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Research review paper

Technical guide for genetic advancement of underdeveloped and intractable *Clostridium*

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ABSTRACT

In recent years, the genus *Clostridium* has risen to the forefront of both medical biotechnology and industrial biotechnology owing to its potential in applications as diverse as anticancer therapy and production of commodity chemicals and biofuels. The prevalence of hyper-virulent strains of *C. difficile* within medical institutions has also led to a global epidemic that demands a more thorough understanding of clostridial genetics, physiology, and pathogenicity. Unfortunately, *Clostridium* suffers from a lack of sophisticated genetic tools and techniques which has hindered the biotechnological exploitation of this important bacterial genus. This review provides a comprehensive summary of biotechnological progress made in clostridial genetic tool development, while also aiming to serve as a technical guide for the advancement of underdeveloped clostridial strains, including recalcitrant species, novel environmental samples, and non-type strains. Relevant strain engineering techniques, from genome sequencing and establishment of a gene transfer methodology through to deployment of advanced genome editing procedures, are discussed in detail to provide a blueprint for future clostridial strain construction endeavors. It is expected that a more thorough and rounded-out genetic toolkit available for use in the clostridia will bring about the construction of superior bioprocessing strains and a more complete understanding of clostridial genetics, physiology, and pathogenicity.

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Abbreviations: AB fermentation, acetone–butanol fermentation; ACE, allele-coupled exchange; bp, base-pair; Dam, DNA adenine methylase; Dcm, DNA cytosine methylase; DNA, deoxyribonucleic acid; EPB, electroporation buffer; FOA, 5-fluoroorotic acid; GC content, guanine + cytosine content; IEP, intron-encoded protein; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ng, nanogram; 6 mA, 6-methyladenine; 6mC, 6-methylcytosine; NGS, next generation sequencing; PCR, polymerase chain reaction; RAM, retrotransposition-activated marker; RM, restriction-modification; RNA, ribonucleic acid; asRNA, antisense RNA; mRNA, messenger RNA; rRNA, ribosomal RNA; sRNA, small non-coding RNA; 16S rRNA, 16 Svedberg unit rRNA; SMRT sequencing, single molecule real-time sequencing; SOE PCR, splicing by overlap extension PCR; UV, ultraviolet.

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Introduction

Clostridium is one of the largest genera of prokaryotes and is comprised of approximately 200 distinct species of bacteria (Wiegel et al., 2006). Prior to the advent of 16S rRNA sequencing, subscription to the genus was traditionally granted based on the fulfillment of three simple criteria, namely the capacity to form endospores, an obligately anaerobic metabolism, and an inability to perform sulfate reduction (Gottschalk et al., 1981). In practice, the clostridia are more generally recognized by distinct rod-shaped cells, the presence of Gram-positive cell walls, and DNA of low guanine + cytosine content (GC content; typically 26–32%), although these characteristics are not strict taxonomic markers. As a result of such simple classification requirements, *Clostridium* is notorious for its extreme phylogenetic heterogeneity and possesses more genetic diversity than almost all other microbial genera (Wiegel et al., 2006). In fact, *Clostridium* contains numerous uncharacteristic phenotypes, including species that are cocci-shaped, asporogenous, aerotolerant, intermediate in GC content, and Gram-negative or Gram-variable (Finegold et al., 2002). As such, 16S rRNA cataloging and next generation genome sequencing have led to several massive proposed rearrangements of *Clostridium* (Gupta and Gao, 2009; Ludwig et al., 2009; Rainey et al., 2009; Yutin and Galperin, 2013). Only 73 species were found to possess sufficient relatedness to the type species, *C. butyricum*, leading to the proposal of a new core genus, termed *Clostridium* sensu stricto (Lawson et al., 1993; Wiegel et al., 2006). The heterogeneity of the genus allows *Clostridium* as a whole to thrive within diverse habitats, as the clostridia are truly ubiquitous in nature. Owing to their ability to form endospores that are resistant to oxygen, heat, desiccation, acid, and alcohol, dormant spores of *Clostridium* are commonly found within soil, aquatic sediments, intestinal tracts of mammals, and unpasteurized or spoiled foods (Jones and Woods, 1986). From this rich diversity many species of *Clostridium* have arisen in recent years in both the medical and industrial sectors of biotechnology.

Clostridium can be divided into pathogenic and apathogenic species. Some of the most notorious and potent human toxins are produced by the pathogenic clostridia, which encompasses *C. botulinum*, *C. difficile*, *C. perfringens*, *C. septicum*, and *C. tetani* (Shone and Hambleton, 1989). *C. botulinum* and *C. tetani* produce two of the most potent neurotoxins and are the causative agents of the well-known diseases botulism and tetanus, respectively. The botulism toxin is the most toxic agent currently known to humans, as type H toxin was recently found to have a lethal dose of 2–13 ng for an average-sized human (Barash and Arnon, 2014). The advent of proper food handling and pasteurization practices in the case of botulism, and access to effective vaccination programs for the prevention of tetanus, have drastically diminished the prevalence of these fatal diseases within the developed world. Still, an estimated 58,000 fatal cases of newborn tetanus were reported globally in 2010 and, as of 2012, 31 countries have yet to effectively eliminate maternal and neonatal tetanus (World Health Organization; http://www.who.int/). *C. difficile*, on the other hand, produces two highly virulent exotoxins and is the leading cause of antibiotic-associated diarrhea and nosocomial infection (McFarland et al., 1989). Dormant spores of *C. difficile* often survive routine hospital sanitation practices, including exposure to alcohol-based hand rubs, and can be ingested through the fecal–oral route, allowing proliferation in the small intestine of patients with compromised gut flora, typically resulting from antibiotic treatment. Infection by *C. difficile* causes a range of intestinal maladies, including severe diarrhea, pseudomembranous colitis, septicemia, and death (Shone and Hambleton, 1989). The occurrence of healthcare-associated *C. difficile* infection has increased drastically over the past decade and has risen to epidemic status (Chalmers et al., 2010; Kelly and LaMont, 2008). Other pathogenic clostridia, particularly *C. perfringens* and *C. septicum*, are associated with food poisoning, gas gangrene, and meningitis, among other ailments (Shone and Hambleton, 1989). It is estimated that *C. perfringens* accounts for more than 10% of the 9.4 million annual cases of foodborne illness in the United States (Scallan et al., 2011).

In addition to the pathogenic clostridia, a number of medically-important species have garnered significant research focus in recent years. Of special interest is the anticancer property exhibited by certain apathogenic species (Van Mellaert et al., 2006). As deoxygenated tissue is generally only found in tumors, spores of *Clostridium* can be injected and targeted to anoxic regions of tumors with impeccable specificity, where they proliferate and promote cytotoxicity and oncolysis (tumor destruction). This idea was first demonstrated in 1955 when only cancerous mice contracted tetanus upon injection of *C. tetani* spores into both healthy and tumor-bearing specimens (Malmgren and Flanigan, 1955). Species of *Clostridium* found to possess significant anticancer properties include *C. acetobutylicum*, *C. butyricum*, *C. novyi*, *C. sporogenes*, and *C. tyrobutyricum* (Dang et al., 2001; Thiele et al., 1964; Van Mellaert et al., 2006). Strains exhibiting natural cytotoxic properties can also be modified genetically to express an anticancer agent, such as cytotoxic proteins, cytokines, and antigens or antibodies (Forbes, 2010). Expression of the corresponding anticancer genes can then be controlled through the use of an inducible genetic promoter, such as one induced by radiation (Nuyts et al., 2001a,b). As such, *Clostridium* anticancer therapy remains a highly active area of research, as reports detailing clostridial therapies lag only behind work with *Salmonella* (Forbes, 2010).

Within the apathogenic clostridia, several species have garnered immense interest in the field of industrial biotechnology as a result of their diversity of substrate utilization and unique metabolic capabilities (Tracy et al., 2012). While many clostridia produce standard fermentation products, including organic acids and carbon dioxide and hydrogen gases, a number of species produce varying amounts of alcohols and solvents, such as acetone, ethanol, 1,3-propanediol, isopropanol, and butanol, which have industrial potential as bulk solvents and prospective biofuels (Gottschalk et al., 1981). In fact, the exploitation of clostridia for large-scale production of commodity chemicals represents one of the first worldwide industrial bioprocesses (Jones and Woods, 1986). Prior to the dominance of the current petrochemical industry, large-scale production of acetone, and later butanol, as solvents (AB fermentation) was carried out by species of *Clostridium*. Fluctuating costs of molasses and maize feedstocks, coupled to the establishment of more economical petrochemical processes during the 1950s, however, led to the eventual downfall of fermentative AB production. A potential revival of the industrial AB fermentation for the production of butanol, this time as a promising biofuel, is currently underway in response to the mounting environmental and political issues surrounding the production and consumption of petroleum-based fuels (Awang et al., 1988). Unfortunately, many process shortcomings have yet to be resolved, including high feedstock costs, poor solvent yields, and product toxicity (Zheng et al., 2009). The revival of a competitive AB fermentation process depends upon the resolution of these fundamental issues. However, it is the capacity to genetically manipulate the solventogenic clostridia that determines the future success of clostridial production of bulk solvents and biofuels (Papoutsakis, 2008).

The overall state of genetic engineering within the *Clostridium* is sparse given the immense medical and industrial biotechnological

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potentials surrounding many species of *Clostridium*. Of the approximately 200 traditionally-classified species, only a small subset of the clostridia has been probed with genome sequencing and manipulated using gene transfer methods (Table 1). Further, numerous landmark advancements have been made since the advent of recombinant DNA technology (Papoutsakis, 2008), yet even the most genetically advanced clostridia lag far behind other microbes, especially the model organisms *Escherichia coli*, *Saccharomyces cerevisiae*, and *Bacillus subtilis*, in terms of access to genetic tools and technologies. In fact, only a small collection of clostridia have experienced any significant degree of genetic advancement. Accordingly, the objective of this review is two-fold. First, we aim to provide a comprehensive review of the genetic tools and methodologies currently available for the manipulation of clostridial species, with an emphasis on significant advancements made in recent years. Second, we present a series of technical guidelines for researchers aiming to genetically manipulate a *Clostridium* species or strain for which few or no previous genetic methodologies have been described. Comprehensive genetic work within the genus, including genome sequencing, the development of gene transfer procedures, host–vector systems, gene overexpression, knockout, and knockdown tools, and advanced genome editing technologies, is required for the advancement of lesser known species, non-type strains, and environmental isolates of *Clostridium*. In-depth experimental aspects of relevant genetic tools and techniques are discussed. A schematic template depicting the workflow involved in the development of clostridial host strains, from establishing plasmid transformation and genome sequencing through to advanced genome editing technologies, is shown in Fig. 1. Readers are also directed toward recent reviews pertaining to various aspects of clostridial genetics, metabolism, and pathogenicity discussed herein (Gheshlaghi et al., 2009; Lee et al., 2008; Lutke-Eversloh and Bahl, 2011; Minton, 2003; Papoutsakis, 2008; Tracy et al., 2012; Van Mellaert et al., 2006). Our hope is that this review facilitates the expansion and development of the genetic engineering tools available to the clostridia, so that the full potential of this important genus can be realized.

