Microbiology Spectrum
American Society for Microbiology Press

Clostridial Genetics: Genetic Manipulation of the Pathogenic Clostridia

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ABSTRACT The past 10 years have been revolutionary for clostridial genetics. The rise of next-generation sequencing led to the availability of annotated whole-genome sequences of the important pathogenic clostridia: *Clostridium perfringens*, *Clostridioides* (*Clostridium*) *difficile*, and *Clostridium botulinum*, but also *Paeniclostridium* (*Clostridium*) *sordellii* and *Clostridium tetani*. These sequences were a prerequisite for the development of functional, sophisticated genetic tools for the pathogenic clostridia. A breakthrough came in the early 2000s with the development of TargeTron-based technologies specific for the clostridia, such as ClosTron, an insertional gene inactivation tool. The following years saw a plethora of new technologies being developed, mostly for *C. difficile*, but also for other members of the genus, including *C. perfringens*. A range of tools is now available, allowing researchers to precisely delete genes, change single nucleotides in the genome, complement deletions, integrate novel DNA into genomes, or overexpress genes. There are tools for forward genetics, including an inducible transposon mutagenesis system for *C. difficile*. As the latest addition to the tool kit, clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technologies have also been adopted for the construction of single and multiple gene deletions in *C. difficile*. This article summarizes the key genetic technologies available to manipulate, study, and understand the pathogenic clostridia.

INTRODUCTION

The *Clostridiaceae* family (and within it the *Clostridium* genus) consists of an extremely diverse group of primarily Gram-positive bacteria that have traditionally been grouped together based on their anaerobic growth requirements and their ability to produce heat-resistant endospores. Historically, the members of the genus were very dissimilar, such that the genus lacked phylogenetic coherence, with over 200 species, at least 35 of which cause disease in humans and animals (1). Two recent studies have proposed a phylogenetic reorganization of the clostridia and subsequently changed the name of *Clostridium difficile* to *Peptoclostridium difficile* (2) and *Clostridioides difficile* (3, 4). However, the earlier name (*P. difficile*) did not comply with the *Internal Journal of Systematic and Evolutionary Microbiology* Bacterial Code and was rejected (3). Also recently, *Clostridium sordellii* has been reclassified as *Paeniclostridium sordellii* (5). For simplicity, in this article pathogenic members of the genera *Clostridium*, *Clostridioides*, and *Paeniclostridium* will all be referred to as pathogenic clostridia.

The most common feature of the pathogenic clostridia is that the cell and tissue damage that they cause primarily results from the production of potent extracellular toxins

Received: 15 August 2018, **Accepted:** 10 December 2018, **Published:** 7 June 2019

Editors: Vincent A. Fischetti, The Rockefeller University, New York, NY; Richard P. Novick, Skirball Institute for Molecular Medicine, NYU Medical Center, New York, NY; Joseph J. Ferretti, Department of Microbiology & Immunology, University of Oklahoma Health Science Center, Oklahoma City, OK; Daniel A. Portnoy, Department of Molecular and Cellular Microbiology, University of California, Berkeley, Berkeley, CA; Miriam Braunstein, Department of Microbiology and Immunology, University of North Carolina-Chapel Hill, Chapel Hill, NC, and Julian I. Rood, Infection and Immunity Program, Monash Biomedicine Discovery Institute, Monash University, Melbourne, Australia

Citation: Kuehne SA, Rood JI, Lyras D. 2019. Clostridial Genetics: Genetic Manipulation of the Pathogenic Clostridia. *Microbiol Spectrum* 7(3):GPP3-0040-2018. doi:10.1128/microbiolspec.GPP3-0040-2018.

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(6, 7). Although it is somewhat artificial in that it crosses family boundaries, it is useful to divide the pathogenic clostridia into three major groups based on their resultant disease pathology. These groups consist of the neurotoxic clostridia, which produce toxins that affect the nervous system, the enterotoxic clostridia, which produce toxins that affect the gastrointestinal tract, and the histotoxic clostridia, whose necrotic pathology results from the production of one or more toxins that affect the structural and functional integrity of host cells located at or near the site of infection. This article focuses on the genetic manipulation of the pathogenic clostridia and is divided by approaches and techniques rather than individual species.

The genetics and, in particular, the genetic manipulation of the clostridia have been revolutionized in the past decade. In large part, this has been made possible by advances in sequencing technology. The genomes of representatives of all of the pathogenic clostridia have now been completely sequenced, assembled, and annotated. The first *Clostridium perfringens* genome was published in 2002 (8), followed by *Clostridium tetani* in 2003 (9), *C. difficile* in 2006 (10), and *Clostridium botulinum* in 2007 (11). Multiple genome sequences are now available for these pathogens (12–19). The availability of these genome sequences has been important in the development of genetic manipulation technologies because precise sequences are required to design homology arms for homologous recombination, complementation, and use of newer technologies such as clustered regularly interspaced short palindromic repeats (CRISPR).

Genetic manipulation can be broken down into two main categories: forward and reverse genetics. Forward genetics involves random screens to identify genes that are responsible for a particular phenotype and can be employed without knowing the target gene. These methods encompass technologies such as transposon mutagenesis and transposon-directed insertion site sequencing, which are tools of random mutagenesis. Reverse genetics involves the inactivation of a particular gene to study the resulting phenotype and requires the target gene to be identified first. The technologies involved in this approach include insertional mutagenesis, allelic exchange to construct deletions and point mutations, and the insertion of genes or regions of DNA to disrupt genes.

EARLY ADAPTATIONS OF MUTAGENESIS TECHNOLOGIES

Genetic manipulation methods have now been established for most pathogenic clostridia but are most refined for *C. difficile* and *C. perfringens*. The clostridia are not naturally competent, but transformation via electroporation has been described for several of the pathogenic species. Electroporation into *C. botulinum* was demonstrated by Zhou et al. in 1983 (20), and *C. tetani* was transformed with the *Enterococcus faecalis* shuttle vector pAT19 in 1998 (21). So far, however, there have been no further reports of successful transformation (via electroporation) into either of these two species. Whether DNA transfer via electroporation is successful partly depends on the restriction and modification status of the organism and the transformation frequency that can be obtained. Electroporation is not currently feasible for *C. difficile*, despite numerous attempts in many laboratories. It has been successful for *C. perfringens* strain 13 (22, 23), but many other strains of *C. perfringens* are not amenable to DNA transformation.

