Using an Endogenous CRISPR-Cas System for Genome Editing in the Human Pathogen *Clostridium difficile*

Anna Maikova,^{a,b,c} Victor Kreis,^c Anaïs Boutserin,^c Konstantin Severinov,^{a,d} Olga Soutourina^c

^aSkoltech Center of Life Sciences, Skolkovo Institute of Science and Technology, Moscow, Russian Federation

^bSorbonne Paris Cité, Université Paris Diderot, Paris, France

^cInstitute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, Université Paris-Saclay, Gif-sur-Yvette, France

^dWaksman Institute for Microbiology, Rutgers, The State University of New Jersey, Piscataway, New Jersey, USA

ABSTRACT The human enteropathogen *Clostridium difficile* constitutes a key public health issue in industrialized countries. Many aspects of *C. difficile* pathophysiology and adaptation inside the host remain poorly understood. We have recently reported that this bacterium possesses an active CRISPR-Cas system of subtype I-B for defense against phages and other mobile genetic elements that could contribute to its success during infection. In this paper, we demonstrate that redirecting this endogenous CRISPR-Cas system toward autoimmunity allows efficient genome editing in *C. difficile*. We provide a detailed description of this newly developed approach and show, as a proof of principle, its efficient application for deletion of a specific gene in reference strain 630 Δ erm and in epidemic *C. difficile* strain R20291. The new method expands the arsenal of the currently limiting set of gene engineering tools available for investigation of *C. difficile* and may serve as the basis for new strategies to control *C. difficile* infections.

IMPORTANCE *Clostridium difficile* represents today a real danger for human and animal health. It is the leading cause of diarrhea associated with health care in adults in industrialized countries. The incidence of these infections continues to increase, and this trend is accentuated by the general aging of the population. Many questions about the mechanisms contributing to *C. difficile*'s success inside the host remain unanswered. The set of genetic tools available for this pathogen is limited, and new developments are badly needed. *C. difficile* has developed efficient defense systems that are directed against foreign DNA and that could contribute to its survival in phage-rich gut communities. We show how one such defense system, named CRISPR-Cas, can be hijacked for *C. difficile* genome editing. Our results also show a great potential for the use of the CRISPR-Cas system for the development of new therapeutic strategies against *C. difficile* infections.

KEYWORDS *Clostridium difficile*, CRISPR, endogenous subtype I-B CRISPR-Cas system, genome editing

The strictly anaerobic spore-forming bacterium *Clostridium difficile* (novel name, *Clostridioides difficile* [1]) is one of the major nosocomial pathogenic clostridia. This enteropathogen causes the majority of cases of antibiotic therapy-associated diarrhea and can lead to pseudomembranous colitis, a potentially lethal disease (2, 3). Over the last few decades, *C. difficile* infections have become one of the most important public health problems due to the emergence of hypervirulent strains (such as the PCR ribotype 027 R20291 strain) (4) and the increased incidence of *C. difficile* antibiotic resistance (5). The disruption of the colonic microflora caused by antibiotic therapy allows *C. difficile* to colonize the intestinal tract after the germination of preexisting or acquired spores (2, 6). Following gut colonization, *C. difficile* produces one or both of

Citation Maikova A, Kreis V, Boutserin A, Severinov K, Soutourina O. 2019. Using an endogenous CRISPR-Cas system for genome editing in the human pathogen *Clostridium difficile*. *Appl Environ Microbiol* 85:e01416-19. <https://doi.org/10.1128/AEM.01416-19>.

Editor Haruyuki Atomi, Kyoto University

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to Olga Soutourina, olga.soutourina@i2bc.paris-saclay.fr.

Received 24 June 2019

Accepted 6 August 2019

Accepted manuscript posted online 9 August 2019

Published 1 October 2019

the large toxins TcdA and TcdB. These toxins trigger alterations in the intestinal cell cytoskeleton, resulting in cell lysis and inflammation (3, 7). Many aspects of *C. difficile* pathogenesis, including the molecular mechanisms of the infection cycle, remain poorly understood. Therefore, it is important to develop new genome editing approaches for further investigations of this emerging human pathogen.

CRISPR (clustered regularly interspaced short palindromic repeat)-Cas (CRISPR-associated) systems protect bacteria and archaea from phages and other mobile genetic elements (8). These adaptive immunity systems are highly diverse (9) and have been discovered in half of the sequenced bacterial genomes and in almost all archaeal genomes (10). CRISPR-Cas systems comprise CRISPR arrays and *cas* gene operons. CRISPR arrays are arranged into short direct repeats (20 to 40 bp) separated by variable spacers. Some spacers are complementary to protospacers, which are sequences within phage and other mobile genetic element genomes (11). CRISPR arrays are transcribed from promoters localized in leader regions into long pre-CRISPR RNAs (pre-crRNAs). Pre-crRNAs are processed into small protective CRISPR RNAs (crRNAs). In complex with Cas proteins, crRNAs serve as guides to recognize and direct the cleavage of foreign genetic elements by Cas nucleases in a process known as “interference” (12). New spacers are acquired into CRISPR arrays from foreign genomes during the adaptation process (13).

For many CRISPR-Cas systems, an important component of immunity mechanism is a protospacer-adjacent motif (PAM). PAMs are short sequences located on the 3′ or 5′ end of the protospacer. PAMs are necessary for protospacer recognition, and they are absent in CRISPR arrays; this allows avoidance of autoimmunity (8).

According to a recent classification based on the *cas* genes involved in interference, the CRISPR-Cas systems are divided into two classes and are further subdivided into six types and 33 subtypes (9). Class 1 includes type I, III, and IV CRISPR-Cas systems, which are characterized by multisubunit effector complexes, while class 2 includes type II, V, and VI CRISPR-Cas systems, which carry single-protein effectors. Recent studies showed that *C. difficile* strains possess an active subtype I-B CRISPR-Cas system (14–17). The *C. difficile* CRISPR-Cas system is characterized by an unusually high number of CRISPR arrays (on average, 8.5 CRISPR arrays per genome, with some arrays being localized in prophages) (16) and the presence of two or three *cas* operons belonging to the same subtype (15, 17). In our previous studies, we demonstrated active expression of all CRISPR arrays for the *C. difficile* 630 and R20291 strains, as well as the ability of *C. difficile* 630 to mount robust CRISPR interference (14, 15). We also bioinformatically predicted and experimentally validated *C. difficile* CRISPR-Cas PAMs (15).