Table 1
Overview of the genus *Clostridium* with respect to available genome sequencing data and gene transfer procedures.

Clostridium species	Available genome sequencing data[a]	Published procedures for gene transfer[b]
Untapped clostridia		
59[c] or 184[d] species	41 species (63 strains)	2 species
Pathogenic clostridia		
C. botulinum	31 strains	+
C. difficile	218 strains	+
C. perfringens	11 strains	+
C. septicum	NA	+
C. tetani	1 strain	+
Medical clostridia		
C. acetobutylicum	3 strains	+
C. butyricum	6 strains	+
C. novyi	1 strain	−
C. sporogenes	2 strains	+
C. tetani	1 strain	+
C. tyrobutyricum	4 strains	+
Industrial clostridia		
C. acetobutylicum	3 strains	+
C. beijerinckii	2 strains	+
C. butyricum	6 strains	+
C. carboxidivorans	2 strains	−
C. cellulolyticum	1 strain	+
C. kluyveri	2 strains	−
C. ljungdahlii	1 strain	+
C. pasteurianum	3 strains	+
C. phytofermentans	1 strain	+
C. saccharoperbutylacetonicum	1 strain	+
C. thermocellum	7 strains	+
C. tyrobutyricum	4 strains	+

[a] Includes finished genomes and draft assemblies. NA: not available.
[b] Includes both transformation and conjugation methods. +: procedures for gene transfer have been developed. −: procedures for gene transfer have not yet been reported.
[c] Based on the core *Clostridium* sensu stricto genus (73 species).
[d] Based on the original *Clostridium* genus (approximately 203 species).

Strain background and phenotypes

Historically, the exploitation of *Clostridium* for the industrial production of acetone and butanol has centered around *C. acetobutylicum* and *C. beijerinckii* (Jones and Woods, 1986), and has led to the widespread investigation of these species with respect to genetics, physiology, and growth and metabolism. Numerous other *Clostridium* species, however, have garnered significant attention in recent years, largely the result of their unique and promising metabolic capabilities (Tracy et al., 2012). Examples include production of biofuels (ethanol or butanol) from diverse substrates, such as cellulose [e.g., *C. cellulolyticum* and *C. thermocellum* (Carere et al., 2008)], crude biodiesel-derived glycerol [*C. pasteurianum* (Taconi et al., 2009)], and synthesis gas [e.g., *C. ljungdahlii* and *C. carboxidivorans* (Munasinghe and Khanal, 2010)]. In each case, the degree of genetic advancement experienced by a *Clostridium* strain is dependent on the biotechnological potential harnessed by the target organism, the number of global research groups interested in its development, and the degree of inherent genetic tractability of the species. Unfortunately, genetic tools and methodologies are frequently strain-dependent within the clostridia, as the application of procedures across species, and even between strains, is difficult and often demands extensive experimentation. For this reason, the clostridia have experienced varying levels of success in genetic tool development and generally only one representative strain of each species has been employed as a host for genetic engineering and strain construction. Nevertheless, species that have proven amendable to extensive genetic manipulation include *C. acetobutylicum*, *C. cellulolyticum*, *C. perfringens*, *C. phytofermentans*, *C. thermocellum*, and *C. tyrobutyricum*.

The benchmark and model *Clostridium* for all strain construction endeavors is *C. acetobutylicum* ATCC 824. Over the past twenty years, work with this organism has led to the development of a host–vector system, electrotransformation protocol, gene reporter system, antisense-RNA gene knockdown and chromosomal gene knockout methodologies, a fully sequenced genome, and, most recently, chromosomal gene integration procedures (Al-Hinai et al., 2012; Heap et al., 2012; Papoutsakis, 2008). The progression of *C. acetobutylicum* as the model *Clostridium* has followed an iterative, bottom-up approach (Fig. 1), which has been broadly applied to the advancement of related clostridia in recent years. The major initial hurdle for genetic manipulation of *C. acetobutylicum* involved the identification and subsequent protection of plasmid DNA against a Type II restriction endonuclease, Cac824I (Mermelstein and Papoutsakis, 1993; Mermelstein et al., 1992). With a high-efficiency electrotransformation procedure in place, efforts were then focused on the selection of both constitutive and inducible promoters for plasmid-based gene expression using a gene reporter system (Tummala et al., 1999). Promoters conferring high-level gene expression were then utilized for antisense-RNA-mediated gene knockdown of key pathways involved in the central metabolism of *C. acetobutylicum* (Desai and Papoutsakis, 1999; Tummala et al., 2003b). The genome of *C. acetobutylicum* ATCC 824 was fully sequenced and annotated in 2001 (Nolling et al., 2001) and signified a major milestone as the first clostridial genome sequence to be completed. A consistent and reliable method for chromosomal gene disruption didn't appear until 2007 in *Clostridium*, which employs Group II intron technology adapted from methods developed for *E. coli* (Heap et al., 2007; Shao et al., 2007). Very recently, methodologies have arisen detailing the introduction of foreign DNA into clostridial chromosomes based on double-crossover homologous recombination (Al-Hinai et al., 2012; Heap et al., 2012), representing the current era of genetic engineering in *Clostridium*.

Owing to the ubiquity of clostridia in nature, there is a growing need for the ability to genetically manipulate non-type strains and environmental isolates in order to fully tap into the metabolic potential of the

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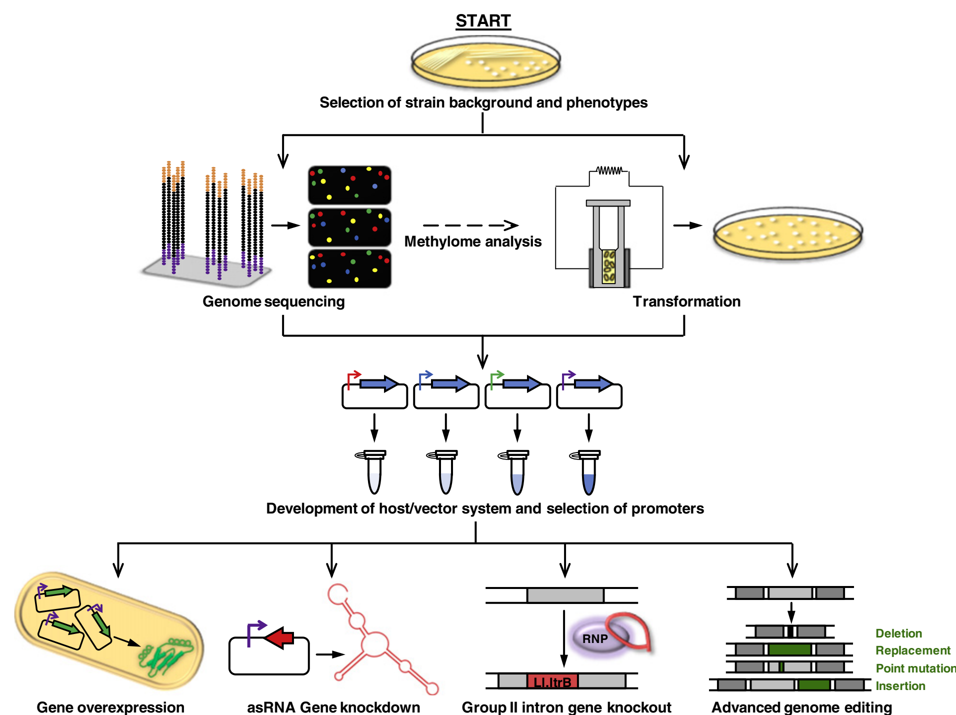

Fig. 1. Schematic diagram depicting the general workflow involved in clostridial strain development using conventional genetic methods. Genetic techniques are organized corresponding to the chronological order of development for *C. acetobutylicum*. The host strain for manipulation is selected based on its background or phenotypic properties. If genome sequencing data is desired and not available, one or a combination of next generation sequencing platforms are utilized. Methylome analysis can be performed from single-molecule real-time sequencing reads to provide methylation data that can aid in elucidating the recognition sites of putative restriction-modification systems. Gene transfer is then performed based on electrotransformation and, to a lesser extent, conjugation using appropriately methylated DNA. Plasmid transformation opens the door to several genetic techniques, all of which require a host/vector system and access to various gene promoters. These applications are gene overexpression, knockdown, and knockout, as well as advanced genome editing, and are selected for use based on the specific genetic or metabolic engineering objectives.

genus. Rather than engineering a heterologous pathway into a common, tractable laboratory organism, it is often advantageous to isolate an environmental strain that naturally expresses the target pathway or phenotype at a high level. Since the clostridia possess an extensive substrate-utilization range and produce an array of valuable metabolites, they provide far superior metabolic frameworks for strain development and metabolic engineering efforts (Tracy et al., 2012). Perhaps the most successful application of this approach involved the genetic development of the Q Microbe® (Qteros, Inc.; http://www.qteros.com/), an environmental isolate of *C. phytofermentans*. The strain was first isolated in 1996 in an attempt to identify superior biomass-degrading, ethanologenic bacteria (Warnick et al., 2002). In an approach analogous to that of *C. acetobutylicum*, *C. phytofermentans* has advanced as a promising industrial host strain for which an impressive repertoire of genetic methodologies have been developed, including conjugative gene transfer, intron-mediated gene knockout, plasmid-based overexpression, and chromosomal integration of non-replicative vectors (Tolonen et al., 2013). Work with *C. phytofermentans* has thus paved the way for the genetic modification of other novel environmental isolates of *Clostridium*.

Genome sequencing

In the current era of genomics and bioinformatics, where genome sequencing, assembly, and annotation can be outsourced for ever-decreasing costs, it is often unjustifiable to undertake genetic studies using an organism lacking genome sequencing data. Thus, as depicted in Fig. 1, a critical step when attempting to manipulate a clostridial host strain entails sequencing the target organism's genome. Recent bacterial genome sequencing projects have involved at least one of three major next generation sequencing (NGS) technologies; namely, Illumina dye sequencing, 454 pyrosequencing, and Pacific Biosciences single molecule real time sequencing (SMRT), all examples of DNA-polymerase-mediated sequencing-by-synthesis (Liu et al., 2012). Illumina dye sequencing and 454 pyrosequencing were developed in the 1990s, whereas SMRT sequencing arrived in 2009. As of 2013, an updated version of the SMRT technology has become available for commercial use. Although all three technologies differ in the length of reads and depth of coverage obtained, generally a single run of any of the three NGS technologies is insufficient to capture a full-length bacterial genome, resulting in a large number of noncontiguous sequences (Mavromatis et al., 2012). In order to move from a fragmented draft assembly to a finished genome, often a hybrid approach comprising more than one NGS platform is utilized, which drives up costs and demands accurate reads for effective error correction (Bashir et al., 2012). A recent exception to this principle, which is expected to exemplify the near future of NGS technology, involves a nonhybrid approach wherein a finished bacterial genome can be generated from a single DNA library using SMRT sequencing (Chin et al., 2013). An important advantage of this technique lies in its ability to resolve long repeat regions of DNA, which largely comprise the noncontiguous gaps found in most bacterial draft genome assemblies.