The first defined mutants in a pathogenic *Clostridium* species were constructed in *C. perfringens* by allelic exchange utilizing double crossover events (22, 24). These studies made use of the fact that derivatives of the gas gangrene strain 13 have an apparent lack of active restriction and modification systems, thereby making them very amenable to genetic analysis (8, 25). There are several well-characterized shuttle plasmids that can reliably and reproducibly be introduced into *C. perfringens* cells by electroporation-mediated transformation (23) or by conjugation from *Escherichia coli* (26). The most widely used *C. perfringens*-*E. coli* shuttle vectors for complementation and other genetic studies are pJIR750 and pJIR751 (27), which are derivatives of the shuttle vector pJIR418 (28). These plasmids all contain the origin of replication *lacZ′* gene and multiple cloning sites of the *E. coli* plasmid pUC18 and the origin of replication and *rep* gene of the *C. perfringens* bacteriocin plasmid pIP404. Detection of recombinants can be achieved by X-Gal screening in *E. coli* and transformants selected on chloramphenicol or erythromycin in either *E. coli* or *C. perfringens*, respectively. Subsequently, derivatives of pJIR750 and pJIR751 were constructed that carried the *oriT* site from plasmid RP4, designated pJIR1456 and pJIR1457, respectively (29). The construction of these vectors facilitated RP4-mediated conjugative transfer of these plasmids from *E. coli* to *C. perfringens*, allowing strains that were not amenable to DNA transformation to be genetically manipulated.

The first genetic manipulation of *C. difficile* was successfully performed using the *oriT*-containing *C. perfringens* vectors, allowing regulatory genes to be introduced *in trans* into this bacterium (30). The first mutants generated in *C. difficile* were derived by homologous recombination from single crossover events. In 2006 O'Connor

et al. (31) constructed a chromosomal mutant in CD3255 (*virR*) using pJIR1456 (26). This replicon is relatively unstable in *C. difficile*, and hence it can act as a pseudo-suicide plasmid in this bacterium. While this development represented an important step forward in the genetic manipulation of *C. difficile*, the mutants were sometimes unstable when selective pressure was removed, which could lead to the generation of revertants (31, 32).

A prerequisite for the mutagenesis of *C. difficile* was the use of an erythromycin resistance marker, and hence an erythromycin-sensitive recipient strain was required. *C. difficile* 630 has become the primary strain for laboratory manipulation, because it is amenable to DNA transfer (via conjugation, as discussed below), and it was the first *C. difficile* strain to have its genome sequenced (10). However, *C. difficile* 630 is erythromycin resistant. After a number of passages two erythromycin-sensitive strains were eventually obtained, namely 630E or JIR8094 (31) and 630Δ*erm* (33). Recently, it was discovered through whole-genome sequencing that both of these erythromycin-sensitive derivatives obtained additional mutations during their passaging, which differed between the two derivatives (34–36). JIR8094 showed a larger number of seemingly important changes compared to the parental strain. In light of these differences, which are described in more detail by Collery *et al.* (34), 630Δ*erm* is now the strain of choice in studies to elucidate pathways of virulence, regulation, and metabolism in *C. difficile*.

THE ROADMAP TO CLOSTRIDIAL GENETICS

Overcoming Restriction and Modification Systems

As mentioned above, complete genome sequences are now available for all of the pathogenic clostridia, which has opened up the field for the development of more sophisticated genetic tools. Technologies such as Illumina sequencing are now quick and affordable; however, obtaining accurate reads in repetitive regions is difficult with these technologies because of the short reads that they produce. This problem can be overcome by the use of long-read technologies such as MinION and, in particular, PacBio. When these latter methods are used in combination with Illumina sequencing, they can provide an accurately assembled genome, which is crucial to locate genes and identify up- and downstream defined genetic elements and, importantly, potential restriction-modification systems (37).

Restriction-modification systems have held back much of the genetic development of the clostridia, because they can protect the recipient bacterial cell from foreign DNA, making the transfer of foreign DNA for genetic manipulation difficult or impossible (38). A common method used to overcome restriction barriers is *in vitro* or *in vivo* methylation of the DNA to be introduced, so that the recipient recognizes it as its own. To this end, it becomes important again to have knowledge of the genome sequence, which can reveal all the methylases and restriction-modification systems present. For many years pAN2, which contains a bacteriophage methyltransferase gene, has been used to methylate DNA before transformation into *Clostridium acetobutylicum* (39). Recently, similar systems have been developed for *Clostridium pasteurianum* (40), *Clostridium saccharobutylicum*, (41, 42), and *Clostridium cellulovorans* (43).

In all fields of bacteriology, researchers work mostly on a few select laboratory strains, which are for one reason or another easy to manipulate. For example, *C. difficile* 630 has no active restriction-modification systems, so it is easier to conjugate with, and its genome was the first to be sequenced, so most molecular studies have been carried out with this strain and its derivatives. Similarly, *C. perfringens* strain 13 is the most widely used strain of that species, with transformation protocols having been successfully developed at an early stage. Finally, ATCC 9714 is the laboratory strain of choice for *P. sordellii* studies. While these strains are great tools to unravel the molecular basis of pathogenicity and metabolism, it has become evident that they often have phenotypic limitations. For this reason, clinical isolates need to be considered and used for research studies, particularly those related to virulence. Different ribotypes of *C. difficile* and different toxinotypes of *C. perfringens*, for example, display very different phenotypes, causing different diseases or disease severity (44, 45). Therefore, it is important to understand and develop ways to overcome the barrier to DNA transfer into these organisms, and the use of the genetics roadmap suggested in reference 37 can help to facilitate such an analysis. In this workflow, the genome sequence of a strain is determined first, which gives insight into its restriction-modification system. This information in turn helps to overcome the barriers to DNA transfer, as well as providing the genetic blueprint of the strain, making genetic manipulations such as gene deletion, substitutions or additions (46), and further investigation possible.