During the last few years, substantial efforts have been concentrated on the development of various CRISPR-based biotechnological tools (18). In particular, the type II Cas9- and type V Cpf1 (Cas12a)-based technologies are widely used for genome editing in different organisms (19, 20). Nevertheless, the application of other types of CRISPR-Cas systems has also attracted the attention of the scientific community. Harnessing of endogenous CRISPR-Cas systems for genome editing in bacteria and archaea appears to be a particularly attractive strategy (18, 21). This approach is based on the use of plasmid vectors containing artificial CRISPR miniarrays with spacers targeting a chromosomal gene (21). crRNAs expressed from a plasmid-borne miniarray utilize the endogenous Cas machinery to form an effector complex which recognizes the protospacer of choice, leading to its cleavage. Destruction of chromosomal DNA leads to the killing of wild-type cells (Fig. 1A). An editing plasmid with sequences homologous to sequences flanking the protospacer triggers homologous recombination and allelic exchange with the targeted chromosomal region (Fig. 1B). This results in elimination of the wild-type allele and preservation of chromosomal mutants since they no longer possess the targeted protospacer (Fig. 1B). The endogenous CRISPR-based method is often easier to set up for editing in prokaryotes than the CRISPR-Cas9 and CRISPR-Cpf1 (Cas12a) technologies. Another advantage of this approach is that there is no need to heterologously express potentially toxic Cas proteins inside bacterial or archaeal cells. The genome editing approach based on an endogenous CRISPR-

FIG 1 General scheme of using endogenous CRISPR-Cas systems for genome editing in bacteria and archaea. (A) The crRNA is expressed from a vector-borne CRISPR miniarray under the control of native or inducible promoters. The crRNA forms a ribonucleoprotein (crRNP) complex with endogenous Cas proteins, which recognizes and directs the cleavage of the PAM-associated protospacer, localized at the target chromosome region. This leads to chromosome disruption and cell death. (B) An editing plasmid, additionally carrying homologous arms (the left arm [LA] and the right arm [RA]), allows recombination between the plasmid and the chromosome to occur before CRISPR interference. The crRNP targets the PAM-protospacer on the plasmid, which leads to the elimination of the plasmid and preservation of the chromosomal mutants.

Cas system was successfully applied in several prokaryotic organisms using CRISPR-Cas subtype I-A and III-B or subtype I-B in archaea, *Sulfolobus islandicus* (21) and *Haloarcula hispanica* (22), respectively, and subtype I-B in several clostridial species, *C. pasteurianum* (23), *C. tyrobutyricum* (24), and *C. saccharoperbutylacetonicum* (25).

In *C. difficile*, various genetic tools for genome manipulation have been established. One of the most widely used methods is the ClosTron technology, based on mobile altered type II introns and the utilization of retrotransposable activated markers (RAM) (26, 27). Though this genome editing technique allows targeting of almost any chromosomal region and RAM enable one to easily identify potential mutants, the method has some disadvantages. Most importantly, ClosTron generates insertion mutations that may cause polar effects on downstream genes. An additional limitation comes from difficulties in finding an efficient insertion site within genes of a small size.

Another popular *C. difficile* genome editing approach is the allele-coupled exchange technique, based on a semisuicidal plasmid vector carrying the *Escherichia coli* cytosine deaminase gene (*codA*) or the *C. difficile* orotate phosphoribosyltransferase gene (*pyrE*) as a counterselection marker (28, 29). This method includes a two-step recombination event between the editing plasmid and the genome and the selection of double-crossing-over clones that lost the plasmid on nutrient-poor medium supplemented with 5-fluorocytosine (for *codA*-based plasmids) or fluoroorotic acid (for the *pyrE* allelic exchange system). The counterselection procedure is based on the generation of highly toxic compounds from these substrates. Despite the fact that this approach allows the creation of *C. difficile* mutants carrying point mutations, deletions, and insertions, it can

be difficult to apply in some cases. First, mutations that result in a growth deficiency phenotype or the inactivation of metabolic genes may affect growth on nutrient-poor medium. Second, there are some difficulties with losing the editing plasmids in mutant strains after editing, which could lead to the spontaneous creation of revertant strains.

Recently, a method based on the DNA double-strand breaks in *C. difficile* has been reported (30). This technology uses site-specific cleavage by the *Saccharomyces cerevisiae* yeast homing endonuclease I-SceI, whose recognition site is introduced in the editing plasmid vector. After the integration of the editing vector into the chromosome, another vector containing the I-SceI endonuclease gene under the control of a constitutive promoter is transferred to the single-crossing-over integrants to induce double-strand breaks and genome editing via homologous recombination. The advantage of this method is the possibility to create markerless deletions and the fast loss of the vector. Nevertheless, this method includes time-consuming two-step conjugations and the expression of I-SceI endonuclease, which could induce side effects.

During the past few years, the successful application of CRISPR-Cas9 and CRISPR-Cpf1 (Cas12a) for genome editing in *C. difficile* has been reported (31–34). These approaches have enhanced the possibilities of genetic manipulation in *C. difficile* and have proven to be efficient. However, the Cas9 and Cpf1 technologies require the design of plasmids harboring specific single guide RNAs (sgRNAs), and the editing plasmid is not automatically cured after the editing is complete. The use of an endogenous CRISPR-Cas system can enhance the possibilities of the genetic manipulation of *C. difficile*. The present work describes the utilization of a native *C. difficile* subtype I-B CRISPR-Cas system to generate deletion mutants in the 630 Δ erm and R20291 strains.

To evaluate the possibility of using an endogenous *C. difficile* CRISPR-Cas system for the targeting of specific sequences on the bacterial chromosome, we have chosen the *hfq* gene. Hfq is a bacterial RNA-binding protein that plays major roles in RNA metabolism and the global posttranscriptional network, in particular, in Gram-negative bacteria (35). The study of Hfq depletion in *C. difficile* 630 Δ erm (36) suggested a pleiotropic role of this protein in *C. difficile* physiology, with the most pronounced effect being on sporulation. The availability of an *hfq* deletion mutant would open new perspectives for further characterization of its role in RNA-based regulation in *C. difficile*. Our previous attempts to inactivate the *hfq* gene using a Clostron gene knockout system were unsuccessful (36). Additionally, we have tried to delete *hfq* using the *codA* allelic exchange approach (28, 29), but also without success (data not shown).

RESULTS

Construction of targeting miniarray plasmids and verification of their functionality. The general strategy for the construction of functional editing plasmids pECrFA_hfq630 and pECrPA_hfqR20291 for use in the 630 Δ erm and R20291 strains, respectively, is shown in Fig. 2. We first constructed two CRISPR miniarray plasmids targeting the *hfq* gene (pECrF_hfq and pECrP_hfq). The miniarray was based on the *C. difficile* 630 Δ erm CRISPR 16 array, which is highly expressed and capable of interference (15). Two variants of the leader sequence upstream of the miniarray were used (see Fig. S1A and B in the supplemental material): the full leader (a 403-bp sequence upstream of the first direct repeat of the CRISPR 16 array) containing all native promoters, which should allow autonomous expression of the miniarray (pECrF_hfq), and a partial leader (a 154-bp region upstream of the first direct repeat of the CRISPR 16 array), which lacked native promoters but which should allow the inducible expression of the miniarray from a vector-borne anhydrotetracycline (ATc)-inducible promoter (P_{tet}) (pECrP_hfq). The repeat-spacer-repeat motif of the synthetic miniarray was also based on 29-bp repeat sequences of the *C. difficile* 630 Δ erm CRISPR 16 array (Fig. 2A and Fig. S1A and B). For successful recognition of protospacers by the *C. difficile* CRISPR-Cas system, a functional PAM-flanking protospacer at the 5' end is necessary (15). Two functional trinucleotide PAMs of the *C. difficile* CRISPR-Cas system, 5' CCA and CCT, have been experimentally validated, and additional alternative motifs, such as CCC,

FIG 2 Strategy for the design of the editing plasmids to delete the *hfq* gene in the *C. difficile* 630 Δ erm and R20291 strains. (A) The coding sequences of the *C. difficile* 630 and R20291 *hfq* genes and a 37-bp sequence associated with the 5' CCT PAM, selected as a protospacer for the miniarray. (B) Construction of the pECrF_hfq and pECrP_hfq miniarray plasmids on the basis of the pRPF185 Δ gus vector. The miniarray sequences were cloned into the BamHI and XhoI restriction sites. (C) Construction of the pECrFA_hfq630, pECrPA_hfq630, and pECrPA_hfqR20291 editing plasmids on the basis of pECrF_hfq and pECrP_hfq. The F in the plasmid names represents the full-length leader region for