The first fully sequenced and annotated clostridial genome appeared in 2001 and was completed prior to the arrival of NGS technologies, thus relying on classical Sanger chain-termination sequencing (Nolling et al., 2001). Since this time, the genomes of many species of *Clostridium* and hundreds of distinct strains have been sequenced and analyzed using NGS platforms. Currently, the genome sequences from at least 22 *Clostridium* species have been fully completed, whereas draft assemblies or raw sequencing data exists for at least 37 additional species, bringing the total to 59 clostridial species, encompassing roughly 374 strains (Table 2). Within some species, only a single strain has been sequenced, whereas up to 218 strains of *C. difficile* have been partially or fully sequenced. A similar situation exists for *C. botulinum*, which includes

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31 whole genome sequencing projects, and embodies the intense medical interest in these notable human pathogens. Clostridial genomes vary drastically with respect to size (2.5 to 6.7 Mbp) and GC content (approximately 26 to 56%), illustrating the tremendous heterogeneity of the genus. Within a species, genome size and GC content can vary by greater than 1 Mbp [*C. acetobutylicum* (Wilkinson and Young, 1993), *C. botulinum* and *C. saccharolyticum*] and 5% (*C. saccharolyticum*), respectively. Several clostridia have been found to harbor extrachromosomal elements, as many as five in *C. botulinum* group II isolates (Skarin et al., 2011), including small, cryptic plasmids and megaplasmids up to several hundred kb in size (Iacobino et al., 2013).

Although genome sequencing is often carried out in conjunction with establishing a means of gene transfer for new or undeveloped host strains (Fig. 1), a fortunate and coincidental secondary outcome of SMRT sequencing can afford a substantial incentive to performing genome sequencing prior to carrying out transformation studies. As a result of inherent DNA polymerase kinetics, fluorescence measurements performed during SMRT sequencing differ reliably between unmodified and modified nucleotides, wherein different types of methylation can be distinguished with single base-pair resolution (Flusberg et al., 2010). In a prokaryotic context, the relevant modified bases that can be detected and differentiated are N6-methyladenine (6 mA) and N4- and N5-methylcytosine (4mC and 5mC, respectively). Accordingly, the ability to probe an organism's unique methylation signature, or methylome, has valuable implications for strain manipulation. Often the chief barrier to genetic manipulation lies in the restriction-modification systems expressed by the host organism, as transfer of foreign DNA into many species is potently hindered by restriction (see Section 4.1 below). Attaining a glimpse into an organism's methylome can allow identification of putative restriction-modification systems and help deduce DNA sequences recognized by such enzymes. Currently, methylome analysis is in its infancy and has only been performed on roughly 45 bacterial species, which includes *C. difficile*, *C. perfringens*, and *C. thermocellum* (Roberts et al., 2010). The utility of this strategy is highly dependent on the accurate detection of both 5mC and 6 mA, respectively considered the fifth and sixth nucleotide bases of DNA. Within current detection limits, 6 mA detection requires only 25× coverage, which is attainable from most SMRT sequencing projects. Unfortunately, 5mC demands at least 250× coverage for reliable determinations. Due to rapid advancement in NGS technology, these process shortcomings are expected to be resolved in the near future, leading to highly accurate de novo profiling of nucleotide modifications.

Establishing transformation

For genetic manipulation of bacterial strains, often the most imminent and difficult step involves the establishment of an efficient means of introducing foreign DNA into host cells, which paves the way to an array of valuable genetic applications (Fig. 1). Thus, establishing a methodology for efficient transfer of plasmid DNA is paramount for the development of superior *Clostridium* strains. Two means of DNA transfer dominate within *Clostridium*: conjugation and transformation. Bacterial conjugation involves direct cell-to-cell transfer of plasmid DNA from one donor species to the target, or recipient, species, while transformation involves the uptake of DNA by competent cells. Transformation is more commonly employed in the clostridia due to its technical simplicity, greater reliability, independence from donor species, and high efficiency of plasmid transfer (Pyne et al., 2013). For these reasons, transformation is typically attempted first and, if unsuccessful, conjugative plasmid transfer is then explored. Many methods of transformation have been utilized for plasmid transfer to bacteria. Within the clostridia, however, transformation via electroporation, or electrotransformation, is the chief method that allows high-level transformation. Electrotransformation was developed in the 1980s and was first used in *Clostridium* for the transfer of plasmid DNA to *C. perfringens* (Allen and Blaschek, 1988) and *C. beijerinckii* (Oultram et al., 1988). This paved the way for numerous other clostridial electrotransformations, leading to the genetic manipulation of approximately fifteen species of *Clostridium*, in addition to at least five other species that rely on conjugative plasmid transfer (*C. butyricum*, *C. phytofermentans*, *C. septicum*, *C. sordellii*, and *C. sporogenes*).

A summary of published clostridial electrotransformation procedures to date is shown in Table 3, which allows delineation of a consensus set of parameters to serve as a starting point when attempting to introduce plasmid DNA to an untransformed strain of *Clostridium*. Such conditions should permit at least low-level electrotransformation, which serves as a foundation for incremental modifications leading to high-level plasmid transfer. This strategy has been utilized effectively in *C. pasteurianum* (Pyne et al., 2013), in which the consensus clostridial conditions in Table 3 generated an initial electrotransformation efficiency of 10^1 transformants μg^{-1} was subsequently elevated to 10^4 transformants μg^{-1} through systematic improvements to the protocol.

Gram-positive bacteria, especially members of *Clostridium*, are among the most difficult bacteria to transform. Rarely can commonalities in protocols be applied between different species or strains without rigorous optimization. Further, some strains appear to be recalcitrant to electrotransformation. For example, the type species of *Clostridium*, *C. butyricum*, has proven refractory to transformation and has only been manipulated using interspecies conjugation with *E. coli* (Gonzalez-Pajuelo et al., 2006). Electrotransformation resistance in *Clostridium* is frequently the result of plasmid restriction by the host cell or from an inability of DNA to penetrate the thick peptidoglycan cell wall of Gram-positive bacteria (Pyne et al., 2013). Restriction can be overcome by appropriate methylation for protection of transforming plasmid DNA, whereas the Gram-positive cell wall can be weakened either through altering the physiological state of recipient cells or by modulating the electrical parameters of the electroporation pulse (Aune and Aachmann, 2010). Strategies to overcome clostridial electrotransformation resistance are discussed in greater detail in Sections 4.1–4.3. Further, a summary flowchart to achieve high-level electrotransformation when working with a new or untransformable strain of *Clostridium* is shown in Fig. 2. Following optimization of electotransformation, efficiencies on the order of 10^3 to 10^6 transformants μg^{-1} should ideally be obtained. High-level efficiencies are required for most genetic engineering applications, such as gene knockout and chromosomal gene integration (refer to Section 5 below). If poor electrotransformation efficiency results following extensive electrotransformation troubleshooting, conjugative plasmid transfer should be employed. Conjugative plasmid transfer has been shown to succeed in some instances where electrotransformation failed, presumably due to the ability of plasmid DNA to evade host restriction (Jennert et al., 2000). Discussion of the experimental factors involved in interspecies conjugation is beyond the scope of this review, as protocols for conjugative plasmid transfer are often more standardized and broadly applicable across different species. Readers are directed toward published protocols for conjugative plasmid transfer to several species of *Clostridium* (Jennert et al., 2000; Lyras and Rood, 1998; Purdy et al., 2002; Tolonen et al., 2009; Williams et al., 1990).

Presence of active restriction-modification systems

The presence of highly active restriction-modification (RM) systems is the most probable factor responsible for hindering electrotransformation. If improperly methylated plasmid DNA is used to transform even highly competent cells, few or no transformants are obtained (Mermelstein et al., 1992; Pyne et al., 2013). As such, an all-or-nothing effect on electrotransformation tends to be indicative of the presence of at least one active RM system. When attempting to transform a new or uncharacterized strain of *Clostridium*, often a key step entails assaying crude cell lysates for the presence of Type II restriction endonucleases by incubation of the active lysate in the presence of

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Table 2
Summary of completed and initiated whole genome sequencing projects. Data is adapted from the National Center for Biotechnology Information (NCBI) and Restriction Endonuclease database (REBASE).

Organism	Strains completed or initiated	Size of completed genomes (Mbp)	GC%	Extrachromosomal elements	M genes[b]
C. acetobutylicum	3 (completed)	4.13–4.15	30.9	1, 2	6
C. acidurici	1 (completed)	3.11	29.9	1	4
C. aldenense	1 (SRA or traces)	–	–	0	–
C. alkalicellulosi	1 (SRA or traces)	–	–	0	–
C. arbusti	1 (scaffolds or contigs)	–	29.8	0	4
C. asparagiforme	1 (scaffolds or contigs)	–	55.6	0	0
C. autoethanogenum	1 (scaffolds or contigs) 1 (SRA or traces)	–	31.0	0	4
C. bartletti	1 (scaffolds or contigs)	–	28.8	0	0
C. beijerinckii	1 (completed) 1 (scaffolds or contigs)	6.00	29.7–29.9	0	2, 4
C. bifermentans	2 (scaffolds or contigs) 1 (SRA or traces)	–	28.1–28.4	0	–
C. bolteae	6 (scaffolds or contigs) 2 (SRA or traces)	6.38[a]	48.6–49.6	0	0
C. botulinum	13 (completed) 16 (scaffolds or contigs) 2 (SRA or traces)	3.21–4.26	27.0–29.0	0, 1, 2, 5	0–7, 9
C. butyricum	6 (scaffolds or contigs)	–	28.5–28.8	0	3
C. cadaveris	1 (scaffolds or contigs) 4 (SRA or traces)	–	31.1	0	–
C. carboxidivorans	2 (scaffolds or contigs)	–	29.7	0	7, 8
C. celatum	1 (scaffolds or contigs)	–	27.7	0	–
C. cellulolyticum	1 (completed)	4.07	37.4	0	10
C. cellulovorans	1 (completed) 1 (scaffolds or contigs)	5.26	31.1–31.2	0	13, 14
C. citroniae	1 (scaffolds or contigs) 1 (SRA or traces)	–	48.9	0	1
C. clariflavum	1 (completed)	4.90	35.7	0	15
C. clostridioforme	10 (scaffolds or contigs) 1 (SRA or traces)	–	48.7–49.2	0	–
C. colicanis	1 (scaffolds or contigs)	–	26.1	0	–
C. difficile	9 (completed) 209 (scaffolds or contigs)	4.05–4.46	28.1–52.8	0, 1	0–5
C. diolis	1 (scaffolds or contigs)	–	29.7	0	2
C. hathewayi	3 (scaffolds or contigs)	–	48.1–50.0	0	1
C. hiranonis	1 (scaffolds or contigs)	–	31.0	0	1
C. hylemonae	1 (scaffolds or contigs) 1 (SRA or traces)	–	48.9	0	–
C. intestinale	1 (scaffolds or contigs)	–	30.2	0	–
C. kluyveri	2 (completed)	3.96–4.02	32.0	1	15
C. lentocellum	1 (completed) 1 (SRA or traces)	4.71	34.3	0	4
C. leptum	1 (scaffolds or contigs) 1 (SRA or traces)	–	50.2	0	5
C. ljungdahlii	1 (completed)	4.63	31.1	0	7
C. methoxybenzovorans	1 (scaffolds or contigs)	–	44.5	0	–
C. methylpentosum	1 (scaffolds or contigs)	–	51.8	0	–
C. nexile	1 (scaffolds or contigs)	–	40.1	0	6
C. novyi	1 (completed)	2.55	28.9	0	3
C. papyrosolvens	2 (scaffolds or contigs)	–	37.0–37.1	0	3, 6
C. paraputrificum	1 (scaffolds or contigs) 1 (SRA or traces)	–	29.6	0	–
C. pasteurianum	1 (completed) 1 (scaffolds or contigs) 1 (SRA or traces)	5.04	29.8–30.6	1	5, 8
C. perfringens	3 (completed) 8 (scaffolds or contigs)	2.96–3.26	28.1–28.6	0, 1, 2, 4	1, 2, 7, 8
C. phytofermentans	1 (completed)	4.85	35.3	0	2
C. ramosum	1 (scaffolds or contigs)	–	31.4	0	0
C. saccharobutylicum	1 (completed)	5.11	28.7	0	–
C. saccharolyticum	2 (completed)	3.77–4.66	45.0–50.2	0	11, 16
C. saccharoperbutylacetonicum	1 (completed)	6.67	29.5	1	3
C. sartagoforms	1 (scaffolds or contigs)	3.98	27.9	0	–
C. scindens	1 (scaffolds or contigs)	–	46.4	0	–
C. spiroforme	1 (scaffolds or contigs)	–	28.6	0	0
C. sporogenes	2 (scaffolds or contigs)	–	27.8–28.0	0	1, 6
C. sporosphaeroides	1 (scaffolds or contigs)	–	53.5	0	–
C. stercorarium subsp. stercorarium	1 (completed)	2.97	42.2–42.3	0	6
C. sticklandii	1 (completed)	2.72	33.3	0	10
C. symbiosum	3 (scaffolds or contigs)	–	47.7–48.2	0	0, 3
C. termitidis	1 (scaffolds or contigs)	–	41.2	0	2
C. tetani	1 (completed)	2.87	28.6	1	3