Plasmid Cloning Vectors

Conjugation as a method of DNA transfer has been very successful in the pathogenic clostridia. *C. perfringens* strains that cannot be transformed by electroporation have successfully been genetically manipulated using conjugation from *E. coli*, using plasmids pJIR1456 or

pJIR1457, as discussed earlier (22, 29, 30). Conjugative manipulation was first established for *C. botulinum* using pJIR1457 (47) with the RP4-carrying *E. coli* strain S17-1 as the donor strain. Conjugation is also used to introduce DNA into *P. sordellii* and has been used successfully to generate numerous mutants in this clostridial species (48–52).

In early studies, *C. perfringens*-based vectors were used for the genetic manipulation of *C. difficile* (30). For example, plasmid pJIR1457 was used to study the functional capacity of the toxin regulator TcdR (then called TxeR) in *C. difficile* employing RP4-mediated conjugative transfer of a *tcdR*-carrying recombinant plasmid from an *E. coli* donor (30). Note that the difficulty of introducing DNA into some strains using the RP4-based system from *E. coli* resulted in the construction of a novel plasmid transfer system that exploited the conjugation apparatus encoded by the broad-host range transposon Tn916 together with the cognate *oriT* site encoded by this element (53). This system, which uses a Tn*916*-carrying *C. perfringens* or *Bacillus subtilis* strain as a donor and $oriT_{Tn916}$-carrying shuttle plasmids, facilitated the genetic manipulation of previously recalcitrant *C. difficile*, *C. perfringens*, *P. sordellii*, and *Clostridium septicum* strains (54). More recently, heat shock has also been used to increase conjugation efficiency in *C. difficile*, and the choice of media was also shown to affect conjugation frequency (55).

The construction of specific *C. difficile* shuttle vectors was realized by Purdy et al. in 2002 (56). The plasmids derived in this work can replicate autonomously in *C. difficile* and contain either replicon CD6, which was amplified from a *C. difficile* plasmid isolated during the same study, the replicon from pCB102, or that from pIP404; the latter two plasmids were derived from *Clostridium beijerinckii* (57) and *C. perfringens* (28), respectively. A much higher frequency of transfer was achieved using the native replicon pCD6, and plasmids carrying this replicon were shown to be maintained more stably in *C. difficile*. The plasmid constructed in this study, containing the pCD6 replicon, a Gram-negative replication site, possibly including *oriT* for transfer and an erythromycin gene for antibiotic selection, was originally called pMTL9301 and was later renamed pMTL960 (56). pMTL960 has been used successfully in numerous studies, including those involving an examination of the function of the *C. difficile* cell wall protein V, the lipoprotein CD0873 (an adhesin), and the cysteine proteases Cwp84 and Cwp13 (58–60).

In 2009, Heap et al. published a modular vector system that has been useful in a number of clostridial species (61). This system includes four main modules: a Gram-negative replicon, a Gram-positive replicon, an antibiotic resistance marker, and an insertion module. All of these modules can easily be swapped and replaced by others, making this system easy to modify depending on the purpose and organism for which it will be used (Fig. 1, Table 1). For example, four different antibiotic resistance markers can be used, and two Gram-negative replicons are included. The use of specific restriction sites that were employed to build the plasmid series allows the straightforward incorporation of new modules incorporating, for example, new Gram-positive replicons. The development of this vector series has helped to standardize the genetic work in many clostridial species. All these vectors can be used as shuttle vectors and can be transferred from *E. coli* via RP4-mediated conjugation, or directly in some species by electroporation, and have successfully been used in many *C. difficile* studies investigating virulence regulation, biofilm formation, colonization, and sporulation (62–64). These plasmids have also been used in other pathogenic clostridial species, including an investigation into the role of two-component systems in neurotoxin regulation of *C. botulinum* (65). If used without the Gram-positive replicon, they act as suicide vectors in the clostridia and have been effectively used in this capacity in studies involving the nonpathogenic species *C. beijerinckii* and *Clostridium autoethanogenum* (66, 67).

FIGURE 1 Illustration of the pMTL80000 modular vector series. The figure highlights the four modules separated by the unique restriction sites: *Sbf*I, *Asc*I, *Fse*I, and *Pme*I. The modules consist of a Gram-positive replicon module, a selectable marker, a Gram-negative replicon unit with optional transfer (*tra*) genes, and an application-specific module.

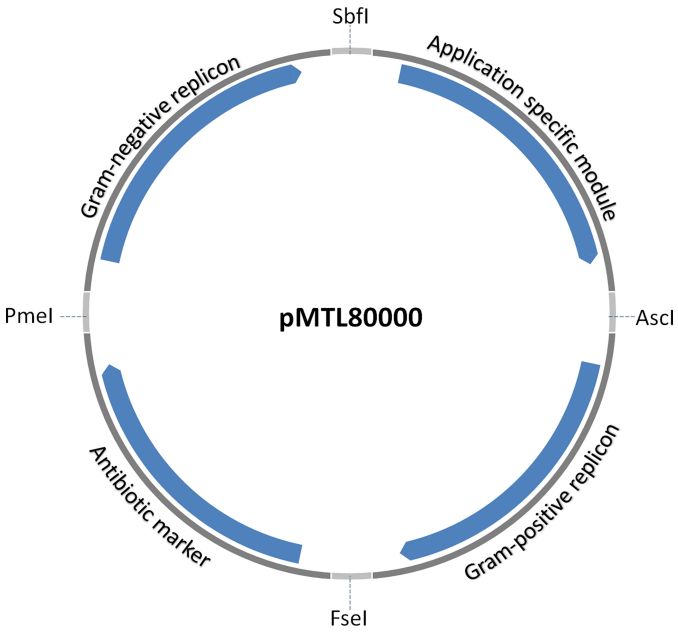

TABLE 1 Module choices for pMTL80000 plasmids

Gram+ replicon	Marker	Gram− replicon	Application-specific
0. Spacer*			0. Spacer*
	1. *catP*	1. P15a	1. MCS
2. pBP1	2. *ermB*	2. p15a + *tra*	2. PthI + MCS
3. pCB102	3. *aad6*		3. Pfdx + MCS
4. pCD6	4. *tetA*	4. ColE1	4. catP reporter
5. pIM13		5. ColE1 + *tra*	

Abbreviations: MCS, multiple cloning site; PthI, thialase promoter; Pfdx, ferrodoxin promoter

*Spacer: a module may consist of a short spacer in place of a functional component

Inducible Vector Systems

In many genetic studies it is essential to be able to control or regulate the expression of cloned genes. The usual method is to place the cloned gene behind an inducible or repressible promoter so that expression of the gene of interest is induced by the addition of a low-molecular-weight effector molecule to the culture medium. However, only a limited number of such systems are available for use in the pathogenic clostridia.