(Continued on next page)

FIG 3 Different effects of the conjugation of the miniarray and editing plasmids into *C. difficile* cells. (A) CRISPR self-cleavage induced by immediate expression of the miniarray from the plasmid pECrF_hfq after conjugation. (B) CRISPR self-cleavage resulted from the ATc-induced expression of the miniarray from the plasmid pECrP_hfq after second plating of the transconjugants. (C) CRISPR self-cleavage induced by the immediate expression of the miniarray from the pECrFA_hfq plasmid after conjugation. (D) Homologous recombination between the chromosome and the pECrPA_hfq plasmid and cleavage of the plasmid resulted from the ATc-induced expression of the miniarray from the plasmid after the second plating of the transconjugants. The effects were tested in the 630 Δ erm strain (B and C), and the effects were tested and gene deletion was performed in both the 630 Δ erm and R20291 strains (A and D).

CCG, and TCA, have been predicted (15). The coding region of the *hfq* gene possesses at least three functional CCW motifs and two alternative TCA motifs. A 37-bp sequence inside the *hfq* gene sequence associated with the 5' CCT PAM was chosen (Fig. 2A; the mean length of the *C. difficile* spacers is 37 bp). The pECrF_hfq and pECrP_hfq plasmids (Fig. 2B) were conjugated to *C. difficile* 630 Δ erm cells using the heat shock method to ensure the highest conjugation efficiency (37). No transconjugants were obtained after conjugation of the pECrF_hfq plasmid in *C. difficile* 630 Δ erm, suggesting CRISPR autoimmunity due to self-targeting (Fig. 3A). The conjugation efficiency of 380 transconjugants/ml was observed after conjugation with pECrP_hfq (approximately 1.9×10^{-6} transconjugants/donor or recipient cell). A control conjugation with the pRPF185 Δ gus vector resulted in 5,480 transconjugants/ml (approximately 27.4×10^{-6} transconjugants/donor or recipient cell). The smaller number of transconjugants in the pECrF_hfq conjugation reaction could be due to P_{tet} promoter leakage leading to autoimmunity caused by self-cleavage in some transconjugants. To check for the efficiency of self-targeting by crRNA expressed from the pECrP_hfq plasmid, eight transconjugant colonies were restreaked on brain heart infusion (BHI) agar plates supplemented with

FIG 2 Legend (Continued)

autonomous expression of the miniarray under the control of native promoters, while the P points out the presence of a partial leader region without native promoters for miniarray expression under the control of an inducible P_{tet} promoter. The presence of homologous arms for recombination within the 630 Δ erm or R20291 strain is indicated by A and the strain name. The pECrFA_hfq630 plasmid carrying the miniarray with the full-length leader region was not efficient for gene deletion in the 630 Δ erm strain; in contrast, pECrPA_hfq630 and pECrPA_hfqR20291 were efficiently used for *hfq* gene deletion in the 630 Δ erm and R20291 strains, respectively.

500 ng/ml ATc to fully induce the expression of the miniarray. No growth was observed on these plates, indicating highly efficient self-targeting by the induced miniarray (Fig. 3B). The same effects were observed after conjugation of the pECrF_hfq plasmid in *C. difficile* R20291 cells, suggesting that the synthetic array based on the *C. difficile* 630 Δ erm CRISPR 16 leader and repeat sequences mimics well native subtype I-B CRISPR arrays in *C. difficile* for at least the 630 and R20291 strains. Therefore, the *C. difficile* endogenous CRISPR-Cas system can recognize and target protospacers on the bacterial chromosome using crRNAs expressed from a plasmid-borne artificial miniarray, and this feature can be utilized for genome editing.

Construction of the genome editing plasmid and deletion of the *hfq* gene of *C. difficile* 630 Δ erm and R20291. We first assessed which miniarray plasmid, pECrF_hfq or pECrP_hfq, is best for *C. difficile* genome manipulation. Approximately 1,200-bp-long regions flanking the *hfq* gene of the 630 Δ erm strain (Fig. S1C) were amplified by PCR and introduced into the SmaI restriction sites of pECrF_hfq or pECrP_hfq using the Gibson assembly reaction (Fig. 2C). No transconjugants were obtained after conjugation of *C. difficile* 630 Δ erm with pECrFA_hfq630, carrying the miniarray with the full-length leader region (Fig. 3C). This may mean that the CRISPR-induced autoimmune degradation of DNA around the targeted protospacer is more efficient than homologous recombination between the chromosome and the homologous region of pECrFA_hfq630. Whatever the reason, the plasmid with the full-length CRISPR array leader sequence is clearly not suitable for genome editing. After conjugation with the pECrPA_hfq630 plasmid carrying the miniarray under the control of the inducible P_{tet} promoter, about 460 transconjugants/ml (approximately 2.3×10^{-6} transconjugants/donor or recipient cell) were obtained. To induce expression of the *hfq*-targeting miniarray, 10 transconjugants were restreaked on BHI agar supplemented with 500 ng/ml ATc. We observed the growth of each transconjugant tested, suggesting that homologous recombination between the chromosome and plasmid had occurred (Fig. 3D) or that CRISPR interference was not efficient. One clone from each plate was then restreaked on BHI plates with or without thiamphenicol (Tm) to check for plasmid loss. Three out of 10 clones lost the plasmid. When analyzed by PCR, these clones turned out to be Δ *hfq* mutants (Fig. 4A). The experiment was independently repeated at least three times. In all cases, when testing 6 to 10 clones, the mutant strains could be reproducibly obtained with an overall efficiency varying from 30% to 100%. Thus, a plasmid containing an inducibly transcribed CRISPR miniarray and arms for homologous recombination at the targeted protospacer allows efficient genome editing in *C. difficile*.

The coding region of the *hfq* gene of the *C. difficile* R20291 strain is identical to that of the 630 Δ erm strain, but the flanking sequences are different. Therefore, to delete the R20291 *hfq* gene, we constructed the pECrPA_hfqR20291 plasmid on the basis of the pECrP_hfq miniarray plasmid with homologous arms of R20291 *hfq* flanking sequences (Fig. 2C and Fig. S1D). Nine out of 10 selected transconjugants had lost the plasmid, and PCR analysis showed that seven out of nine clones without the plasmid were Δ *hfq* mutants (Fig. 4A).

Validation and complementation of *hfq* deletion strains. To validate the *hfq* deletion, we assessed *hfq* mRNA expression in the wild-type and Δ *hfq* mutant strains carrying an empty pRPF185 Δ gus vector (the wt/p and Δ *hfq*/p strains, respectively) as well as in complemented *C. difficile* Δ *hfq* strain Δ *hfq*/p-*hfq* expressing plasmid-borne *hfq*. Quantitative reverse transcription-PCR (qRT-PCR) analysis confirmed the absence of *hfq* expression in the *C. difficile* 630 Δ erm Δ *hfq* and R20291 Δ *hfq* strains and the presence of the transcript in the wild-type strains (Fig. 4B). A high 400- to 500-fold increase in *hfq* mRNA abundance compared to that in the wild type was detected in complemented strains due to strong P_{tet} induction in the presence of ATc (Fig. 4B). Western blotting with polyclonal anti-Hfq antibodies confirmed the lack of the Hfq protein in the Δ *hfq*/p strains (Fig. 4C; InstantBlue dye-stained protein gels, used as loading controls, are shown in Fig. S2).

