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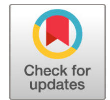
MEETING REVIEW



Expanding the *Clostridioides difficile* Genetics Toolbox

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ABSTRACT *Clostridioides difficile* genetics has rapidly advanced in recent years thanks to the development of tools for allelic replacement and transposon mutagenesis. In this *Journal of Bacteriology* issue, Müh et al. extend the genetics toolbox by developing a CRISPR interference (CRISPRi) strategy for gene silencing in *C. difficile* (U. Müh, A. G. Pannullo, D. S. Weiss, and C. D. Ellermeier, 2019, J Bacteriol 201: e00711-18. https://doi.org/10.1128/JB.00711-18). The authors demonstrate the tunability and robustness of their CRISPRi system, highlight its utility in studying essential gene function, and discuss exciting new possibilities for dissecting *C. difficile* physiology.

KEYWORDS CRISPRi, *Clostridioides difficile*, genetics

Clostridioides (formerly *Clostridium*) *difficile* has long been considered a genetically intractable organism. Early methods for genetically manipulating *C. difficile* were relatively limited, with the first plasmids being conjugated into *C. difficile* in 2002 (1, 2) and the first gene disruption mutant being constructed in 2006 (3). The development of a TargeTron-based gene disruption system (ClosTron [4]) brought *C. difficile* genetics out of the Dark Ages, but the off-target effects of this system for some genes limited its utility, and ClosTron disruption was typically limited to a single gene. Around this time, *C. difficile* was emerging as a major nosocomial pathogen, stimulating a small group of dedicated researchers to usher in a Renaissance in *C. difficile* genetics. These efforts led to the development of methods for inducing gene expression (5, 6), silencing gene expression using antisense methods (5, 7), conducting genome-wide transposon screens (8), constructing multiple and precise gene deletions using allelic exchange (9–11), and, most recently, using CRISPR-based methods to repress gene expression (12, 13).

Development of a CRISPR-based gene silencing system in *C. difficile*. While these methods have transformed the ability of researchers to study *C. difficile*, most of the methods for manipulating the chromosome have been limited to the most genetically tractable strain of *C. difficile*, 630Δ*erm* (14), which derives from a clinical isolate (15) but is less pathogenic than the epidemic, so-called "hypervirulent," strains (16, 17). A recent report by Müh et al. in this *Journal of Bacteriology* issue describes the development of a CRISPR interference (CRISPRi) method that promises to overcome some of these limitations and opens up exciting new possibilities for studying *C. difficile* biology (18).

Müh et al. (18) show that their optimized CRISPRi system can be used to study the function of multiple essential genes while also bypassing the lengthy and often difficult process of constructing mutants in the epidemic strain R20291 (19). They demonstrate that their CRISPRi system is inducible, titratable, highly effective at repressing gene expression (20- to 100-fold), and robust, achieving tight repression with all 8 guide RNAs tested and no off-target effects being observed.

They based their CRISPRi method off a system developed for *Bacillus subtilis* (20) that involves constitutively expressing a single guide RNA (sgRNA) to target a nuclease-deactivated mutant of Cas9 (dCas9) to bind a specific gene sequence and block transcription by RNA polymerase. The targeted dCas9 can be used to repress down-

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stream as well as upstream gene transcription of operons in the *B. subtilis* system (20) and, thus, to study the function of operons as well as individual genes.

To tune the level of dCas9-mediated gene silencing, Müh et al. placed a codon-optimized *dCas9* gene, derived from *Streptococcus pyogenes*, under the control of a xylose-inducible promoter (P_{xyl}), similar to the *B. subtilis* system (20). This xylose-inducible system uses the xylose repressor XylR to repress the transcription of the P_{xyl} promoter in the absence of xylose, while the addition of xylose alleviates repression and induces gene expression. Using *mCherry* as a reporter gene, the authors demonstrate that the xylose-inducible system induces titratable, uniform gene expression and is only mildly affected by catabolite repression.