In *C. perfringens*, an inducible expression system was made available by the development of the vector pKRAH1 (68). This shuttle plasmid encodes the *C. perfringens* BgaR transcriptional regulator and the inducible promoter, P_{bgaL}, which is activated by BgaR in the presence of lactose. The addition of lactose to the culture medium therefore leads to the expression of genes cloned downstream of P_{bgaL}. This system initially was validated by cloning the *gusA* reporter gene behind P_{bgaL} and measuring β-glucuronidase (β-GusA) activity in *C. perfringens* SM101 cells with and without lactose induction. Substituting isopropyl β-d-1-thiogalactopyranoside (IPTG) for lactose did not result in increased activity, suggesting that IPTG is either not an inducer or cannot be transported into the cell (68). Subsequently, pKRAH1 was constructed and used to express a *yfp-pilB* construct, which allowed the resultant fusion protein to be localized (68). pKRAH1 has been used in other studies of the biogenesis of type IV fimbriae in *C. perfringens* (69), and the BgaR/P_{bgaL} system was also used to study the role of the endonuclease RNaseY (70) and carbonic anhydrase (71) in *C. perfringens* and for the analysis of acetogenesis in *Clostridium ljungdahlii* (72).

Other workers developed a xylose-inducible gene system for use in *C. perfringens* (73). The vectors, pXCH (confers chloramphenicol resistance) and pXEH (confers erythromycin resistance), encode the XylO transcriptional repressor and the *xylB* promoter from *C. difficile*. Genes are cloned downstream of P_{xylB}, and XylO repression is subsequently relieved by the addition of xylose. The vectors were validated in *C. perfringens* strain 13 by using them to regulate the expression of chloramphenicol acetyltransferase and α-toxin reporter constructs. The xylose-inducible system has been used by other workers in combination with the lactose system (69).

Classic IPTG-inducible systems are not effective in *C. difficile*, but in 2011 Fagan et al. developed an anhydrous tetracycline-inducible system for this bacterium (74). The system is based on pRMC2 from *Staphylococcus aureus* (75), combining the tetracycline-inducible promoter system with a codon-optimized *gusA* gene from pCBR023 on plasmid pRPF185, including the *fdx* terminator from *C. pasteurianum*. The plasmid backbone is taken from the *E. coli-C. difficile* shuttle vector pMTL960. When GusA activity was monitored as a measure for leakiness of the system, no GusA activity was measured in the absence of anhydrous tetracycline (the inducer), providing supporting evidence that the system is tightly regulated (74). This system has been used successfully in other studies involving *C. difficile* to determine the functional role of TcdE (76) and to investigate the spore differentiation pathway, including the creation of an inducible *spo0A* gene most recently (77, 78). Additionally, it has been used to study spore coat proteins of *C. difficile* (79) and has been employed in other clostridial species, such as *P. sordellii*, in which it was used to examine a new conjugation system (51). A second inducible system has also been described and employed in *C. difficile*. Purcell et al. created a nisin-inducible system in which the *cpr* promoter, responsible for the transcription of an ABC transporter, which confers resistance against nisin, is cloned upstream of the target gene. This system was used to study the role of cyclic di-GMP in motility and aggregation of *C. difficile* (80).

Some clostridial genes are toxic when overexpressed in *E. coli* and therefore are difficult to clone, making complementation studies in the original clostridial host problematic. This problem can be overcome by use of the vector pJIR3422 (81, 82). This vector exploits the phenotypic features of the clostridial Tn*4451/3* site-specific recombinase TnpX, specifically, the specificity of binding of this protein to a transposon-derived promoter. TnpX represses expression from its own promoter, P_{attCI}, which is usually located at the joint of the circular form of the Tn*4451/3* elements (83). This promoter is present in pJIR3422, and expression from this promoter is repressed in the presence of a catalytically inactive derivative of TnpX, present in the *E. coli* strain used for cloning, but is not repressed in the clostridial host because of the absence of TnpX. The use of this system facilitated the

cloning of the *tcpG* conjugation gene and the *feoB* ferrous uptake gene in *E. coli* and the complementation of mutants of these genes in *C. perfringens*, which could not be achieved without this system (81).

Reporter Assays

Historically, protein reporter systems for the analysis of protein localization have been lacking in most pathogenic clostridia, particularly in *C. difficile*. Widely used reporter proteins such as green fluorescent protein for fluorescence or luciferase for chemiluminescence require oxygen for correct reporter protein folding or full enzymatic activity and hence have been thought to be of limited value in anaerobic bacteria. In the past 5 years, however, a number of assay systems have been established (77, 84, 85), which have made the functional characterization of genes and their encoded products more feasible. Reporters can be split into two main categories: visualizable reporters and enzymatic reporters. The former are utilized to study phenotypic heterogeneity and localizing proteins, whereas the latter serve as read-outs on transcription.