FIG 4 Validation of *hfq* deletion mutants. (A) PCR analysis of the *C. difficile* clones which lost the plasmid after genome editing. The 2,151-bp PCR bands correspond to the wild-type genotype; the 1,893-bp PCR bands correspond to the mutant genotype. For the R20291 strain, both the wild-type and mutant copies were detected with clone 4 (lane 4); this clone was discarded from further analysis. Lanes m, molecular mass markers. (B) qRT-PCR analysis of the wild-type and Δ hfq mutant strains carrying an empty pRPF185 Δ gus (the wt/p and Δ hfq/p strains, respectively) and the complemented Δ hfq *C. difficile* strain (the Δ hfq/p-hfq strain). mRNA levels are relative to those of 16S rRNA. (C) Western blot analysis of the wt/p, Δ hfq-p, and Δ hfq/p-hfq *C. difficile* strains. As loading controls, InstantBlue dye-stained protein gels were used (see Fig. S2 in the supplemental material).

Sporulation assay of *C. difficile* 630 Δ erm Δ hfq mutants. Sporulation represents one of the crucial features of *C. difficile* as a successful pathogen. In our previous work, we revealed that the Hfq protein likely controls the sporulation rates in *C. difficile* 630 Δ erm-derived strains (36). The Hfq-depleted strain demonstrated higher levels of sporulation than the control strain. To analyze the effect of the *hfq* gene deletion on this phenotype, we compared the sporulation rates in the 630 Δ erm wt/p, Δ hfq/p, and Δ hfq/p-hfq strains. After 24 h and 48 h in BHIS medium supplemented with Tm and ATc, the mutant strain (the Δ hfq/p strain) demonstrated a higher level of sporulation than the wild-type strain (the wt/p strain) (Fig. 5). In addition, the complemented strain (the Δ hfq/p-hfq strain) showed a reversion of sporulation efficiency to a level close to that seen in the wild type (Fig. 5). Thus, these results are consistent with previously obtained data and confirm the potential involvement of the Hfq protein in the control of sporulation in *C. difficile* (36).

DISCUSSION

Over the last decade, the rapid development of various biotechnological tools based on prokaryotic adaptive immune CRISPR-Cas systems has occurred (18). In addition to the most popular CRISPR tools, based on class 2 Cas9 and Cpf1 (Cas12a) proteins (19, 20), other CRISPR-Cas systems are also being actively explored for genetic manipulation purposes. One of the most promising applications is the use of endogenous CRISPR-Cas systems for genome editing and engineering of bacteria and archaea (18, 21).

In the present work, we utilized the endogenous CRISPR-Cas system for genome editing of enteropathogenic *C. difficile*. Although other techniques for genome manipulation in this bacterium are available (26–34), they present some limitations in their

FIG 5 Sporulation levels in the *C. difficile* 630 Δ erm wt/p, Δhfq /p, and Δhfq /p-*hfq* strains (as numbers of spores) and the total amount of bacteria (as numbers of CFU) (control) after 24 h (A) and 48 h (B) of growth in BHI supplemented with Tm and ATc. The serial dilutions of the cultures spotted on BHI plates supplemented with taurocholate are indicated (ND, not diluted). Spore samples were heated to kill all cells other than spores, while the control samples were not heated to estimate the total amount of bacteria.

applications. Harnessing the native subtype I-B CRISPR-Cas system for genome editing in *C. difficile* allowed us to create deletion mutants of the *hfq* gene, encoding the RNA chaperone Hfq. Attempts to inactivate this gene using other approaches, including the ClosTron technology (36) and *codA* allelic exchange, were not successful (data not shown). Though a strain depleted of Hfq by expression of antisense RNA is available, the construction of the *hfq* deletion mutant opens up interesting possibilities for future studies of the regulatory role of Hfq and its RNA network in *C. difficile*.

The general work flow for the application of native CRISPR-Cas genome editing method in *C. difficile* is presented in Fig. 6. To repurpose the endogenous CRISPR-Cas system for deletion of the *hfq* gene, we designed plasmid vectors carrying targeting miniarray and editing plasmids carrying, in addition, homologous arms for recombination (Fig. 2B and C). The *C. difficile* 630 Δ erm CRISPR 16 array was chosen as a basis for synthetic miniarray construction, since it is functional for interference (15). The repeat-spacer-repeat motif for the artificial miniarray was composed of 29-bp repeat sequences and a 37-bp spacer sequence associated with a functional 5' CCT PAM inside the *hfq* gene coding region. To facilitate the genome editing procedure, we used the pECrPA_*hfq*630 plasmid containing the miniarray under the control of the inducible P_{tet} promoter. This strategy allowed us to successfully generate *hfq* deletion mutants in both *C. difficile* 630 Δ erm and epidemic R20291 strains. The CRISPR repeats in the 630 Δ erm and R20291 strains have similar consensus sequences (15). Moreover, both strains possess homologous complete and partial subtype I-B *cas* operons also present in the majority of sequenced *C. difficile* strains (15). The Cas machineries of the R20291 strain (and, by extension, those of other *C. difficile* isolates) can successfully recognize and utilize crRNAs expressed from a 630 Δ erm-based miniarray. Thus, the artificial miniarray designed from the *C. difficile* 630 Δ erm CRISPR 16 leader and repeat sequences is suitable for targeting specific chromosomal protospacer sequences and can be used for genome editing in at least two *C. difficile* strains. The general conservation of subtype I-B *cas* operons in *C. difficile* makes it likely that the same targeting arrays will be suitable for the majority of *C. difficile* strains, though this conjecture remains to be experimentally verified.

Repurposing of native CRISPR-Cas systems for genome editing in *C. difficile* has considerable advantages over other techniques applied to this bacterium. First of all,

FIG 6 General work flow for application of endogenous CRISPR-Cas-based genome editing method in *C. difficile*.

this method does not require the expression of heterologous proteins inside *C. difficile* cells, which may have toxic or other unpredictable effects. A miniarray localized on an editing plasmid mimics the natural *C. difficile* CRISPR array and should not have an undesirable impact during genome manipulation. Second, this approach includes only one conjugation round and fewer plating steps, giving significant time savings (Fig. 6). For example, the *codA* allelic exchange method requires at least three more colony plating steps than the method with the miniarray editing plasmid, increasing the time needed to complete the editing experiment by at least 3 days. Finally, the miniarray editing plasmid is readily lost after the editing process, preventing the spontaneous emergence of revertants.

Among the possible challenges for the application of the method could be the choice of the best protospacer on the target genome region. The presence of a functional PAM upstream of the protospacer is imperative for successful targeting. For this reason, the choice of the genome sequence for editing should be guided by the availability of PAMs. For the moment, only two PAMs (CCA and CCT) have been experimentally confirmed for *C. difficile* CRISPR-Cas target recognition (15). At the same time, general *in silico* analysis of CRISPR spacer homology to phage protospacers revealed a rather unconstrained PAM consensus CCN/TCN for the *C. difficile* CRISPR-Cas system (15). These data increase the possibilities of target sequence selection. In addition, type I CRISPR-Cas systems can recognize protospacers on both strands of the target DNA, which expands the opportunities to find functional PAMs in the target region (21).