To maximize the expression of the sgRNA, the authors identified P_{gdh} as an optimal promoter for driving sgRNA-mediated transcriptional repression. The sgRNA and *dCas9* genes were cloned into a *C. difficile-Escherichia coli* shuttle plasmid vector (5) and conjugated into R20291. By simply exchanging the sgRNA encoded, the authors knocked down the expression of three genes predicted to play an important role in the biogenesis and integrity of the cell envelope (8). They validated the ability of their system for studying gene essentiality by showing that knockdown of *ftsZ*, which encodes a key division protein conserved in almost all bacteria (21), and *cdr20291_0712* (*pbp-0712*), a monocistronic gene encoding a previously uncharacterized bifunctional (class A) penicillin-binding protein (22), markedly decreased cell viability and induced distinct cell division defects. Their analyses also confirmed that repressing the expression of the gene encoding the major S-layer protein, *slpA*, renders cells susceptible to lysozyme, similar to the *slpA* mutant isolated from bacteriocin-based selection (23).

Impact of the research. The utility of the CRISPRi system in *C. difficile* was also recently shown by Marreddy et al., who developed a similar xylose-inducible CRISPRi system to show that the fatty acid biosynthesis gene *fabK* is essential in *C. difficile* (24). The CRISPRi system developed by the Hurdle group was not necessarily optimized for *C. difficile*, which may explain why their antisense RNA approach for knocking down *fabK* expression was more effective at repressing gene expression than their CRISPRi system. Since antisense repression systems can have limited utility due to variable efficacy (25), and since Müh et al. observed a 100% success rate for gene repression with their CRISPRi system, the optimized CRISPRi system described in this special issue is likely to be more broadly applicable to studying gene function in *C. difficile*.

The xylose-inducible system developed by Müh et al. also represents an advance in *C. difficile* genetics, since the existing (anhydro)tetracycline (5)- and nisin (6)-based inducible systems can exhibit toxicity at high levels of the inducer. Furthermore, their system expands the options for studying gene function in *C. difficile*, since the different inducible systems can be combined to titrate the expression of multiple gene targets.

CRISPRi also enables multiple genes to be simultaneously targeted, which facilitates the study of genetic redundancy, particularly for essential pathways; obviates the need for complex strain construction (20, 26); and minimizes the chance that gene deletions cause compensatory changes in gene expression. Furthermore, the ease with which CRISPRi can generate targeted and genome-wide libraries of gene knockdowns has been exploited in phenotype screens for determining gene function (20, 27–29) and identifying drug targets, since strains producing smaller amounts of the drug targets are sensitized to the inhibitor (20, 29). Remarkably, large-scale libraries of CRISPRi have recently been used to conduct genome-wide screens with even greater efficacy than transposon sequencing (TnSeq) libraries (28). While these larger-scale approaches will likely require additional advances in *C. difficile* genetics due to their low conjugation efficiencies (see below), the construction of targeted libraries is an exciting possibility.

One of the most important applications of the *C. difficile* CRISPRi system is its potential to be used for studying gene function in *C. difficile* strains that have largely been resistant to genetic manipulation. It is generally much easier to acquire transconjugants than to generate strains that require even single recombination events, and

heat shock can increase plasmid uptake in the epidemic R20291 strain (30). Thus, it seems likely that their system will have broad utility in *C. difficile*.

Conclusions and future directions. In demonstrating that their optimized xylose-inducible CRISPRi system is tunable and robust, Müh et al. have markedly expanded the tools available for genetically manipulating *C. difficile*. Their plasmid system should permit the study of less genetically tractable *C. difficile* clinical isolates, since CRISPRi was recently applied to difficult-to-manipulate *Staphylococcus aureus* clinical isolate strains (31).

Nevertheless, modifying the *C. difficile* CRISPRi plasmid-based system to permit integration onto the chromosome would allow gene function to be studied during infection, since antibiotic maintenance of the plasmid can be challenging. A recent study described the development of a "mobile CRISPRi" system that allows for the ready integration of the gene silencing system onto the chromosomes of diverse bacteria (32, 33). While it remains to be seen whether this type of system could be adapted for use in *C. difficile*, there are clearly many applications possible for their CRISPRi system in studying *C. difficile* physiology and drug susceptibility. Improving the methods for reliably introducing DNA into *C. difficile* would further advance the utility of this system.

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