Visualizable reporters

Several of these systems are based on fluorescence. Ransom et al. successfully developed a codon optimized cyan-fluorescent protein and mCherry (mCherryOpt) in *C. difficile* to perform a number of subsequent studies (84, 86). They created two vectors using mCherryOpt, one to study localization, which they used to examine septal location of two cell division proteins, MldA and ZapA, in fixed cells (86). The other vector allows cloning of a promoter upstream of the mCherryOpt gene to study gene expression and has been exemplified by studying the *pdaV* operon, which is required for lysozyme resistance. (84). The variants were successfully expressed in *C. difficile* but required exposure to oxygen for chromophore maturation (84). Recently, work by Ribis et al. used mCherry fusions to establish the importance of SpoIVa and SipL in spore coat assembly and to elucidate the mechanism of engulfment of the forespore by the mother cell in *C. difficile* (87, 88). Other recent examples of the use of mCherry in *C. difficile* include a study of flagellum and toxin phase variation, demonstrating the potential to study single-cell heterogeneity, and work demonstrating that toxin gene expression is bistable in *C. difficile* (89, 90).

Yellow fluorescent protein fusions have recently been used successfully in *C. perfringens* to examine localization and function of PilB, an ATPase responsible for pili assembly (69).

iLOV, a light oxygen voltage domain, has also been optimized for use in *C. difficile*. LOV domains are plant or bacterial blue light receptors and are part of flavin mononucleotide-based fluorescent proteins (FbFPs) (91, 92). These fluorescent reporters can be utilized with and without oxygen, and they are smaller (13 kDa) than green fluorescent protein (25 kDa), which makes them ideal fusion proteins with a reduced probability of disrupting native protein function. iLOV has been used as a fluorescent reporter in *C. difficile* (85), as a translational fusion to study the cell division protein FtsZ. This study also proved that iLOV fusions can be successfully transported out of the cell by constructing a fusion to FliC, the flagella subunit. Functional iLOV fusions in *P. sordellii* and the nonpathogenic *C. acetobutylicum* were also generated in this study. The brightness of FbFPs is lower than that of green fluorescent protein, and hence a few technical modifications have been suggested to improve their usefulness, such as lowering background noise and improving the quality of the output signal. Flavin-based media, for example, can lead to highly fluorescent backgrounds, which can be reduced by eliminating yeast and beef extract (93). Also, several clostridial species, such as *C. difficile* and *C. acetabutylicum*, have a natural green autofluorescence that can increase the background signal (85).

SNAP-tag technology has also been adapted for *C. difficile*. The 20-kDa peptide tags are fused to target genes, and fusions are visualized using a fluorescent microscope by adding the cell-permeable fluorescent substrate TMR-star. This technology has been used to elucidate the role of the sigma factor cascade in the sporulation process in *C. difficile* and, furthermore, a split SNAP-tag has been employed to study protein interactions between the forespore protein SpoIIQ and the mother cell protein SpoIIIAH (77, 94). SNAP-tags have been proven to be versatile and have worked effectively on fixed cells, demonstrating the temporally regulated gene expression of the sigma factors involved in *C. difficile* spore formation and establishing the interactions of *C. difficile* SpoIIQ and SpoIIIA proteins and their control over forespore engulfment and integrity (77, 95).

Enzymatic reporter systems

The widely used β-GusA reporter assay has been used to study the regulation of the large toxins in *C. difficile*. To elucidate the function of the sigma factor TcdR, Mani et al. created *gus*-fusions in *C. perfringens* and then, in a follow-up publication, *gus*-fusions in *C. difficile*, the study of which showed that TcdR positively regulates *tcdA* and *tcdB* expression (30, 96).

An alternative reporter system used in *C. difficile* involves a luciferase gene fusion to the signal sequence of the secreted protein PPEP-1. PPEP-1 is a zinc metalloprotease

that has been shown to be one of the most abundant secreted proteins of *C. difficile* (97). Two reporters have been constructed, AmyEopt and sLucopt (98). AmyEopt can be used to measure promoter activity in liquid or plate-based assays due to the production of α-amylase, a codon optimized enzyme from *B. subtilis*, which results in starch degradation. sLucopt is a codon-optimized luciferase based on NanoLuc (99) and has been shown to be detectable in culture supernatants when fused to an inducible promoter (98). Both reporters are assayed under aerobic conditions once the samples have been taken from the anaerobically grown culture (98).

Finally, the *E. faecalis* alkaline phosphatase gene, *phoZ*, has been successfully used in *C. difficile* for the qualitative and quantitative measurement of gene expression using an alkaline phosphatase assay. PhoZ was shown to be active anaerobically and is therefore presumed to fold correctly under these conditions, which makes it a useful reporter gene for anaerobic bacteria (100).

A promoter probe plasmid based on pJIR418 has also been constructed for use in *C. perfringens*. This plasmid, pPSV, was constructed by deletion of the *catP* gene from pJIR418 followed by the addition of a promoterless *catP* gene downstream of the multiple cloning site (101). The *catP* gene has also been used as a reporter system in the promoter probe shuttle vector pTCATT, which contains a promoterless *catP* gene flanked by transcriptional terminators. However, PCR-mediated regeneration of the 5′ terminus of the *catP* gene is required during the construction of promoter fusions in pTCATT, limiting its usefulness (102). Other studies have attempted to develop reporter systems for use in *C. perfringens*. One system involved the construction of a plasmid that contains the *luxAB* genes from *Vibrio fischeri* under the control of the *plc* promoter. Luciferase activity and bioluminescence were obtained from this plasmid in *C. perfringens* (103). In another study, reporter plasmids using the *E. coli gusA* gene cloned into pJIR750 were constructed, and β-GusA production was used successfully to monitor the sporulation-specific regulation of the *cpe* gene in *C. perfringens* (104). More recently, high-level expression of the *C. perfringens* NanI sialidase was achieved by using a ferredoxin promoter-based plasmid, pFF, in *C. perfringens* (105). This system was particularly useful for purification of NanI since the *C. perfringens* expression strain secreted the enzyme, as does the wild type, and seemed to efficiently express the protein, resulting in a 60-fold increased yield in comparison to protein isolation from an *E. coli* host. These results suggest that in *C. perfringens* the pFF plasmid may be very useful for the expression of AT-rich genes from other clostridia and other bacteria (105).