The applications of endogenous CRISPR-Cas system for genome editing in *C. difficile* could be potentially larger than those for the generation of deletion mutants. This technique could be readily applied for introducing other types of mutations, i.e., point mutations and insertions (21). For a point mutation, the homologous arms on the editing plasmid could be designed to introduce changes in the functional PAMs at the editing region to a nonfunctional motif. Alternatively, substitutions could be introduced into a seed region, the first 8 nucleotides of the protospacer, crucial for CRISPR targeting (38). As a priority choice, a point mutation design could be achieved by introducing changes at the first or second position of PAMs. Combining the changes within PAMs and the seed region could even increase the efficiency of editing, as reported for other endogenous CRISPR editing tools (21, 25). We have previously shown that a nonfunctional PAM and mutation in the first position of protospacer within the seed region abolished or considerably impaired CRISPR interference (15). Genome insertions designed to make a break in the integrity of the chosen protospacer or/and PAM of the targeted genome sequence (21) or to insert a mutation to knock out the PAM (25) could be introduced by the homologous arms.

The role of essential genes cannot be easily investigated since no deletion mutant can be generated. Therefore, the CRISPRi method (utilizing CRISPR interference), which allows repression of the expression of target genes, has recently been developed (39). This technology is primarily based on CRISPR-Cas9 systems with a mutated catalytic site of the Cas9 protein (catalytically dead Cas9 [dCas9]) (40). The dCas9-based method has already been used in *C. difficile* (41). In addition, it was shown that an *E. coli* native subtype I-E CRISPR-Cas system lacking *cas3* could be repurposed for programmable transcriptional repression (42). Furthermore, a recent study showed that the subtype I-B CRISPR-Cas system of *Haloferax volcanii* lacking the *cas3* and *cas6* genes could be used for gene repression in this archaeon (43). Altogether, these data suggest that the *C. difficile* native CRISPR-Cas system may be used for this goal, too, in a particular context. However, about 90% of the sequenced *C. difficile* strains possess two subtype I-B *cas* operons, each carrying the *cas3* nuclease gene. An additional partial *cas* operon with the *cas3* gene is present in the majority of the multilocus sequence type 3 group of *C. difficile* strains, including the PCR ribotype 027 strains (15). Thus, depending on the strain, the creation of a double- or triple-*cas3*-mutant background would be necessary to consider application of this CRISPRi method.

CRISPR self-targeting could lead to bacterial cell death. This feature of CRISPR-Cas systems can be applied for the development of new antimicrobial agents (44). Among the suggested strategies reside the use of phage particles and phagemids as vectors to deliver all the necessary autotargeting CRISPR-Cas components inside the cell of the targeted pathogen (44). In the present study, we showed the active killing of *C. difficile* cells by CRISPR self-targeting via expression of the miniarray from a plasmid vector. Therefore, in perspective, this approach could be promising for the future development of alternative strategies for the treatment of *C. difficile* infections.

In conclusion, the repurposing of the endogenous CRISPR-Cas system for genome editing in *C. difficile* extends the range of biotechnological techniques available for this enteropathogenic bacterium and could be valuable in further studies.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
<i>E. coli</i>		
NEB-10 beta	$\Delta(\text{ara-leu})7697 \text{ araD139 fhuA } \Delta\text{lacX74 galk16 galE15 e14 mutant } \phi80\text{dlacZ}\Delta\text{M15}$	New England Biolabs
HB101(RP4)	$\text{recA1 relA1 endA1 nupG rpsL (Str}^r\text{) rph spoT1 } \Delta(\text{mrr hsdRMS-mcrBC})$ $\text{supE44 aa14 galk2 lacY1 } \Delta(\text{gpt-proA})62 \text{ rpsL20 (Str}^r\text{) xyl-5 mtl-1 recA13}$ $\Delta(\text{mcrC-mrr}) \text{ hsdS}_{\text{B}}(\text{r}_{\text{B}}^- \text{ m}_{\text{B}}^-)$ RP4 ($\text{Tra}^+ \text{ IncP Ap}^r \text{ Km}^r \text{ Tc}^r$)	Laboratory stock
<i>C. difficile</i>		
630 Δerm	Sequenced reference strain, ΔermB	Laboratory stock (52)
R20291	PCR ribotype 027 epidemic strain	Laboratory stock
wt/p	630 Δerm or R20291 carrying the pRPF Δgus plasmid	This work
$\Delta\text{hfq/p}$	630 $\Delta\text{erm } \Delta\text{hfq}$ or R20291 Δhfq carrying the pRPF Δgus plasmid	This work
$\Delta\text{hfq/p-hfq}$	630 $\Delta\text{erm } \Delta\text{hfq}$ or R20291 Δhfq carrying the p-hfq plasmid	This work
Plasmid		
pRPF185 Δgus	pRPF185 Δgus vector derivative	14, 47
pECrF_hfq	pRPF185 Δgus carrying the hfq gene targeting the CRISPR miniarray with the full leader sequence	This work
pECrP_hfq	pRPF185 Δgus carrying the hfq gene targeting the CRISPR miniarray with the partial leader sequence under the control of the P_{tet} promoter	This work
pECrFA_hfq630	pECrF_hfq carrying arms for recombination in the 630 Δerm strain	This work
pECrPA_hfq630	pECrP_hfq carrying arms for recombination in the 630 Δerm strain	This work
pECrPA_hfqR20291	pECrP_hfq carrying arms for recombination in the R20291 strain	This work
p-hfq	pRPF185 Δgus carrying the hfq gene under the control of the P_{tet} promoter	This work

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All the plasmids and bacterial strains used in this study are listed in Table 1. *C. difficile* strains were grown in brain heart infusion (BHI; Difco) or tryptone, yeast extract (TY) (45) medium at 37°C under anaerobic conditions (5% H₂, 5% CO₂, 90% N₂) in an anaerobic chamber (Jacomex). BHI medium supplemented with yeast extract (5 mg/ml) and L-cysteine (0.1%) (BHIS) was used in the sporulation experiments. When needed, thiamphenicol (Tm) at a final concentration of 15 µg/ml was added to the *C. difficile* cultures. The *E. coli* strains (Table 1) were grown in LB medium (46) supplemented with ampicillin (100 µg/ml) and chloramphenicol (15 µg/ml) when it was suitable. The nonantibiotic analog anhydrotetracycline (ATc) was used for induction of the P_{tet} promoter of pRPF185 vector derivatives in *C. difficile* (47).

Plasmid construction and conjugation into *C. difficile*. All the oligonucleotides used in this work are listed in Table 2. To create artificial CRISPR miniarrays targeting the *C. difficile* hfq gene, the full leader sequence (positions –403 to –1 relative to the first nucleotide of the first repeat in the array) and a partial leader sequence (positions –154 to –1 relative to the first nucleotide of the first repeat in the array) of the *C. difficile* 630 Δerm CRISPR 16 array were amplified by PCR of genomic DNA (see Fig. S1A and B in the supplemental material). The artificial repeat-spacer-repeat motif was amplified by PCR from synthetic oligonucleotides to generate the double-stranded fragment. The full or partial leader sequence and the repeat-spacer-repeat motif were assembled and cloned into the BamHI and XhoI sites of the pRPF185 Δgus plasmid vector (14) using the Gibson assembly reaction (48), giving the pECrF_hfq and pECrP_hfq miniarray plasmids (Fig. 2B).