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Table 2 (*continued*)

Organism	Strains completed or initiated	Size of completed genomes (Mbp)	GC%	Extrachromosomal elements	M genes[b]
C. thermocellum	2 (completed) 5 (scaffolds or contigs)	3.56–3.84	39.0–39.1	0	5–7, 12
C. tunisiense	1 (scaffolds or contigs)	–	31.2	0	4
C. tyrobutyricum	4 (scaffolds or contigs)	–	30.8	0	8, 9
C. ultunense	1 (scaffolds or contigs)	–	40.9	0	9
Summary	59 species 374 strains	2.55–6.67	26.1–55.6	0–5	0–16

SRA: Sequence Read Archive.
[a] One strain (90A9) contains a single scaffold, allowing accurate genome size determination.
[b] Genes annotated as methyltransferases.

unmethylated plasmid DNA. Using this technique, Type II RM systems have been detected in *C. acetobutylicum* (Mermelstein et al., 1992), *C. cellulolyticum* (Jennert et al., 2000), *C. difficile* (Purdy et al., 2002), *C. pasteurianum* (Richards et al., 1988), and *C. thermocellum* and *C. thermosaccharolyticum* (Klapatch et al., 1996a). Analysis of cell lysates for restriction activity can also be coupled to methylome analysis via SMRT sequencing (see Section 3) to decipher the recognition sequence of putative restriction barriers.

Once a putative recognition sequence is identified, efforts can be made to protect, via methylation, the recognition sequences on transforming plasmids. For example, many *Clostridium* species produce isoschizomers of the *E. coli* Dam (*D*NA *a*denine *m*ethylase) RM system (Roberts et al., 2010), and therefore restrict DNA prepared from Dam^- *E. coli* hosts. Most common laboratory *E. coli* strains are Dam^+ and, therefore, such barriers are easily overcome. Other identified Type II RM systems from *Clostridium* possess more unique recognition sequences and DNA protection requires methylation from a specific methyltransferase. DNA methylation can be performed in vivo by expressing the methyltransferase in *E. coli* cloning strains or, ideally, in vitro if the methyltransferase is available commercially. Most clostridial Type II RM systems possess recognition sequences rich in GC [e.g., 5′-GCNGC-3′ for Cac824I from *C. acetobutylicum* (Mermelstein et al., 1992) and 5′-CGCG-3′ for CpaAI from *C. pasteurianum* (Richards et al., 1988)], owing to the low GC content that is a typical characteristic of the clostridia. In the case of *C. cellulolyticum*, a Type II RM system, CceI, was identified within cell lysates and found to cleave the sequence 5′-CCGG-3′, which can be conveniently protected by the commercial methyltransferase, M.MspI (5′-5mCCGG-3′), but not M.HpaII (5′-C5mCGG-3′), demonstrating the importance of methylation pattern within a given recognition sequence (Jennert et al., 2000). Unfortunately, few methyltransferases are commercially available and cloning of an appropriate methyltransferase for in vivo methylation is necessary. If genome sequencing data exists for the target species, the simplest in vivo methylation strategy involves expressing in *E. coli* the putative methyltransferase gene corresponding to the host restriction activity. This method can prove difficult, however, as clostridial genomes encode up to 16 methyltransferase genes (Table 2). Since restriction endonuclease genes are located adjacent to their respective methyltransferase, the list of candidate methyltransferases can be reduced to a smaller subset by identifying ones associated with a restriction endonuclease. The resulting methyltransferase genes can then be expressed individually in *E. coli* in an attempt to identify which gene product imparts protection of transforming plasmid DNA from host restriction. If the proper methyltransferase gene cannot be identified

Table 3
Summary of established clostridial electrotransformation procedures.

Organism	Growth phase (OD_{600})	EPB[a]	kV	kV cm^{-1}	μF	Ω	Transformants $μg^{-1}$	Reference
C. acetobutylicum ATCC 824	Early stat. (1.2)	SP (7.4)	2.0	5.0	25	∞	5.0×10^4	Harris et al. (2002); Mermelstein et al. (1992)
C. acetobutylicum DSM 792	Mid log. (0.7)	SP (6.0)	1.8	4.5	50	600	6.0×10^2	Nakotte et al. (1998)
C. beijerinckii NCIMB 8052	Mid log. (0.6)	SMP (7.4)	2.0	5.0	25	NR	2.9×10^3	Oultram et al. (1988)
C. beijerinckii NRRL B-592	Mid log.	SMH (7.4)	2.5	6.25	25	∞	NR	Birrer et al. (1994)
C. botulinum Hall A	Mid log. (0.8)	PEG	2.5	6.25	25	∞	3.4×10^3	Zhou and Johnson (1993)
C. botulinum ATCC 25765	(0.8)	SMP	2.0	10	25	400	0.8×10^4	Davis et al. (2000)
C. cellulolyticum ATCC 35319	Late log. (0.5–1.0)	SMP (7.4)	1.5	7.5	25	100	2.0×10^2	Jennert et al. (2000)
C. difficile P-881	NR	SMP (7.4)	2.5	6.25	25	200	Integration[b]	Ackermann et al. (2001)
C. ljungdahlii DSM 13528 (ATCC 55383)	Early log. (0.2–0.3)	SMP (6.0)	0.625	6.25	25	600	1.7×10^4	Leang et al. (2013)
C. paraputrificum M-21	(0.4–0.6)	S	0.5	5.0	25	400	5.6×10^3	Sakka et al. (2003)
C. pasteurianum ATCC 6013	Mid log. (0.6–0.8)	SMP (6.0)	1.8	4.5	25	∞	7.5×10^4	Pyne et al. (2013)
C. perfringens 3624A	Late log./stat. (0.85–1.3)	SMP (7.4)	2.5	6.25	25	NR	9.2×10^4	Allen and Blaschek (1988, 1990); Kim and Blaschek (1989)
C. saccharoperbutylacetonicum NI-4	(0.6)	MOPS (6.5)	NR	2.5	25	350	1.2×10^5	Nakayama et al. (2007)
C. sporogenes	NR	PEG	1.25	6.25	25	100	$1.0–2.0 \times 10^2$	Liu et al. (2002)
C. tetani CN655	Mid log.	SMP (7.4)	2.5	NR	25	200	NR	Marvaud et al. (1998a)
C. thermocellum DSM 1313	NR	CB	5.0[c]	25	NR	NR	2.2×10^5[d]	Tyurin et al. (2004)
C. thermocellum ATCC 27405							5.0×10^4[d]	
C. thermocellum DSM 4150							1.0×10^3[d]	
C. thermocellum DSM 7072							1.0×10^3[d]	
C. thermosaccharolyticum ATCC 31960	(1.05)	G	2.0	10	25	800	5.2×10^1	Klapatch et al. (1996b)
C. tyrobutyricum ATCC 25755	(0.8)	SMP (7.4)	2.5	6.25	25	600	Integration[b]	Zhu et al. (2005)
C. tyrobutyricum JM1	Late log.	SMP (7.4)	2.5	6.25	NR	NR	$3.0–4.0 \times 10^0$	Jo et al. (2010)
Consensus	Mid log. (0.5–0.8)	SMP (7.4)	2.0–2.5	5.0–6.25	25	200–600	$10^3–10^4$	Pyne et al. (2013)

NR: not reported.
[a] S: 270–272 mM sucrose, M: 1 mM $MgCl_2$, P: 5–7 mM sodium phosphate, H: 7 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), PEG: 10% polyethylene glycol 8000, MOPS: 65 mM 3-(*N*-morpholino)propanesulfonic acid, CB: 200 mM cellobiose, G: 20% glycerol.
[b] Non-replicating (i.e., integrative) plasmid was used.
[c] Square-wave pulse was administered.
[d] Induction of antibiotic selection prior to plating on selective media.