Allelic Exchange and Homologous Recombination

Homologous recombination has been used extensively as a mutagenesis tool for many bacterial species and may involve either single- or double crossover events. Since two crossovers are required for allelic exchange, it is a less efficient process than obtaining mutants by a single crossover event using insertional mutagenesis. However, the disadvantage of single crossover events is that wild-type genes can be regenerated from the mutants by further homologous recombination events. This issue is not a problem with allelic exchange or double crossovers, which result in inherently stable mutants. It is worth noting that the efficiency with which mutants are obtained by allelic exchange depends on the size of the homologous regions that flank the gene of interest, and it is recommended that there be at least 2 kb of flanking DNA present on either side of the gene to be replaced (106). Additionally, a large number of mutants can be generated using homologous recombination, in contrast to a limited number that can be generated with insertional mutagenesis, because of the small number of antibiotic cassettes available, which are required for insertional mutagenesis.

Homologous recombination and allelic exchange methods for the clostridia have also been developed and improved significantly during the past 2 decades. In early studies, allelic exchange was used successfully for genetic analysis in *C. perfringens* (22), and the first defined genetic mutants constructed in the pathogenic clostridia were the *plc* and *pfoA* toxin gene double-crossover mutants that were constructed in *C. perfringens* (22, 24). Since that time many *C. perfringens* mutants have been constructed by allelic exchange, too many to list here, and homologous recombination has become a standard genetic tool for the construction of mutants in this bacterium.

Early homologous recombination studies of *C. difficile* involved the construction of mutants by single crossover events using suicide plasmids that were introduced by conjugation from *E. coli* (31). Subsequently, two improved systems (107, 108) were developed for *C. difficile*, both of which are based on the use of negative selection markers. The first system uses cycloserine deaminase (107) as a counter selection marker and relies on the presence of the *codA* gene on a pseudo-suicide vector (107). Single crossovers are selected on the basis that cells that have the plasmid, which contains a heterologous *codA* gene from *E. coli*, integrated into their chromosome are able to grow much faster than cells carrying the resistance gene on a plasmid. This is because the plasmid used in this approach is inherently unstable in *C. difficile* and is lost easily from cells. The faster-growing colonies

are selected and tested (via PCR) to determine if a single crossover event has occurred. They are then plated on *C. difficile* minimal medium containing 5-fluorocytosine. Only clones in which a second crossover event has occurred, resulting in the loss of the *codA* gene, are able to grow, because the cells still carrying the *codA* gene are killed when 5-fluorocytosine is converted to toxic 5-fluorouracil (97) by CodA. Double crossover events result either in a reversion back to wild type or in the construction of the desired mutant and loss of the plasmid. This system can be used for the construction of DNA deletions or point mutations. Genes can also be complemented by going through another cycle of single and double crossover events, using a different plasmid which contains the intact gene of interest. In this case, genes are reinserted into their original position on the chromosome. It is hence advisable to introduce a watermark or sequence signature into the gene to differentiate it from the wild type (107). This allelic exchange system has successfully been used in a number of studies, including work characterizing two sortase substrates of *C. difficile* and their mechanism, involving cyclic di-GMP, of anchoring proteins to the peptidoglycan layer (109). Another study investigated a dipicolinic acid release mechanism during *C. difficile* spore germination (110).

The second system for allelic exchange in *C. difficile* is based on creating a *pyrE* mutant, carrying a 3′ deletion of the gene, using allele-coupled exchange (111) in the strain of choice and then using a heterologous *pyrE* gene as a negative/counter selection marker. The *pyrE* gene encodes orotate phosphoribosyltransferase, which is involved in pyrimidine biosynthesis. This enzyme is essential for bacterial growth in the absence of exogenous pyrimidines and also acts on 5-fluoro-orotate to generate a toxic derivative of this compound. The *pyrE* mutants are auxotrophs for uracil. A functional *pyrE* gene from *Clostridium sporogenes* is supplied on an allelic exchange plasmid as the counterselection (108). *pyrE*-mutants were generated in *C. difficile* 630Δ*erm* and R20291 (108) and were then used to generate deletion mutants using a process similar to that described for the *codA* methodology. Single crossover clones are selected on the basis of faster-growing colonies once the vector has integrated into the chromosome, since the vector is unstable due to a frameshift mutation in the *oriR*. Double crossover mutants in which the plasmid has been excised from the chromosome are then selected on *C. difficile* minimal medium supplemented with 5-fluoro-orotate and uracil. Once 5-fluoro-orotate-resistant uracil auxotrophs have been isolated and shown to have lost the plasmid, they can easily be restored to prototrophy by conjugative transfer of a plasmid that contains part of the *pyrE* gene that can be utilized to repair the *pyrE* gene in the chromosome using allelic exchange. The resultant transconjugants are streaked on *C. difficile* minimal medium without the addition of uracil, so only bacteria with a repaired *pyrE* gene are able to grow. It is also possible to complement the mutation at the same time as repairing the *pyrE* gene by cloning a copy of the disrupted gene into the plasmid either under the control of its native promoter or through the use of a constitutive promoter for overexpression. Using allele-coupled exchange, the complementation gene is then integrated simultaneously during *pyrE* repair at the *pyrE* locus (108). This technique is very powerful and rapid, allowing for the generation of complementation strains within 5 to 6 days, opposed to obtaining an allelic exchange mutant in 2 to 3 weeks. This approach has been used in several studies; for example, it was used to generate a complete pathogenicity locus deletion in *C. difficile* R20291 to study the role of binary toxin in the absence of the overpowering potency of TcdA and TcdB (112). It was used in a recent study that suggested that the introduction of trehalose into our diets has favored the emergence of so-called hypervirulent *C. difficile* ribotypes, such as RT027 (113). Ribis et al. created the first published quadruple mutant in *C. difficile*, using the *pyrE*-system, in their work studying the roles of putative engulfment regulators, IID (CD0126), IIP (CD2469), and IIM (CD1221), and the known engulfment regulator and putative endopeptidase, IIQ (CD0125) (88).