To construct editing plasmids, approximately 1,200-bp-long regions flanking the hfq gene of the 630 Δerm and R20291 strains (Fig. S1C and D) were amplified by PCR and introduced into the SmaI restriction site of pECrF_hfq or pECrP_hfq using the Gibson assembly reaction, resulting in the pECrFA_hfq630, pECrPA_hfq630, and pECrPA_hfqR20291 plasmids (Fig. 2C).

To construct a plasmid for complementation of the hfq deletion, the hfq gene sequence, including the ribosome-binding site (positions –50 to +397 relative to the translational start site), was amplified by PCR and cloned into the StuI and BamHI sites of pRPF185 Δgus under the control of the ATc-inducible P_{tet} promoter, giving the p-hfq plasmid.

DNA sequencing was performed to verify the plasmid constructs. pRPF185 Δgus is a shuttle vector that replicates both in *E. coli* (ColE1 origin) and in *C. difficile*. All resulting plasmids were transformed into the *E. coli* HB101(RP4) strain and further transferred into *C. difficile* cells by conjugation. The heat shock method with incubation for 15 min at 50°C was used to get the highest conjugation efficiency (37). *C. difficile* transconjugants were selected on BHI agar containing Tm (15 µg/ml), D-cycloserine (25 µg/ml), and cefoxitin (8 µg/ml).

Deletion of the hfq gene and validation of Δhfq mutants. To induce the expression of the CRISPR miniarrays under the control of the P_{tet} promoter, *C. difficile* transconjugants containing the pECrP_hfq, pECrPA_hfq630, or pECrPA_hfqR20291 plasmid were subsequently restreaked onto BHI agar supplemented with ATc (500 ng/ml). The resulting *C. difficile* colonies were then restreaked in parallel onto BHI agar supplemented or not with Tm (15 µg/ml) to check for plasmid loss. Subsequently, selected clones without plasmids were analyzed by PCR to detect the chromosomal deletion of the hfq gene. The resulting PCR fragments were sequenced to confirm the gene deletion.

TABLE 2 Oligonucleotides used in this study

Primer purpose and name	Sequence (5'–3') ^a	Description ^b
Construction of CRISPR miniarray plasmid		
AM81	TAACAGATCTGAGCTCCAGGCCCTT CAATTATATGATAGGTTTTTTATTAAGCATACTAGCTGGTGTATATC	Full leader CR16 F
AM82	GTTAATCTAAAC CCCCAAATAAAGCTTAGTATTTCCAATATCTACACATACAC	Leader CR16-R
AM83	TAACAGATCTGAGCTCCAGGCCCTT CTGAGCAATATTTGCGATAAATTGAAGTTTAAACAATTG	Partial leader CR16-F
AM91	GTTTAGATTAAGTATATGGAATGTAATGTTACTATATATTTAGTTAATGGAGTGCAAGTTAAAGGTT TTAGATTAAGTATATGGAATGTAAT	<i>hfq</i> repeat-spacer-repeat motif
AM92	AGTTTATTTTGGG GTTTTAGATTAAGTATATGGAATGTAATGTTACTATATATTTAGTTAATGGAGTG	Repeat-spacer-repeat F
AM93	TTTAAAGTTTATTA AAACTTATAGATTACATCCATATAGTTAATCTAAACCTTTAACTTGCAC	Repeat-spacer-repeat R
Construction of editing plasmids		
AM158	GAACACTTGCCGAAAAAGAAAACTGCCGGGTACGTACCCG ATATTGAAATAAAAAAGTTTATTG	Left arm 630 and R20291 F
AM159	TCTTAAATTAATAATTAT TAGATTGTACCTCCCAAG	Left arm 630 and R20291 R
AM160	CTTGGGAGGGTACAAATCTA ATAATTAATTTAATTAAGATGATTGAG	Right arm 630 and R20291 F
AM161	GAGCGAGGAAGCGGAAGAGCGCTCGCGGGGATCGATCCCG GAACAGGTTTTACATAAGAATC	Right arm 630 and R20291 R
Δhfq mutant detection		
AM106	ACTAAAAGGGTCATAAGAGC	Δhfq F
AM169	TATAAGGAGGCTTATTGGAGC	Δhfq R
Construction of plasmids for complementation		
HFQ1	GAAGGCTGGTAGGAATTTTAGAAGT	5' <i>hfq</i> <i>Stu</i> I
HFQ2	GGGGATCCCATTAAGCATTTTATCACCTGTC	3' <i>hfq</i> <i>Bam</i> HI
qRT-PCR		
QRTBD37	GGGAGACTTGAGTGCAGGAG	16S RNA F
QRTBD38	GTGCCTCAGCGTCAGTTACA	16S RNA R
IMV447	AGGGCTTGTAAGGGGTTTG	qRT-PCR <i>hfq</i> F
IMV448	TTGTTGTTTGGTTTGAATTGTT	qRT-PCR <i>hfq</i> R

^aOverlapping regions are indicated in boldface, and underlined sequences represent those of the restriction endonucleases.^bCR16, CRISPR 16 array; F, forward; R, reverse.

RNA extraction and qRT-PCR. For total RNA extraction, *C. difficile* 630 Δ erm- and R20291-derived pRPF185 Δ gus- and p-*hfq*-carrying strains were grown for 6 h or 8 h in TY medium supplemented with Tm (7.5 μ g/ml) and Atc (250 ng/ml). Total RNA isolation was performed as previously described (49). cDNA synthesis by reverse transcription and quantitative reverse transcription PCR (qRT-PCR) was performed as previously described (50) using a Bio-Rad CFX Connect real-time system. The expression level of the *hfq* gene relative to that of the 16S RNA gene was calculated (51).

Protein extract preparation and Western blotting. To extract total proteins, *C. difficile* 630 Δ erm- and R20291-derived pRPF185 Δ gus- and p-*hfq*-carrying strains were grown for 6 h or 16 h in TY medium supplemented with Tm (7.5 μ g/ml) and Atc (250 ng/ml). Cell lysis and protein extraction were performed as previously described (36).

For each sample, 30 μ g of protein extract was loaded onto two 15% SDS polyacrylamide gels in parallel. After the electrophoresis, proteins from the 1st gel were transferred to a polyvinylidene fluoride membrane. Membrane hybridization with primary and secondary antibodies was then performed as described before (36). The bioluminescent signal from the secondary antibodies was detected using the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and a Fusion FX (Vilber Lourmat) digital camera. The 2nd gel was stained with InstantBlue dye (Expedeon) and used as a loading control (Fig. S2).

Sporulation assay. *C. difficile* strains harboring the pRPF185 Δ gus and p-*hfq* plasmids were grown overnight in TY medium containing Tm (15 μ g/ml). Overnight cultures were used to inoculate the strain at an optical density at 600 nm (OD₆₀₀) of 0.1 in fresh TY medium supplemented with taurocholate (0.1%), D-fructose (0.5%), Tm (7.5 μ g/ml), and Atc (10 ng/ml) to get only vegetative cells. When the cultures had reached an OD₆₀₀ of 1.0 to 1.5, they were diluted to an OD₆₀₀ of 0.01 in BHIS medium containing Tm (7.5 μ g/ml) and Atc (10 ng/ml) and grown at 37°C. After 24 h and 48 h of growth, 1 ml of each culture was divided into two samples. To determine the total amount of bacteria (in number of CFU), the first sample was serially diluted and spotted (10 μ l per spot) onto BHI agar containing 0.1% taurocholate. The second sample was incubated at 65°C for 30 min to eliminate vegetative cells. Subsequently, the sample was serially diluted and spotted (10 μ l per spot) onto BHI agar containing 0.1% taurocholate to estimate the number of spores.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01416-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

This work was supported by grants from the Agence Nationale de la Recherche (CloSTARn; grant ANR-13-JSV3-0005-01 to O.S.), the Institut Universitaire de France (to O.S.), the University Paris-Sud, the Institute for Integrative Biology of the Cell, the DIM-1HEALTH regional Ile de France program (LSP grant no. 164466), the CNRS-RFBR PRC 2019 (grant no. 288426) to O.S. and K.S., a Vernadski fellowship to A.M., and a Skoltech Biomedical Initiative grant (grant no. SBI RF-0000000136) to K.S.