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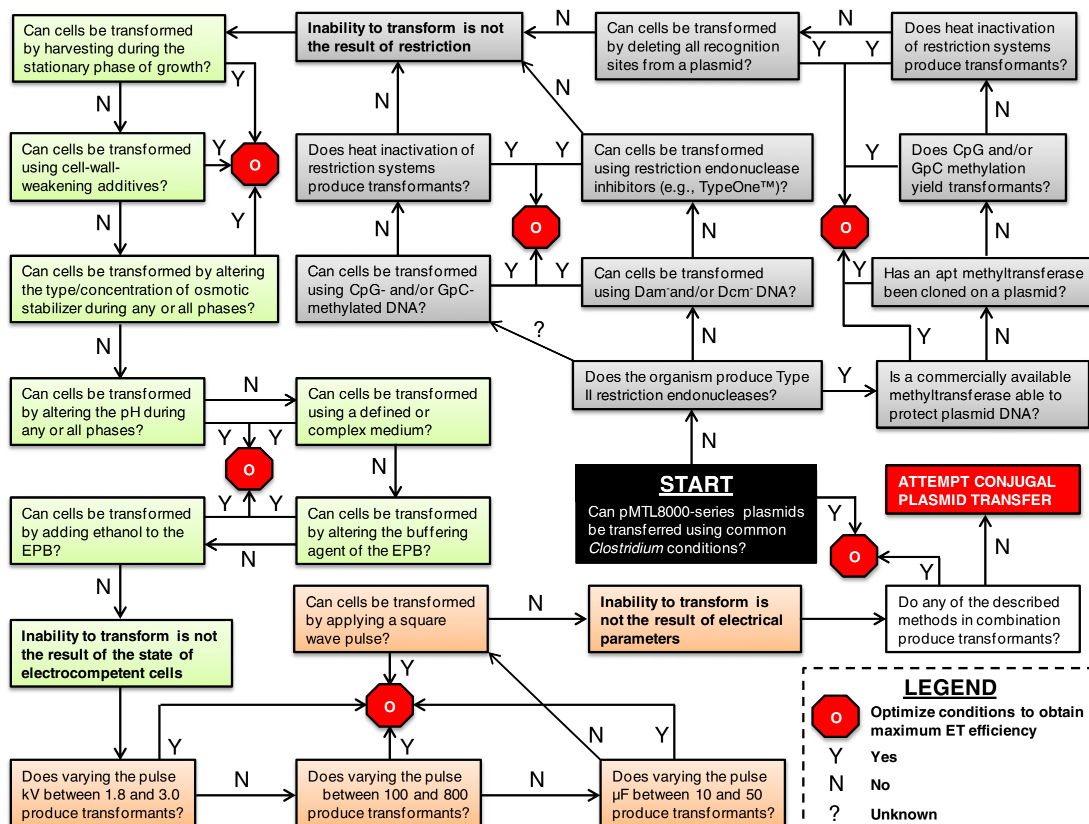

Fig. 2. Flow chart, modified from Saunders et al. (1984), for establishing an electrotransformation protocol in an untransformed or intractable *Clostridium* species. Factors affecting efficient transformation are grouped according to common electroporation barriers; namely, restriction (blue shading), competent cell physiology (green shading), and electrical parameters (red shading).

or if genome sequencing data is not available, an isoschizomeric methyltransferase from an alternative bacterium or bacteriophage can be utilized for in vivo methylation. For this purpose researchers at New England Biolabs have cloned a selection of methyltransferases in *E. coli* (Lunnen et al., 1988) and have compiled REBASE (Roberts et al., 2010), a comprehensive restriction-modification database, which can be used to identify sources of suitable methyltransferase genes. Restriction endonuclease or methyltransferase protein sequences can be used as search queries against the entire REBASE to identify unknown recognition sequences and identify putative isoschizomeric methyltransferases for in vivo methylation. The above strategy has been employed for electrotransformation of *C. acetobutylicum* and *C. pasteurianum*, whereby methyltransferase genes from bacteriophage ϕ3T and *Fusobacterium nucleatum*, respectively, are expressed from p15A-based *E. coli* vectors for methylation of co-resident ColE1-based *E. coli*–*Clostridium* shuttle vectors (Mermelstein et al., 1992; Pyne et al., 2013).

In cases where neither commercial methyltransferases nor suitable methyltransferase genes are available, other methods have found utility in evading RM systems in a range of bacteria (Fig. 2). For example, it has been shown that RM systems in some organisms can be temporarily heat-inactivated, allowing transformation to occur unimpeded by restriction. Inactivation regimens differ widely among bacteria with respect to duration of exposure, including 55 °C for 15 min for *C. acetobutylicum* (polyethylene-glycol-mediated protoplast transformation) (Lin and Blaschek, 1984) and 56 °C for 2 min for *Staphylococcus carnosus* (Lofblom et al., 2007). This method has not been demonstrated in a clostridial electrotransformation context, but should prove effective in some mesophilic clostridia. An alternative method involves methylation of transforming DNA with the commercially available CpG (M.SssI) and/or GpC (M.CviPI) methyltransferases, which yield 5′-5mCG-3′ and 5′-G5mC-3′ target sequences, respectively. Theoretically, methylation from one or both of these methyltransferases should confer protection against most known clostridial Type II restriction endonucleases as a result of the high occurrence of G and C bases in clostridial recognition sequences [REBASE (Roberts et al., 2010)]. Many bacteria, however, including *E. coli*, potently restrict DNA possessing abundant 5mC residues (Raleigh and Wilson, 1986). Consequently, extensively CpG- and GpC-methylated substrates failed to transform *C. pasteurianum*, despite adequate protection against the CpaAI (5′-CGCG-3′) Type II endonuclease (Pyne et al., 2013). Although RM systems generally lead to an all-or-nothing effect on transformation, some transforming plasmids can escape restriction and become methylated by the endogenous methyltransferase, allowing propagation. Therefore, if at least one transformant can be obtained, plasmid DNA can then be isolated for re-transformation into the host strain. If a Type II endonuclease is responsible for inhibiting electrotransformation, utilizing plasmid DNA isolated from the same host should transform several orders of magnitude better than *E. coli*-prepared plasmid DNA, as demonstrated in *C. septicum* (Kennedy et al., 2005) and *C. acetobutylicum* (Mermelstein et al., 1992). This strategy represents a classical approach for determining the presence of RM systems and, if practical, for overcoming the transformation barriers posed by such RM systems. Finally, all known restriction recognition sequences can be mutated from a transforming plasmid to evade host restriction. However, the recognition sites of many clostridial Type II endonucleases occur frequently, especially within the *E. coli* regions of *E. coli*–*Clostridium* shuttle vectors, making this method less practical.

While most bacterial RM systems acting on transforming DNA substrates are Type II, Types I, III, and IV restriction endonucleases can also drastically reduce electrotransformation efficiencies. Type I and III restriction endonucleases are more complex than Type II systems, as

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they cleave non-specifically and at regions remote from their recognition sequences. Nevertheless, at least one mode of overcoming Type I restriction is commercially available. Named TypeOne™ Restriction Inhibitor (Epicentre; http://www.epibio.com/), the bacteriophage T7 Ocr protein has been shown to increase electrotransformation efficiency by several orders of magnitude in *E. coli*, *Salmonella typhimurium*, and *C. ljungdahlii* (Kopke et al., 2010; Walkinshaw et al., 2002). Alternatively, Type IV systems possess a reversed RM specificity by cleaving methylated recognition sequences, such as restriction of 5′-S5mCNGS-3′ (where S = G or C; N = A, C, G, or T) by SauUSI from *Staphylococcus aureus* subsp. *aureus* USA300 (Xu et al., 2011). Compared to Type II enzymes, the respective mechanisms and recognition sequences of Type I, III, and IV RM systems remain largely unknown. Improvements in SMRT sequencing and subsequent methylome analysis are expected to decipher the recognition sites of many Type I, III, and IV RM systems and determine the relative contribution of such systems to various bacterial transformations.

Physiological state of electrocompetent cells

Inability to transfer plasmid DNA to a recipient cell can also be the result of the physical barrier imposed by the Gram-positive cell wall (Aune and Aachmann, 2010). Since the cell wall is continuously remodeled throughout the course of bacterial growth, the growth phase of cells at the time of harvest is often critical. Whereas it is usually optimal to use mid-logarithmic-phase cells for electrotransformation (Table 3), some species of *Clostridium* undergo autolysis during stationary phase, which has been found to enhance permeabilization. Electrotransformation of *C. perfringens* is optimum using cells harvested from the late logarithmic or stationary phase, as mid-logarithmic-phase cells cannot be electrotransformed (Kim and Blaschek, 1989). The structure and density of the cell wall can also be altered by the formulation of the growth medium and wash and electroporation buffer (EPB), including the pH and buffer type and strength, in addition to the presence of cell-wall-disrupting agents and associated osmotic stabilizers (Aune and Aachmann, 2010). Work with *C. perfringens* has demonstrated the importance of maintaining a favorable and consistent pH during all phases of electrotransformation, as adjusting the pH of the recovery and plating media to match that of the growth medium afforded a 2- to 6-fold enhancement in electrotransformation efficiency (Allen and Blaschek, 1990). On the other hand, the majority of clostridia are best electrotransformed using an EPB consisting of 5–7 mM sodium phosphate buffer, pH 7.4, 1 mM magnesium chloride, and isotonic (270 mM) sucrose (Table 3). However, in addition to 10–20% glycerol or polyethylene glycol solutions, it is often beneficial to assess the effect of alternative EPBs, such as HEPES and MOPS buffering systems, which have been shown to affect permeabilization of the clostridia (Birrer et al., 1994; Klapatch et al., 1996b; Nakayama et al., 2007; Zhou and Johnson, 1993).

The most robust method for enhancing electrotransformation of *Clostridium* and other Gram-positive organisms entails the application of cell-wall-weakening additives, muralytic enzymes, and membrane-solubilizing agents. Various cell-wall-weakening compounds, including glycine, isonicotinic acid hydrazide (isoniacin), penicillin G and ampicillin, and DL-threonine, have all been investigated for stimulating clostridial electrotransformation (Cui et al., 2012; Pyne et al., 2013; Tyurin et al., 2004; Zhu et al., 2005). Glycine (Hammes et al., 1973) and DL-threonine (Chassy, 1976) weaken the cell wall through incorporation and subsequent disruption of cross-linking, while isoniacin (Hermans et al., 1990) and penicillin G and ampicillin (Blumberg and Stroming, 1974) inhibit key enzymes involved in cell wall synthesis. Glycine was found to significantly stimulate electrotransformation of *C. pasteurianum* (Pyne et al., 2013) and *C. cellulolyticum* (Cui et al., 2012), and DL-threonine is indispensable for plasmid DNA transfer to *C. tyrobutyricum* (Zhu et al., 2005) and, to a lesser extent, *C. ljungdahlii* (Kopke et al., 2010). Further, muralytic enzymes, such as lysostaphin and lysozyme, have proven effective for the partial removal of cell walls prior to transformation in *C. perfringens* (Reysset, 1993; Scott and Rood, 1989). Still, many species of *Clostridium* do not require any special compound or enzyme treatments to achieve high-level electrotransformation and the effect of such additives on electrotransformation cannot be foreseen without experimentation. Since these additives are strongly detrimental to growth, effective cell wall disruption must be carefully balanced with cell viability (Aune and Aachmann, 2010). Thus, cell-wall-weakening compounds and muralytic enzymes are added during cell growth, typically during logarithmic phase, or for a minimal span of time immediately prior to electrotransformation. Regardless of timing, exposure to cell-wall-weakening additives creates an increased requirement for osmotic stabilization, as cells with disrupted cell walls are highly susceptible to growth inhibition, cytoplasmic leakage, and death. In fact, the concentration and duration of exposure of such additives are dependent on the amount of osmotic stabilizer present in the growth medium, as typically a greater concentration of osmoprotectant permits more extensive cell wall disruption (Aune and Aachmann, 2010). Sucrose, ranging from 0.25 to 0.5 M, is the most common osmotic stabilizer and is supplied to yield an iso- or hypertonic environment. Osmotic stabilization has proven vital not just during exposure to cell-wall-weakening additives, but also during all stages of electrotransformation, including washing, pulse delivery, and recovery (Pyne et al., 2013). Finally, in an analogous manner to cell wall disruption, the cellular membrane of bacteria can be disturbed via partial solubilization in the presence of ethanol for *E. coli* (Sharma et al., 2007), *Oenococcus oeni* (Assad-Garcia et al., 2008), and *C. pasteurianum* (Pyne et al., 2013), and Tween 80 for *Bacillus amyloliquefaciens* (Zhang et al., 2011).