ClosTron and TargeTron

Two related technologies based on the exploitation of a mobile group II intron were developed for insertional mutagenesis in the clostridia. Mobile group II introns are found in bacterial genomes and are site-specific retroelements. They use a mobility mechanism termed retrohoming to create insertions in DNA, and they do this by inserting an excised intron lariat RNA directly into a DNA target site, which is then reverse transcribed by an intron-encoded enzyme protein (114). The DNA target site is primarily recognized by base pairing of intron RNA, and these bases can be modified to allow intron insertion into any specific DNA target (4). As a result, a mobile group II intron from *Lactococcus lactis, L1.LtrB,* was developed commercially (Sigma-Aldrich) and was used in the development of TargeTron and ClosTron technologies. The use of the TargeTron system in a clostridial species was first described in 2005, when it was used to inactivate the *plc* gene in *C. perfringens* (115). TargeTron was also used as a tool to introduce the simian immunodeficiency virus p27 gene into the *pfoA* gene

on the *C. perfringens* chromosome, thereby simultaneously inactivating *pfoA* and introducing the simian immunodeficiency virus p27 gene, which was subsequently shown to be expressed within the mouse gastrointestinal tract (116).

ClosTron technology is an insertional mutagenesis tool similar to TargeTron, but it incorporates the use of a retrotransposition-activated marker (RAM), based on the *ermB* gene, for selective purposes, with successful insertion indicated by the acquisition of resistance to erythromycin (117). The presence of a RAM makes mutant selection much more efficient and less laborious. The retargeted region is designed using an online retargeting algorithm and is then incorporated into the plasmid of choice for subsequent introduction into the clostridial strain (118).

Although the acquisition of a resistance marker in the bacterial genome via ClosTron or similar systems makes mutant selection simpler, it can also be a drawback. In particular, this means that a second mutation using the same marker cannot be obtained. Furthermore, the use of a strong promoter, driving the transcription of the RAM, can lead to unwanted polar effects. While the ClosTron system is very efficient and easy to use, it can, on occasion, be nonspecific, requiring the empirical testing of several insertion sites. To be able to construct double mutants, it is necessary either to use another marker or to remove the initial marker, both of which have been attempted. No other RAM marker other than erythromycin resistance has been described for the clostridia, but markerless ClosTrons or ClosTrons with markers that are not retroposition activated have been used successfully (115, 119). The alternative and preferred option is marker removal. Flp recombinase has been used successfully in *C. acetobutylicum* to remove the retargeted *ermB* gene from a ClosTron mutant (120). The ability of the Tn*4451/3* site-specific recombinase TnpX to recognize and excise specific DNA fragments has also been exploited for antibiotic resistance marker recycling. Specifically, TnpX was used to remove the *ermB* gene from a marked chromosomal *C. perfringens* mutant, enabling the construction of a double mutant by allowing the removal and subsequent reuse of *ermB* to construct the second mutation (81).

Transposon-Mediated Random Mutagenesis

Transposon mutagenesis has been established in *C. perfringens* for several decades, although the early systems were not optimized and lacked efficiency (22). The most successful early mutagenesis method utilized the conjugative enterococcal tetracycline-resistance transposon Tn*916* (121). In these studies, *C. perfringens* cells were transformed with a suicide plasmid carrying Tn*916*, and transformants were selected on medium containing tetracycline. Hybridization analysis showed that Tn*916* had inserted at different sites on the chromosome but that multiple insertion events were common. However, mutants that had a single Tn*916* insertion and that abrogated the ability to produce perfringolysin O were detected. The insertion site was shown to be located within the *virS* sensor histidine kinase gene, leading to the identification of the VirSR two-component signal transduction system (121). Subsequent studies involved the isolation of Tn*916* insertions in several toxin genes (122), and conjugative transfer or mobilization of Tn*916* from *E. faecalis* and *E. coli*, respectively, has been used to isolate auxotrophic mutants of *C. perfringens*, but again, multiple insertion events were observed (123). Tn*916* mutagenesis also has been used to isolate mutants that have an altered response to oxidative stress (124). In summary, the use of Tn*916* for mutagenesis in *C. perfringens* has been useful, but it is hampered by the presence of multiple insertion and deletion events and subsequently has not been widely adopted.

Other transposon mutagenesis options include transposon mutagenesis of cloned genes in *E. coli* and subsequent transformation and screening in *C. perfringens*. This approach has been used to identify the replication protein of the *C. perfringens* plasmid pCW3 (125). Other workers (126) developed an EZ-Tn*5*-based random mutagenesis system that involved the electroporation of a transposome complex into *C. perfringens* and selection (erythromycin resistance) for the insertion of a modified Tn*5* element. The erythromycin-resistant transformants appeared to contain random single insertions. These experiments led to the mutagenesis and identification of the *agrB* gene, which is involved in the regulation of perfringolysin O production by quorum-sensing (126). Similarly, the bacteriophage Mu-based transposon delivery system has been successfully adapted for *C. perfringens* (127). Electroporation was used to introduce a Mu transpososome complex, and a strain library that contained single transposon inserts was obtained. Finally, the *Himar1* mariner transposon system has been adapted for *C. perfringens* and used to show that the *sagA* gene is required for gliding motility in *C. perfringens* (128). This method utilizes a replicating plasmid that carries an inducible mariner element, but it requires a strain with *galK* and *galT* mutations to ensure that the replicating delivery plasmid can subsequently be selected against in the presence of galactose.

Despite in-depth studies of transposons in *C. difficile* (129), there is only one system for random mutagenesis in