We are grateful to P. Boudry, E. Semenova, and J. Peltier for helpful discussions during the preparation of the manuscript.

REFERENCES

- Oren A, Rupnik M. 2018. *Clostridium difficile* and *Clostridioides difficile*: two validly published and correct names. *Anaerobe* 52:125–126. <https://doi.org/10.1016/j.anaerobe.2018.07.005>.
- Rupnik M, Wilcox MH, Gerding DN. 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nat Rev Microbiol* 7:526–536. <https://doi.org/10.1038/nrmicro2164>.
- Abt MC, McKenney PT, Pamer EG. 2016. *Clostridium difficile* colitis: pathogenesis and host defence. *Nat Rev Microbiol* 14:609–620. <https://doi.org/10.1038/nrmicro.2016.108>.
- Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC. 2005. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* 366:1079–1084. [https://doi.org/10.1016/S0140-6736\(05\)67420-X](https://doi.org/10.1016/S0140-6736(05)67420-X).
- Banawas SS. 2018. *Clostridium difficile* infections: a global overview of drug sensitivity and resistance mechanisms. *Biomed Res Int* 2018: 8414257. <https://doi.org/10.1155/2018/8414257>.
- Seekatz AM, Young VB. 2014. *Clostridium difficile* and the microbiota. *J Clin Invest* 124:4182–4189. <https://doi.org/10.1172/JCI72336>.
- Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M, Aktories K. 1995. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* 375:500–503. <https://doi.org/10.1038/375500a0>.
- Sorek R, Lawrence CM, Wiedenheft B. 2013. CRISPR-mediated adaptive immune systems in bacteria and archaea. *Annu Rev Biochem* 82: 237–266. <https://doi.org/10.1146/annurev-biochem-072911-172315>.
- Koonin EV, Makarova KS, Zhang F. 2017. Diversity, classification and evolution of CRISPR-Cas systems. *Curr Opin Microbiol* 37:67–78. <https://doi.org/10.1016/j.mib.2017.05.008>.
- Grissa I, Vergnaud G, Pourcel C. 2007. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. *BMC Bioinformatics* 8:172. <https://doi.org/10.1186/1471-2105-8-172>.
- Shmakov SA, Sitnik V, Makarova KS, Wolf YI, Severinov KV, Koonin EV. 2017. The CRISPR spacer space is dominated by sequences from species-specific mobilomes. *mBio* 8:e01397–17. <https://doi.org/10.1128/mBio.01397-17>.
- Garneau JE, Dupuis M-V, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH, Moineau S. 2010. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 468:67–71. <https://doi.org/10.1038/nature09523>.
- Marraffini LA. 2015. CRISPR-Cas immunity in prokaryotes. *Nature* 526: 55–61. <https://doi.org/10.1038/nature15386>.
- Soutourina O, Monot M, Boudry P, Saujet L, Pichon C, Sismeiro O, Semenova E, Severinov K, Le Bouguenec C, Coppée JY, Dupuy B, Martin-Verstraete I. 2013. Genome-wide identification of regulatory RNAs in the human pathogen *Clostridium difficile*. *PLoS Genet* 9:e1003493. <https://doi.org/10.1371/journal.pgen.1003493>.
- Boudry P, Semenova E, Monot M, Datsenko KA, Lopatina A, Sekulovic O, Ospina-Bedoya M, Fortier LC, Severinov K, Dupuy B, Soutourina O. 2015. Function of the CRISPR-Cas system of the human pathogen *Clostridium difficile*. *mBio* 6:e01112–15. <https://doi.org/10.1128/mBio.01112-15>.
- Andersen JM, Shoup M, Robinson C, Britton R, Olsen KEP, Barrangou R. 2016. CRISPR diversity and microevolution in *Clostridium difficile*. *Genome Biol Evol* 8:2841–2855. <https://doi.org/10.1093/gbe/evw203>.
- Hargreaves KR, Flores CO, Lawley TD, Clokie RJ, Trevor D, Hargreaves KR, Flores CO, Lawley TD, Clokie RJ. 2014. Abundant and diverse clustered regularly interspaced short palindromic repeat spacers in *Clostridium difficile* strains and prophages target multiple phage types within this pathogen. *mBio* 5:e01045–13. <https://doi.org/10.1128/mBio.01045-13>.
- Barrangou R, Horvath P. 2017. A decade of discovery: CRISPR functions and applications. *Nat Microbiol* 2:17092. <https://doi.org/10.1038/nmicrobiol.2017.92>.
- Hsu PD, Lander ES, Zhang F. 2014. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157:1262–1278. <https://doi.org/10.1016/j.cell.2014.05.010>.
- Safari F, Zare K, Negahdaripour M, Barekati-Mowahed M, Ghasemi Y. 2019. CRISPR Cpf1 proteins: structure, function and implications for genome editing. *Cell Biosci* 9:36. <https://doi.org/10.1186/s13578-019-0298-7>.
- Li Y, Pan S, Zhang Y, Ren M, Feng M, Peng N, Chen L, Liang YX, She Q. 2016. Harnessing type I and type III CRISPR-Cas systems for genome editing. *Nucleic Acids Res* 44:e34. <https://doi.org/10.1093/nar/gkv1044>.
- Cheng F, Gong L, Zhao D, Yang H, Zhou J, Li M, Xiang H. 2017. Harnessing the native type I-B CRISPR-Cas for genome editing in a polyploid archaeon. *J Genet Genomics* 44:541–548. <https://doi.org/10.1016/j.jgg.2017.09.010>.
- Pyne ME, Bruder MR, Moo-Young M, Chung DA, Chou CP. 2016. Harnessing heterologous and endogenous CRISPR-Cas machineries for efficient markerless genome editing in *Clostridium*. *Sci Rep* 6:25666. <https://doi.org/10.1038/srep25666>.
- Zhang J, Zong W, Hong W, Zhang Z-T, Wang Y. 2018. Exploiting endogenous CRISPR-Cas system for multiplex genome editing in *Clostridium tyrobutyricum* and engineer the strain for high-level butanol production. *Metab Eng* 47:49–59. <https://doi.org/10.1016/j.ymben.2018.03.007>.
- Atmadjaja AN, Holby V, Harding AJ, Krabben P, Smith HK, Jenkinson ER. 2019. CRISPR-Cas, a highly effective tool for genome editing in *Clostridium saccharoperbutylacetonicum* N1-4(HMT). *FEMS Microbiol Lett* 366: fnz059. <https://doi.org/10.1093/femsle/fnz059>.
- Heap JT, Kuehne SA, Ehsaan M, Cartman ST, Cooksley CM, Scott JC, Minton NP. 2010. The Clostron: mutagenesis in *Clostridium* refined and streamlined. *J Microbiol Methods* 80:49–55. <https://doi.org/10.1016/j.mimet.2009.10.018>.
- Kuehne SA, Heap JT, Cooksley CM, Cartman ST, Minton NP. 2011. Clostron-mediated engineering of *Clostridium*. *Methods Mol Biol* 765: 389–407. https://doi.org/10.1007/978-1-61779-197-0_23.
- Cartman ST, Kelly ML, Heeg D, Heap JT, Minton NP. 2012. Precise manipulation of the *Clostridium difficile* chromosome reveals a lack of association between the *tcdC* genotype and toxin production. *Appl Environ Microbiol* 78:4683–4690. <https://doi.org/10.1128/AEM.00249-12>.
- Ng YK, Ehsaan M, Philip S, Coltery MM, Janoir C, Collignon A, Cartman ST, Minton NP. 2013. Expanding the repertoire of gene tools for precise manipulation of the *Clostridium difficile* genome: allelic exchange using *pyrE* alleles. *PLoS One* 8:e56051. <https://doi.org/10.1371/journal.pone.0056051>.
- Theophilou ES, Vohra P, Gallagher MP, Poxton IR, Blakely GW. 2018. Generation of markerless deletions in the nosocomial pathogen *Clostridium difficile* by induction of DNA double-strand breaks. *Appl Environ Microbiol* 85:e02055–18. <https://doi.org/10.1128/AEM.02055-18>.
- McAllister KN, Bouillaut L, Kahn JN, Self WT, Sorg JA. 2017. Using CRISPR-Cas9-mediated genome editing to generate *C. difficile* mutants defective in selenoproteins synthesis. *Sci Rep* 7:14672. <https://doi.org/10.1038/s41598-017-15236-5>.
- Ingle P, Groothuis D, Rowe P, Huang H, Cockayne A, Kuehne SA, Jiang W, Gu Y, Humphreys CM, Minton NP. 2019. Generation of a fully erythromycin-sensitive strain of *Clostridioides difficile* using a novel CRISPR-Cas9 genome editing system. *Sci Rep* 9:8123. <https://doi.org/10.1038/s41598-019-44458-y>.