Electrical parameters

Since electrotransformation depends on the development of transient pores in cellular membranes through the delivery of an electric pulse, the electrical parameters of the administered pulse are critical for effective permeabilization and plasmid transfer. Electroporation pulses are typically described by the voltage (kV) or field strength (kV cm^{-1}), capacitance (μF), and resistance (Ω). In addition, the duration of the pulse is described using the time constant, which is the product of the total resistance and capacitance of the circuit, or the time it takes for a pulse to exponentially decrease to 37% of its initial peak voltage (Bio-Rad; http://www.bio-rad.com/). While eukaryotic cells require moderate field strengths for efficient electrotransformation, smaller bacterial cells necessitate high field strengths up to 18 kV cm^{-1}. The majority of the clostridia, however, are transformed optimally with field strengths between 5.0 and 6.25 kV cm^{-1}, generally corresponding to voltages of 2.0–2.5 kV (Table 3). For high-level electrotransformation to occur, it is often assumed that a significant proportion of the cell population might not survive pulse delivery. Therefore, the strength of the electric pulse must be balanced with cell viability. The strength of the electric pulse is also dependent on the physiological state of the recipient cells at the time of electroporation, as cells possessing weakened cell walls require lower voltages to achieve adequate electrotransformation compared to untreated cells (Aune and Aachmann, 2010). Glycine-treated *C. pasteurianum* (Pyne et al., 2013) and DL-threonine-treated *C. ljungdahlii* (Kopke et al., 2010), therefore, utilize low voltages of 1.8 and 0.625 kV, respectively. In terms of capacitance, all clostridial electrotransformation protocols described to date, except one developed for *C. acetobutylicum* (Nakotte et al., 1998), utilize capacitors of 25 μF, although this parameter can be modulated to optimize the pulse time constant. In a similar manner, the pulse resistance can be varied to alter the duration of the electric pulse. Parallel resistors between 100 to 800 Ω, or ∞ Ω (absence of parallel resistors), are effective for transforming *Clostridium* species (Table 3), and should be investigated when developing an electrotransformation methodology for a new or untransformed strain. Finally, rather than traditional exponential-

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decay pulses, which are the most commonly employed electroporation pulses, square wave pulses have found utility within the *Clostridium*. Square wave pulses maintain a consistent voltage throughout the duration of the pulse and have led to increased electrotransformation efficiencies in *C. acetobutylicum* (Tyurin et al., 2000) and *C. thermocellum* (Tyurin et al., 2004).

Development and application of genetic tools and techniques

Development of host/vector system and selection of promoters

With an efficient method of DNA transfer in place, development of a host–vector system, encompassing a broad selection of antibiotic-resistance determinants and functional replication origins, is paramount for effective strain manipulation in the clostridia. Recombinant strains of most species are selected using either erythromycin/clarithromycin (from *ermB*) or thiamphenicol/chloramphenicol (from *catP*), although determinants for spectinomycin- and tetracycline-resistance have been effective for some species (Heap et al., 2007). Recent work has also led to the construction of a series of vectors containing four functional and distinct origins of replication and four common clostridial selectable markers (Heap et al., 2009). Different replication origins can then be easily assessed, as the superior origin has been shown to differ between species. Segregational stabilities of the four modular clostridial origins were found to vary between 69.0 and 99.9% per generation for *C. acetobutylicum*, *C. botulinum*, *C. difficile*, and *C. tyrobutyricum* hosts (Heap et al., 2009; Yu et al., 2012). Yu et al. (2012) demonstrated the importance of proper replicon selection by heterologously expressing the *C. acetobutylicum adhE2* gene in *C. tyrobutyricum* from four shuttle vectors, each harboring a different modular clostridial origin. While segregational stability was greater than 95% for three of the four vectors, conjugative plasmid transfer efficiency varied by an order of magnitude between the vectors. More importantly, butanol titers differed by more than 7-fold (0.90 to 6.87 g L^{-1}), suggesting profound implications for plasmid-based metabolic engineering strategies. While segregational stability is often regarded as a desirable feature of host/vector systems, pronounced plasmid stability in the absence of antibiotic selection hinders certain strain construction applications in which plasmid loss is a necessary requirement (refer to Section 5.4).

In addition to selectable markers and replication origins, evaluation and selection of strong gene promoters are needed. The genetic techniques depicted in Fig. 1, specifically plasmid-based gene overexpression, antisense-RNA-mediated gene knockdown, chromosomal gene knockout, and advanced genome editing technologies, demand access to a repertoire of both constitutive and inducible promoter systems. In order to assess promoter function and strength, numerous gene reporter systems have been adapted or developed for use in *Clostridium* (Table 4). Among these, *lacZ* and *gusA* have been most widely used for genetic studies. In each case, the selected promoterless reporter gene is cloned into a shuttle vector for transcriptional fusion with various promoters in order to evaluate relative promoter strength. Promoters from genes involved in the central carbon metabolism of the clostridia are presumed to be strong and are commonly chosen for investigation. Acetoacetate decarboxylase (*adc*), phosphotransbutyrylase (*ptb*), and thiolase (*thl*) gene promoters (Cui et al., 2012; Tummala et al., 1999; Yu et al., 2011) have been extensively applied for gene expression in *C. acetobutylicum* and related clostridia. Promoters from other central fermentative genes leading to high-level gene expression include ones from ferredoxin [*fdx*; *C. pasteurianum* and *C. sporogenes* (Heap et al., 2007, 2010)], butanol dehydrogenase [*bdh*; *C. saccharoperbutylacetonicum* (Nakayama et al., 2008)] and pyruvate:ferredoxin oxidoreductase [*pfo*; *C. phytofermentans* (Tolonen et al., 2009)].

Certain genetic applications require the use of inducible promoter systems, resulting from either the toxicity or the desire for transient expression of the encoded protein. As a result, inducible gene expression systems have recently been adapted for use in the clostridia, with induction arising from either biotic or abiotic inducers (Table 5). These systems include a xylose-inducible promoter (Girbal et al., 2003), a *lac* operator-containing ferredoxin promoter (*fac* promoter) induced by isopropyl β-D-1-thiogalactopyranoside (Heap et al., 2007), a UV-inducible *recA* promoter (Nuyts et al., 2001a,b), a *tetO1* operator-containing promoter induced by anhydrotetracycline (Dong et al., 2012), and, most recently, a lactose-inducible promoter (Al-Hinai et al., 2012; Hartman et al., 2011). Clostridial promoter–repressor systems developed to date vary drastically with respect to stringency, as expression in the presence of inducer has been found to increase from 1.4- up to approximately 120-fold. For an optimum inducible expression system, promoter activity should be efficiently repressed in the absence of inducer and activated to a high level upon induction. Of all clostridial inducible promoters, it appears the anhydrotetracycline-inducible Pcm-2tetO1 promoter (Dong et al., 2012) is the most stringent system currently available (Table 5). Also, another recent inducible gene expression system, based on lactose induction in both *C. acetobutylicum* (Al-Hinai et al., 2012) and *C. perfringens* (Hartman et al., 2011), also exhibits marked repression in the absence of inducer and has been utilized successfully for the propagation of a plasmid harboring a functional *mazF* gene, the expression of which is lethal. The clostridial inducible promoters should prove indispensable for future genetic applications requiring transient gene expression or strict control of intracellular protein levels, particularly for processes involving toxic or lethal gene products.

Plasmid-based gene overexpression

Gene overexpression applications encompass both homologous and heterologous expression strategies. While homologous overexpression aims to amplify the activity of a resident chromosomal gene through increasing gene dosage, heterologous strategies are commonly utilized to impart a foreign or non-native activity to the host strain. Many clostridial plasmid-based gene overexpression strategies have been reported in the past twenty years, most of which involve *C. acetobutylicum* (Table 6). Several genes involved in the central fermentative metabolism of *C. acetobutylicum* have been overexpressed individually and in combination, including the aldehyde/alcohol dehydrogenases, *aad*/*adhE1* (Nair and Papoutsakis, 1994; Sillers et al., 2009) and *adhE2* (Sillers et al., 2008), thiolase, *thl* (Sillers et al., 2009), and acetone/solvent formation operons, *adc-ctfAB* (Mermelstein et al., 1993) and *aad-ctfAB* (Lee et al., 2009). These strategies have led to both positive and negative outcomes in terms of butanol and total solvent titers, demonstrating the limitations of rational metabolic engineering approaches in clostridia with highly complex and branched fermentations. Non-solventogenic pathway genes that have been overexpressed in *C. acetobutylicum* include the master sporulation transcriptional regulator, *spo0A* (Alsaker et al., 2004; Harris et al., 2002), and the class I heat shock response operon, *groESL* (Tomas et al., 2003), yielding important mutant strains with enhanced solvent production and tolerance. Homologous gene expression has also been performed in related clostridia. In analogous studies, regulatory proteins controlling botulism [*botR*/*A*; (Marvaud et al., 1998b)] and tetanus [*tetR*; (Marvaud et al., 1998a)] toxin production were overexpressed in their respective hosts, *C. botulinum* and *C. tetani*, allowing characterization of their previously unknown roles in toxin production. Clostridial hydrogenases, encoded by *hydA*, have also been common targets for homologous gene overexpression due to their central roles in redox balance during fermentative growth. Hydrogenases from *C. acetobutylicum* (Girbal et al., 2005; Klein et al., 2010), *C. paraputrificum* (Morimoto et al., 2005), and *C. tyrobutyricum* (Jo et al., 2010) have been homologously overexpressed in their respective hosts. As shown with other central fermentative genes, overexpression of hydrogenases in clostridial species often yields unpredictable outcomes, ranging from drastic effects on redox balance and product distribution (Jo et al., 2010; Morimoto et al., 2005) to no detectable changes in growth and metabolism (Klein et al., 2010).

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Table 4
Summary of clostridial gene-reporter systems developed to date.