this organism. Transposons such as Tn*5397* and Tn*916* (33, 130, 131) have been well characterized, but they either lead to multiple insertion events or show a strong bias to particular target sites. However, the mariner transposon system has been successfully developed for *C. difficile* (132, 133). The transposable element of the mariner system *Himar1* inserts randomly into a TA target site, which lends itself for use in AT-rich organisms such as the clostridia. Furthermore, only the cognate *Himar1* transposase is required for transposition to occur. As mentioned earlier, no transformation system has yet been developed successfully for *C. difficile*, which means that the transposon vectors cannot rely on a plasmid suicide mechanism for their introduction into cells. To overcome this problem, Cartman and Minton (132) developed a pseudosuicide vector by creating a segregationally unstable plasmid, using the replicon pBP1. This method allowed them to obtain transposition events at a frequency of 4.5×10^{-4} per cell. Drawbacks of this method were the lack of effective control of the time of transposition and lack of plasmid loss, which meant that the size of the library that could be obtained was limited. Library size is important for experiments such as transposon-directed insertion site sequencing, which can be used to determine essential genes under specific conditions. Subsequently, the system was improved by integrating the previously described anhydrous tetracycline system (74) with the transposon vector, generating a plasmid that has tetracycline-dependent conditional replication. The new transposon vector not only has a conditional replication phenotype, but it also has tight control over transposition of the *ermB* transposon. This tetracycline-inducible system consists of two promoters (133); one promoter drives expression of *tetR* (74), and the other promoter drives expression of the codon-optimized Hinmar1 transposase. Upon induction, Dembek et al. showed near complete plasmid loss within 13 generations, whereas 40% of bacteria in the uninduced culture retained the plasmid. (133). The transposon system has been demonstrated in *C. difficile* 630Δ*erm* and in the epidemic strain R20291. Transposon libraries have been created during *in vitro* growth and during sporulation, followed by transposon-directed insertion site sequencing, which resulted in the identification of 404 and 798 essential genes, respectively, for these conditions (133).

Newer Technologies for Genetic Manipulation

New and improved technologies for genetic analysis and manipulation of the pathogenic clostridia are being reported all the time. Most *C. difficile* genomes contain prophages and lysogenic bacteriophages, which have been characterized in some detail (134–136), but to date, no lytic phages have been described. There has been one report of transduction, showing that a novel transposon, Tn*6215*, carrying an erythromycin resistance gene, could be transferred from a donor strain to a recipient strain and integrated into the recipient genome if the bacteriophage ΦC2 was integrated into the recipient genome. The study was unable to show transduction into another three recipient strains, which were all susceptible to the phage, suggesting that conditions for transduction need to be optimized further before it can be used as a reliable genetic tool (137).

Antisense RNA technology has successfully been used to repress gene expression in *C. difficile* and to show that the S-layer proteins and cell wall protein V are translocated across the cell membrane through an accessory Sec-system (SecA2). Most recently, CRISPR-gene editing technologies were successfully used in the clostridia. Initially developed in nonpathogenic clostridia, such as *C. pasteurianum* (138), *C. beijerinckii* (139), and *C. cellulolyticum* (140), this emerging technology was used in 2017 to generate deletion mutants in *C. difficile* (141). The authors developed a CRISPR-Cas9 system with a reported mutation frequency of 20 to 50%, exemplifying it by deleting *selD*, a selenophosphate synthetase that is essential for the specific incorporation of selenium into selenoproteins. They showed that deletion of *selD* leads to growth reduction and a lack of selenium incorporation, suggesting that the Stickland reaction could be a target for future antimicrobial therapy development. Since then, two more papers were published (142, 143), further developing and refining the technology for use in *C. difficile*. Wang et al. used a plasmid-based CRISPR-Cas9 system for mutagenesis, reporting 100% mutation efficiency and knocking out the sporulation master regulator Spo0A (142). The latest study by Hong et al. developed a CRISPR-Cpf1 system capable not only of targeted deletion from the *C. difficile* genome, including the largest deletion to date of 49.2 kb, but also of simultaneous deletion of at least two targets located on different parts of the genome. This new toolkit could greatly improve the genetic analysis of *C. difficile* and advance the development of new therapeutics and diagnostics by identifying new targets for these strategies (143).

CONCLUSIONS

The pathogenic clostridia are a diverse group of anaerobic, Gram-positive spore formers, all of which can cause human disease. Pathogenesis is toxin mediated, but many auxiliary virulence factors have been described and are

still being discovered. Historically, it has been extremely difficult to study the clostridia at a molecular level owing to their anaerobic nature, but also because of a number of other difficulties such as the presence of multiple restriction modification systems, which preclude the uptake of foreign DNA into the bacterial cell. With the advent of next-generation sequencing and, consequently, the availability and analysis of whole-genome sequences, the development of clostridial molecular tools has taken an unprecedented leap. The technologies presented and reviewed in this article open up unique opportunities to study the pathogenic clostridia and their disease mechanisms in detail, with the objectives of improved diagnostics and the development of novel disease therapies.

There is, however, more work to be done. Recent years have seen a surge in interest in the nonpathogenic clostridia, particularly because of their utility in producing a range of biochemicals. Technologies have been developed that could be adapted for use in the pathogenic clostridia, such as a range of allelic exchange systems. Examples include the I-SceI system used in *C. acetobutylicum* and *C. beijerinckii* (144) and the MazF toxin-antitoxin system, which has been used in *C. acetobutylicum* and *C. cellulolyticum* (145, 146).

Additionally, there is an ever-growing interest in the healthy microbiota and in particular clostridial species within that microbiota (147, 148). To date, no mechanistic studies have been conducted, despite studies showing compelling data that suggest benefits to the host or microflora from certain of these species (147). The main reason for this information shortfall is the lack of available genetic tools. With the newest approaches, described in this article, we are likely to see this area of research develop rapidly in the very near future (38, 142).

In conclusion, the past 10 years have seen a rapid increase in mechanistic studies of the pathogenic clostridia owing to the development of novel, sophisticated tools, the outcomes of which are being translated into the clinic through improved diagnostics, prevention strategies, and novel treatments. The next decade will undoubtedly see even more significant advances in clostridial genetics and their subsequent application to provide a more detailed understanding of this important genus of bacteria.

ACKNOWLEDGMENTS

Research in S.A.K.'s laboratory is supported by grants from the British Society of Periodontology (RCFW20100) and DEBRA UK (Chapple1-20711). Research in J.I.R.'s laboratory is supported by grants from the National Health and Medical Research Council (GNT1082401, GNT1104502, and GNT1125349) and the Australian Research Council (DP160102680). Research in D.L.'s laboratory is supported by grants from the National Health and Medical Research Council (GNT1107812, GNT1107969, GNT1103024, and GNT1145760) and the Australian Research Council (DP150104670 and DP180102449).

We thank Andrew Dempster for help with Fig. 1.

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