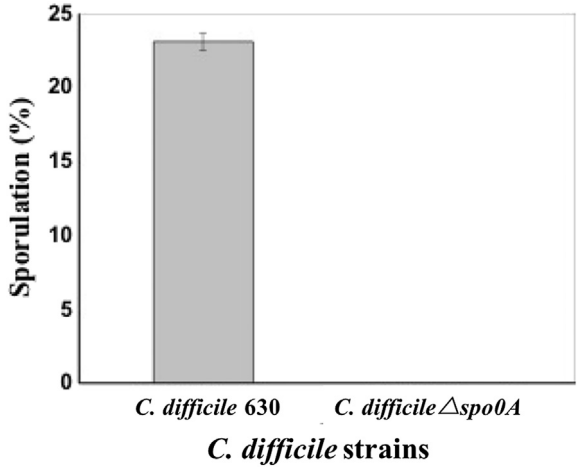

Fig. 2. The sporulation frequency of *C. difficile Δspo0A* in comparison to *C. difficile* 630. The sporulation frequency was determined as the ratio of heat-resistant colony-forming units (cfu; grown from the culture with heat treatment) out of the total cfu (grown from the culture without heat treatment). Reported values represent the means ± standard deviation of triplicates.

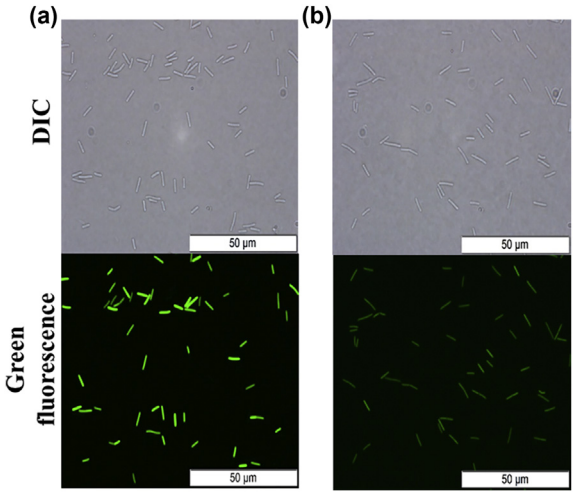

Fig. 3. The detection of green fluorescence signal in *C. difficile*::*PpFbFPm* (a) in comparison to *C. difficile* 630 (b). In each panel: top, the image from the differential interference contrast (DIC) microscopy; bottom, the image from the green fluorescence microscopy. Cells from the logarithmic growth phase were harvested, washed twice with phosphate-buffered saline (PBS), resuspended in 1/10 volume of PBS, and then a microscope slide was prepared for fluorescence detection under an Olympus BX51 microscope.

engineering purposes. This paves the way for the systematic understanding of the host–pathogen interaction and the pathogenesis of the CDI-related diseases, which will further facilitate the development of strategies for CDI diagnostics and therapies.

Interestingly, for the deletion of the *spo0A* gene, a mutation rate of 100% was achieved based on the original transformants without subculturing or the induction for the Cas9 expression. This suggested that the lactose-inducible promoter was not stringently regulated in *C. difficile*; with the limited leaked expression of Cas9, a high selection power can be achieved for the desirable mutants, demonstrating the high efficiency of the developed CRISPR-Cas9 system. Similar to the previous reports [4,26], *C. difficile Δspo0A* failed to form spores (Fig. 2). This mutant will facilitate the study of mechanisms of CDI with regard to sporulation because spores are the primary infectious form of *C. difficile* [23].

Furthermore, an oxygen-independent GFP gene, *PpFbFPm* was successfully integrated into the chromosome of *C. difficile*. Compared to the *spo0A* deletion, subculturing and the induction of Cas9 expression with lactose was required to obtain the *PpFbFPm* insertion mutant, with a lower mutation rate (80%). One reason that might have led to such a difference is that, for the *PpFbFPm* integration, shorter homology arm sequences (less than 500 bp in length for each vs 1 kb for each for *spo0A* deletion) were used, and generally a lower homologous recombination efficiency can be achieved with shorter homology arms [27]. On the other hand, the gene integration might be generally more difficult to realire than a simple gene deletion. *PpFbFPm* was designed to be inserted into a non-coding intergenic region between two genes, and thus, technically, the expression of neither gene would be influenced. Since the protospacer adjacent motif (PAM; NGG in this case, for gRNA to guide Cas9 to target onto the specific site on the chromosome) is widely distributed over the genome of *C. difficile*, it is easy to find such intergenic regions to integrate the desirable gene using the developed CRISPR-Cas9 system. The fluorescence microscopy illustrated that *C. difficile*::*PpFbFPm* showed much stronger green fluorescence than *C. difficile* 630 which could demonstrate weak intrinsic green autofluorescence (Fig. 3). *C. difficile*::*PpFbFPm* is a very useful strain for the study of the host-pathogen interaction since its green fluorescence signal can be monitored and tracked in real time.

In this study, for both the gene knockout and gene integration with the CRISPR-Cas9 system, high efficiencies have been obtained. However, we want to point out that these results were obtained based on only a small set of colonies for the cPCR screening in each case. In addition, the exact genome editing efficiency could be variable depending on the different target sites, target genes, the guiding sequence used for the gRNA, the length of the homology arms, and etc. Despite the high efficiency for genome editing as demonstrated here, there are still limitations for the developed CRISPR-Cas9 system in this study, which warrants further investigation and improvement. For example, comparing to other bacterial strains, the deletion or integration of large gene fragments in *Clostridia* has never been demonstrated, and could be still challenging even using the CRISPR-Cas9 system (8,12). Moreover, the developed CRISPR-Cas9 system has only been tested with *C. difficile* 630 type strain in this study. The effectiveness of this system in other *C. difficile* strains, especially for the various clinical isolates, is still questionable and needs to be further investigated.

In conclusion, an efficient CRISPR-Cas9 system has been developed for *C. difficile* which can be used to realise versatile genome engineering purposes. Based on this system, *spo0A* was deleted with a 100% mutation rate without subculturing or induction for Cas9 expression. Conversely, the anaerobic GFP gene, *PpFbFPm* was integrated into the genome of *C. difficile*, also with a very high efficiency (80%). *C. difficile Δspo0A* can be used to facilitate the study of mechanisms of CDI related to sporulation, and *C. difficile*::*PpFbFPm* will be useful for the study of the host–pathogen interaction. The developed CRISPR-Cas9 system can serve as a powerful tool to simplify the functional genomic studies in *C. difficile* such as virulence factor discovery as well as mechanism studies on host–bacteria interaction and pathogenesis. This will provide essential references for the development of effective strategies for CDI diagnostics and therapies.

Transparency declaration

This work was supported by the Start-up fund from Auburn University to YW. No external funding was received to support this research. The authors report no conflicts of interest.

Acknowledgements

We thank Dr. Shonna McBride (Emory University) for providing the *C. difficile* 630 strain, Dr. Mike Young (Aberystwyth University, UK) for proving *E. coli* CA434, Dr. Hans Blaschek (University of Illinois at Urbana-Champaign) for providing pYW34, and Dr. Nigel Minton (University of Nottingham, UK) for providing pMTL82151.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cmi.2018.03.026.

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