Gene	Expression host	Native organism	Reference
Chloramphenicol acetyltransferase (*catP*)	*C. perfringens*	*C. perfringens*	Matsushita et al., 1994
β-Galactosidase (*lacZ*)	*C. acetobutylicum*	*Thermoanaerobacterium thermosulfurogenes*	Tummala et al., 1999 Feustel et al., 2004
β-1,4-Endoglucanase (*eglA*)	*C. beijerinckii*	*C. acetobutylicum*	Quixley and Reid, 2000
Luciferase (*luxAB*)	*C. perfringens*	*Vibrio fischeri*	Phillips-Jones, 2000
(*lucB*)	*C. acetobutylicum*	*Photinus pyralis*	Feustel et al., 2004
β-GLUCURONIDASE (*gusA*)	*C. acetobutylicum*	*E. coli*	Girbal et al., 2003
Green fluorescent protein (*gfp*)	*C. cellulolyticum*	*Pseudomonas putida*	Cui et al., 2012

In contrast to homologous overexpression strategies, expression of foreign or non-native activities in *Clostridium* is a relatively new avenue of research. Nevertheless, work from the past decade has led to the engineering of *C. acetobutylicum* for the expression of a range of heterologous activities, including most notably components of a functional minicellulosome from *C. cellulolyticum* and *C. thermocellum* (Chanal et al., 2011; Kovacs et al., 2013; Mingardon et al., 2005, 2011; Perret et al., 2004a), a 1,3-propanediol production pathway from *C. butyricum* (Gonzalez-Pajuelo et al., 2005, 2006), a *C. beijerinckii* primary/secondary alcohol dehydrogenase (Lee et al., 2012) and acetoin reductase (Siemerink et al., 2011) for conversion of acetone to isopropanol or 2,3-butanediol, respectively, and transaldolase from *E. coli* (Gu et al., 2009) for improved xylose utilization. A number of prokaryotic and eukaryotic hydrogenases have also been heterologously expressed in *C. acetobutylicum*, including ones of clostridial (Klein et al., 2010) and algal (Girbal et al., 2005) origins. In other instances, a genetically-tractable species is utilized to study the genetics of a related strain for which genetic tools are not available, as has been shown through the investigation of *C. difficile* pathogenicity regulatory elements in *C. perfringens* [e.g., (Mani and Dupuy, 2001; Mani et al., 2002; Matamouros et al., 2007)]. Conversely, solventogenic genes from *C. acetobutylicum*, the most tractable *Clostridium*, have been heterologously expressed in hopeful alternative hosts for which genetic tools have been recently developed, such as *C. ljungdahlii* (Kopke et al., 2010) and *C. tyrobutyricum* (Yu et al., 2011, 2012). While efforts with *C. ljungdahlii* have led to only modest titers of butanol (approximately 0.15 g L^{-1}) and demand more extensive metabolic engineering strategies, mutants of *C. tyrobutyricum* expressing a heterologous *adhE2* gene from *C. acetobutylicum* have been reported to produce butanol with titers of 10–20 g L^{-1}. Finally, *C. cellulolyticum* has shown promise as a host for the production of ethanol and isobutanol from cellulose through construction and expression of synthetic, chimeric operons harboring heterologous genes from *E. coli*, *B. subtilis*, *Lactococcus lactis*, and *Zymomonas mobilis* (Guedon et al., 2002; Higashide et al., 2011).

Owing in part to the low GC content of most industrially- and medically-significant clostridia (roughly 27–32%; Table 2), codon bias is an important consideration for heterologous gene expression. *C. perfringens* exhibits one of the strongest codon biases of all bacteria analyzed to date (Musto et al., 2003; Sharp et al., 2005). Of the six codons encoding arginine, *C. perfringens* favors AGA by a factor of approximately 10–260× relative to four of the other degenerate codons, while the remaining CGG codon is essentially unused (Musto et al., 2003). Genes that are highly expressed have been found to exhibit a greater degree of codon bias, whereas poorly expressed genes tend to follow a more random selection of codons (Gouy and Gautier, 1982). Nevertheless, codon optimization is commonly avoided in clostridial heterologous gene expression approaches [e.g., (Girbal et al., 2005; Gu et al., 2009)], especially those involving large operons and multi-subunit gene products (Guedon et al., 2002; Higashide et al., 2011). Instead, genes or operons of interest are often amplified from the native organism's DNA and cloned directly to the host strain for heterologous expression. Although this strategy is often adequate when cloning genes between related clostridia, which are expected to possess similar codon biases, poor expression is expected to arise when expressing genes from distantly related organisms (Gustafsson et al., 2004). Therefore, codon optimization via continuously-improving commercial gene synthesis will allow the generation of superior clostridial strains through the improved expression of heterologous genes, operons, and metabolic pathways.

asRNA gene knockdown

In contrast to plasmid-based overexpression strategies, antisense RNA (asRNA) molecules possessing complementarity to the mRNA transcript of a target gene can be utilized to decrease, yet not entirely abolish, protein expression. Accumulating evidence from transcriptome studies suggests that asRNA transcription occurs naturally in the bacterial kingdom. Approximately 1000 different asRNAs have been identified in *E. coli* (Dornenburg et al., 2010) and the transcriptome of *B. subtilis* encompasses asRNAs for 18% of the 506 genes analyzed (Lee et al., 2001). The basic premise of asRNA techniques lies in the interaction of the asRNA with its complementary mRNA target, forming duplex RNA with altered the secondary structure and potentially reduced stability and half-life. Antisense RNA, therefore, allows inhibition

Table 5
Summary of clostridial inducible promoter–repressor systems developed to date.

Organism	Basal promoter	Repressor	Operator	Inducer	Maximum inducibility[a]	Reference
C. acetobutylicum ATCC 824	*recA* (*C. acetobutylicum*)	*dinR* (endogenous)	Cheo box	2 Gy (radiation)	1.4- to 4.1-fold	Nuyts et al. (2001a) Nuyts et al. (2001b)
C. acetobutylicum ATCC 824	*xylA* (*Staphylococcus xylosus*)	*xylR* (*S. xylosus*)	xylO	10 g/l xylose (in absence of glucose)	18-fold	Girbal et al. (2003)
C. botulinum ATCC 3502 *C. difficile* 630 *C. sporogenes* NCIMB 10696	*fdx* (*C. pasteurianum*)	*lacI* (*E. coli*)	lacO	1 mM IPTG	NR	Heap et al. (2007)
C. acetobutylicum ATCC 824	*catP* (*C. perfringens*)	*tetR* (*E. coli*)	2× tetO1	100 ng/ml anhydrotetracycline	41- to 119-fold	Dong et al. (2012)
C. acetobutylicum ATCC 824 *C. perfringens* SM101 *C. perfringens* JGS4143 *C. perfringens* 13	bgaL (*C. perfringens*)	*bgaR* (*C. perfringens*)	NR	10 mM lactose	10- to 15-fold (*C. acetobutylicum*) 80-fold (*C. perfringens*)	Al-Hinai et al. (2012) Hartman et al. (2011)

[a] Calculated by dividing reporter enzyme activity under induction by the corresponding activity under uninduced conditions.

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Table 6
Summary of notable clostridial homologous and heterologous gene overexpression strategies.

Host organism	Gene(s)/operon	Phenotype relative to control strain(s)	Reference
Homologous overexpression			
Clostridium acetobutylicum ATCC 824/DSM 792	Acetone operon; *adc-ctfAB*	Higher yield of solvents	Mermelstein et al. (1993)
	Sporulation transcriptional regulator; *spo0A*	Enhanced solvent formation and tolerance	Alsaker et al. (2004), Harris et al. (2002)
	Class I heat shock genes; *groESL*	Increased solvent titers and tolerance	Tomas et al. (2003)
	Hydrogenase; *hydA*	NR	Girbal et al. (2005)
		Unaltered hydrogen yield and productivity	Klein et al. (2010)
	Aldehyde/alcohol dehydrogenase; *aad*	Increased ethanol and acetate production	Nair and Papoutsakis (1994) Sillers et al. (2009)
	Thiolase; *thl*	Increased acid and decreased solvent production	Sillers et al. (2009)
	Aldehyde/alcohol dehydrogenase; *adhE2*	Partially rescued butanol production[a]	Sillers et al. (2008)
	Solvent operon; *aad-ctfAB*	Fully rescued butanol production with acetate accumulation[a]	Lee et al. (2009)
C. botulinum A strain 62	Botulism neurotoxin regulatory protein; *botR/A*	Increase in botulism neurotoxin production	Marvaud et al. (1998b)
C. paraputrificum M-21	Hydrogenase; *hydA*	Increased hydrogen gas productivity and acetic acid production	Morimoto et al. (2005)
C. tetani CN655	Tetanus toxin regulatory protein; *tetR*	Increase in tetanus toxin production	Marvaud et al. (1998a)
C. tyrobutyricum JM1	Hydrogenase; *hydA*	Increased hydrogen yield	Jo et al. (2010)
Heterologous overexpression			
Clostridium acetobutylicum ATCC 824/DSM 792	Minicellulosome components (*C. cellulolyticum* + *C. thermocellum*)	Secretion of active, heterologous and chimeric cellulases and scaffoldin proteins	Chanal et al. (2011) Kovacs et al. (2013) Mingardon et al. (2011) Mingardon et al. (2005) Perret et al. (2004a)
	1,3-Propanediol operon; *dhaB1-dhaB2-dhaT* (*C. butyricum*)	1,3-Propanediol as major fermentation product[a]	Gonzalez-Pajuelo et al. (2005)
	Hydrogenase; *hydA* (algal) (*C. butyricum*)	NR	Girbal et al. (2005)
		Unaltered hydrogen yield and productivity	Klein et al. (2010)
	Transaldolase; *talA* (*E. coli*)	Enhanced xylose utilization and solvent production	Gu et al. (2009)
	Acetoin reductase; *acr* (*C. beijerinckii*)	Conversion of acetone to 2,3-butanediol	Siemerink et al. (2011)
	Primary/secondary alcohol dehydrogenase; $adh_{B\text{-}593}$ (*C. beijerinckii*)	Conversion of acetone to isopropanol	Lee et al. (2012)
C. cellulolyticum H10	Synthetic ethanol formation operon; *pdc-adhII* (*Zymomonas mobilis*)	Improved growth and ethanol production	Guedon et al. (2002)
	Chimeric isobutanol operon; *kivD-yqhD-alsS-ilvC-ilvD* (*E. coli*, *B. subtilis*, and *L. lactis*)	Small amounts of isobutanol produced	Higashide et al. (2011)
C. ljungdahlii DSM 13528	Synthetic butanol formation operons; *bcd-hbd-crt* and *thlA-bdhA-adhE* (*C. acetobutylicum*)	Small amounts of butanol produced	Kopke et al. (2010)
C. perfringens SM101	Alternative RNA polymerase sigma factor; *txeR* (*C. difficile*)	Activation of *toxA* and *toxB* promoters	Mani and Dupuy (2001)
	Negative regulator of toxin synthesis; *tcdC* (*C. difficile*)	Decreased toxin gene expression	Mani et al. (2002) Matamouros et al. (2007)
C. tyrobutyricum ATCC 25755	Aldehyde/alcohol dehydrogenase; *aad* (*C. acetobutylicum*)	Significant production of butanol and ethanol	Yu et al. (2011)
	Aldehyde/alcohol dehydrogenase; *adhE2* (*C. acetobutylicum*)		Yu et al. (2012)

NR: not reported.
[a] Host strain was a solvent-defective, degenerate mutant of *C. acetobutylicum*, either strain M5 or DG1

of gene expression by either initiating degradation of the mRNA transcript or by restricting ribosome-mediated translation (Desai and Papoutsakis, 1999; Georg and Hess, 2011; Wagner and Simons, 1994).