33. Inés I, Cañ C, Groothuis D, Zygouropoulou M, Rodrigues R, Minton NP. 2019. RiboCas: a universal CRISPR-based editing tool for *Clostridium*. *ACS Synth Biol* 8:1379–1390. <https://doi.org/10.1021/acssynbio.9b00075>.
34. Hong W, Zhang J, Cui G, Wang L, Wang Y. 2018. Multiplexed CRISPR-Cpf1-mediated genome editing in *Clostridium difficile* toward the understanding of pathogenesis of *C. difficile* infection. *ACS Synth Biol* 7:1588–1600. <https://doi.org/10.1021/acssynbio.8b00087>.
35. Sobrero P, Valverde C. 2012. The bacterial protein Hfq: much more than a mere RNA-binding factor. *Crit Rev Microbiol* 38:276–299. <https://doi.org/10.3109/1040841X.2012.664540>.
36. Boudry P, Gracia C, Monot M, Caillet J, Saujet L, Hajnsdorf E, Dupuy B, Martin-Verstraete I, Soutourina O. 2014. Pleiotropic role of the RNA chaperone protein Hfq in the human pathogen *Clostridium difficile*. *J Bacteriol* 196:3234–3248. <https://doi.org/10.1128/JB.01923-14>.
37. Kirk JA, Fagan RP. 2016. Heat shock increases conjugation efficiency in *Clostridium difficile*. *Anaerobe* 42:1–5. <https://doi.org/10.1016/j.anaerobe.2016.06.009>.
38. Semenova E, Jore MM, Datsenko KA, Semenova A, Westra ER, Wanner B, van der Oost J, Brouns SJJ, Severinov K. 2011. Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. *Proc Natl Acad Sci U S A* 108:10098–10103. <https://doi.org/10.1073/pnas.1104144108>.
39. Peters JM, Colavin A, Shi H, Czarny TL, Larson MH, Wong S, Hawkins JS, Lu CHS, Koo BM, Marta E, Shiver AL, Whitehead EH, Weissman JS, Brown ED, Qi LS, Huang KC, Gross C. 2016. A comprehensive, CRISPR-based functional analysis of essential genes in bacteria. *Cell* 165:1493–1506. <https://doi.org/10.1016/j.cell.2016.05.003>.
40. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA. 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152:1173–1183. <https://doi.org/10.1016/j.cell.2013.02.022>.
41. Müh U, Pannullo AG, Weiss DS, Ellermeier CD. 2019. A xylose-inducible expression system and a CRISPRi-plasmid for targeted knock-down of gene expression in *Clostridioides difficile*. *J Bacteriol* 201:e00711–18. <https://doi.org/10.1128/JB.00711-18>.
42. Luo ML, Mullis AS, Leenay RT, Beisel CL. 2015. Repurposing endogenous type I CRISPR-Cas systems for programmable gene repression. *Nucleic Acids Res* 43:674–681. <https://doi.org/10.1093/nar/gku971>.
43. Stachler A-E, Marchfelder A. 2016. Gene repression in *Haloarchaea* using the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas I-B system. *J Biol Chem* 291:15226–15242. <https://doi.org/10.1074/jbc.M116.724062>.
44. Bikard D, Barrangou R. 2017. Using CRISPR-Cas systems as antimicrobials. *Curr Opin Microbiol* 37:155–160. <https://doi.org/10.1016/j.mib.2017.08.005>.
45. Dupuy B, Sonenshein AL. 1998. Regulated transcription of *Clostridium difficile* toxin genes. *Mol Microbiol* 27:107–120. <https://doi.org/10.1046/j.1365-2958.1998.00663.x>.
46. Bertani G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* 62:293–300.
47. Fagan RP, Fairweather NF. 2011. *Clostridium difficile* has two parallel and essential Sec secretion systems. *J Biol Chem* 286:27483–27493. <https://doi.org/10.1074/jbc.M111.263889>.
48. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6:343–345. <https://doi.org/10.1038/nmeth.1318>.
49. André G, Even S, Putzer H, Burguière P, Croux C, Danchin A, Martin-Verstraete I, Soutourina O. 2008. S-box and T-box riboswitches and antisense RNA control a sulfur metabolic operon of *Clostridium acetobutylicum*. *Nucleic Acids Res* 36:5955–5969. <https://doi.org/10.1093/nar/gkn601>.
50. Saujet L, Monot M, Dupuy B, Soutourina O, Martin-Verstraete I. 2011. The key sigma factor of transition phase, SigH, controls sporulation, metabolism, and virulence factor expression in *Clostridium difficile*. *J Bacteriol* 193:3186–3196. <https://doi.org/10.1128/JB.00272-11>.
51. Metcalf D, Sharif S, Weese JS. 2010. Evaluation of candidate reference genes in *Clostridium difficile* for gene expression normalization. *Anaerobe* 16:439–443. <https://doi.org/10.1016/j.anaerobe.2010.06.007>.
52. Hussain HA, Roberts AP, Mullany P. 2005. Generation of an erythromycin-sensitive derivative of *Clostridium difficile* strain 630 (630Δ*erm*) and demonstration that the conjugative transposon Tn916ΔE enters the genome of this strain at multiple sites. *J Med Microbiol* 54:137–141. <https://doi.org/10.1099/jmm.0.45790-0>.