Prior to the major advancement of gene knockout technology in the clostridia, asRNA gene knockdown was the most common tool for reducing expression of native chromosomal genes for metabolic engineering of *C. acetobutylicum*. Desai and Papoutsakis (1999) separately targeted asRNAs to butyrate kinase (*buk*) and phosphotransbutyrylase (*ptb*) genes, resulting in enhanced production of acetone and butanol for the *buk* knockdown mutant, but deteriorated solvent production for the *ptb* downregulated strain. Similarly, Tummala et al. (2003b) demonstrated effective knockdown of coenzyme A-transferase (*ctfAB*) involved in acetone production, resulting in significant reduction in acetone titer and mildly lower levels of butanol. Downregulation of *ctfB* has also been combined with overexpression of an alcohol-aldehyde dehydrogenase (*aad*) gene to return butanol yield to control levels with a 23-fold increase in ethanol titer, while maintaining low acetone selectivity (Tummala et al., 2003a). In addition, Sillers et al. (2009) overexpressed the thiolase gene of *C. acetobutylicum* along with *ctfAB* knockdown and *aad* overexpression to further increase butanol production. Thus, asRNA approaches can work effectively with overexpression approaches for optimal metabolic engineering of *Clostridium*.

Antisense-RNA-mediated gene knockdown has also been employed in *Clostridium* beyond *C. acetobutylicum*. In *C. saccharoperbutylacetonicum*, asRNA was applied effectively to downregulate expression of the hydrogenase gene cluster, *hupCBA*, and alter solvent selectivity, leading to a butanol/acetone ratio of 1.3, compared to 2.9 for the control strain (Nakayama et al., 2008). Antisense RNA was also employed successfully in *C. beijerinckii* to reduce the expression of a glycerol dehydrogenase gene, *gldA*, and increase butanol tolerance (Liyanage et al., 2000), and in *C. cellulolyticum* to downregulate *cel48F*, a major component of the organism's cellulosome (Perret et al., 2004b). Finally, within the medical clostridia, asRNA-mediated gene knockdown has been utilized for downregulation of alpha- and beta-type small acid-soluble spore proteins to investigate the relationship between these proteins and their roles in resistance of *C. perfringens* spores to moist heat and UV radiation (Raju et al., 2007).

For clostridial species with limited availability of genetic tools, asRNA can be the simplest genetic technique to carry out (Table 7). Gene knockdown requires only access to a functional and stable shuttle vector, a constitutive or inducible promoter, a means of plasmid

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Table 7
Summary of chromosomal gene knockout, knockdown, and genome editing strategies for use in *Clostridium*.

Technique	Outcome(s)	Advantages	Potential limitations	References
Antisense RNA downregulation	Gene knockdown	• Up to 92% downregulation • Simple construction and procedure	• Effectiveness difficult or impossible to predict • Difficult to assay	Desai and Papoutsakis (1999) Tummala et al. (2003b)
Transposon mutagenesis	Random gene disruption	• Mutant library construction	• Difficult to control single vs. multiple insertions • Site preference • Requires sensitive assay	Lanckriet et al. (2009) Vidal et al. (2009) Cartman and Minton (2010)
Ll.ltrB group II intron retrohoming	Targeted gene disruption	• Independent of host functions • Streamlined retargeting and construction • RAM-based selection	• Polar effects • Ectopic insertions • Low efficiency (without RAM) • Difficult for small genes (<400 bp)	Heap et al. (2007) Shao et al. (2007) Heap et al. (2010)
Allele-coupled exchange (ACE)	Gene insertion Gene replacement	• Versatile and broadly applicable • Integration of large sequences (up to 28 kb)	• Must be coupled to a strong chromosomal promoter • Requires a *pyrE* mutant background or access to two antibiotic markers	Heap et al. (2012)
Heterologous counter-selective integration	Gene insertion Gene replacement	• Independent of auxotrophic strains • Integration at any genomic loci • Target gene <300 bp • asRNA-mediated plasmid curing • Versatile and broadly applicable	• Laborious enrichment procedure (up to 10 days) • Low efficiency • Demands highly efficient transformation	Al-Hinai et al. (2012)
Recombinogenic engineering (oligo-mediated)	Point mutation	• Immense potential • Straightforward procedure	• Proof-of-principle only → repair of mutation in antibiotic marker • Low efficiency	Dong et al. (2014)

transfer, and sequence data for the target gene to be downregulated. Being less drastic than gene knockouts, asRNA gene knockdown strategies are less likely to be lethal and often allow isolation of a viable mutant where harsher knockout methods fail. Additionally, careful promoter selection can facilitate asRNA knockdown experiments through strict control over the degree of downregulation by fine tuning of inducible promoters or selection of appropriate constitutive promoters (Desai and Papoutsakis, 1999). Antisense RNA strategies may be more conducive to a high-throughput screening approach than more complicated approaches, such as group II intron gene knockout (refer to Section 5.4).

Effective downregulation of a target gene by asRNA can be unpredictable due to many complex factors involved in the design of the asRNA. Care should be taken in terms of size of the asRNA molecule, degree of homology to the target mRNA, region of mRNA to be targeted (e.g. coding sequence, untranslated region, ribosome binding site, etc.), and asRNA secondary structure which can be predicted using RNA structural prediction software (Tummala et al., 2003b). Many successful asRNA approaches have included the ribosome binding site, in addition to a small 5′ portion of the target coding sequence (Perret et al., 2004b; Tummala et al., 2003b). Unfortunately, few studies have systematically investigated asRNA design for the effectiveness of gene downregulation in the clostridia. Tummala et al. (2003b) evaluated the design of asRNA constructs in an attempt to illuminate design parameters fundamental to the success of asRNA downregulation in *C. acetobutylicum*. This study employed RNA prediction software to design several different asRNAs targeting the acetoacetate decarboxylase (*adc*) and coenzyme A-transferase (*ctfAB*) mRNAs. Antisense RNAs were structurally analyzed and the normalized metrics of these structural features were plotted against the degree of downregulation. Two structural features, referred as components (regions of intramolecular complementarity within an asRNA molecule) and free nucleotides (which do not reside within a component but are available for interaction with the target mRNA), were analyzed for correlation with in vivo asRNA downregulation (Patzel and Sczakiel, 1998; Tummala et al., 2003b). Experimental data suggest that the normalized number of components correlates well with the degree of asRNA downregulation. Specifically, the ratio of the number of components to the number of total free nucleotides can be used as a predictor of asRNA effectiveness, whereby maximal downregulation is achieved using the asRNA construct having the lowest component/nucleotide ratio (Tummala et al., 2003b).

Finally, in terms of the broader role of non-coding RNAs as tools for modulating gene expression, RNA deep sequencing has revealed an abundance of small non-coding RNAs (sRNAs) in *C. acetobutylicum* (Venkataramanan et al., 2013), at up to 7.5% of the total sequencing reads. As the *Clostridium* sRNome continues to be deciphered, sRNAs may become an important part of the clostridial genetic engineering toolkit.

Transposon- and group-II-intron-mediated gene knockout

Gene knockouts in *Clostridium* can be performed with either a random mutagenesis approach or in a site-specific manner. Random mutagenesis has the advantage of not requiring gene sequence information or extensive knowledge of metabolic pathways within the *Clostridium* under study and much progress has been made in the advancement of powerful random mutagenesis techniques involving transposable elements (Table 7). Stable libraries of random chromosomal mutants have been generated in *C. difficile* and *C. perfringens* using the Tn916 (Awad and Rood, 1997), bacteriophage Mu (Lanckriet et al., 2009), EZ-Tn5 (Vidal et al., 2009), and *Himar1* mariner-based (Cartman and Minton, 2010) transposons. Transposons can be selected to preferentially yield mutants with predominantly single [e.g., *Himar1* mariner transposon (Cartman and Minton, 2010)] or multiple insertions [e.g., bacteriophage Mu transposons (Lanckriet et al., 2009)]. Mutants in toxin production (Awad and Rood, 1997), quorum sensing (Vidal et al., 2009), and sporulation/germination (Cartman and Minton, 2010) have been isolated using transposition-based mutagenesis techniques.

Site-specific gene knockouts offer the advantage of performing rational genetic engineering to eliminate competing product pathways, and to formulate and test specific hypotheses regarding metabolic and physiological pathways in *Clostridium*. It took approximately 15 years after the first reports of plasmid transfer for the first broadly-applicable gene knockout methodology to reach widespread use in the *Clostridium* community. The current established method of gene knockout in clostridia was derived from group II intron technology, termed TargeTron gene knockouts, originally developed and commercialized for use in *E. coli* and related bacteria (Sigma-Aldrich; http://www.sigmaaldrich.com/). The clostridial TargeTron system, or ClosTron, was developed independently by two groups in 2007 (Heap et al., 2007; Shao et al., 2007) and employs the mobile *L. lactis* Ll.ltrB group II intron and its cognate intron-encoded protein (IEP), LtrA, which is essential for intron function. According to the Ll.ltrB intron splicing mechanism, the bacterial intron can be programmed to insert into precise regions of bacterial genomes (Mohr et al., 2000). The predominant factor dictating site-specific intron insertion was found to be minor base-pairing (generally 11–16 bp for group II introns) between the intron RNA and host target

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DNA (Guo et al., 2000; Karberg et al., 2001). Once target site recognition has occurred, the IEP nicks one strand of the host's DNA and integrates the Ll.ltrB intron into the genome through reverse transcriptase activity (Matsuura et al., 2001). The LtrA IEP thus exhibits a tripartite functionality, acting as a maturase, endonuclease, and reverse transcriptase (Matsuura et al., 1997). Since the intron homing mechanism utilizes an RNA intermediate to generate a chromosomal DNA disruption via reverse transcription, the intron mechanism has been termed retrohoming. Further, by identifying the regions of the intron RNA involved in base-pairing and generating a pool of introns randomly mutated in such regions, the rules governing intron insertion were systematically elucidated in *E. coli* (Zhong et al., 2003). From these efforts, a computer algorithm was then designed to utilize the rules for Ll.ltrB insertion site recognition and splicing to predict putative insertion sites within a target gene query (Perutka et al., 2004). PCR primers are then generated to allow mutation of the plasmid-encoded Ll.ltrB intron, via splicing by overlap extension PCR (SOE PCR), for retargeting the intron to the selected chromosomal gene. Since the targeting portion of the intron is less than 400 bp, it is generally more cost-effective to have retargeted introns synthesized commercially in order to minimize labor involved in generating gene knockout mutants (Heap et al., 2010).

Perhaps the most promising aspect of the Ll.ltrB retrohoming mechanism is that targeted insertion occurs essentially independent of host functions (Table 7). This has allowed the technology to be broadly applied to at least 11 species of *Clostridium* (Table 8), including *C. acetobutylicum*, *C. beijerinckii*, *C. botulinum*, *C. butyricum*, *C. cellulolyticum*, *C. difficile*, *C. perfringens*, *C. phytofermentans*, *C. sordellii*, *C. sporogenes*, and *C. thermocellum* (Cai et al., 2011; Cui et al., 2012; Heap et al., 2007, 2010; Li et al., 2012; Mohr et al., 2013; Shao et al., 2007; Tolonen et al., 2009). The ClosTron technology has also been significantly advanced in recent years [http://clostron.com/; (Heap et al., 2010)]. The entire intron retargeting procedure, from selection of putative insertion sites through to intron mutation and vector construction, has been automated and streamlined. ClosTrons have also been revamped to better suit the modular clostridial shuttle vector repertoire. Still, targeting frequencies have varied drastically, ranging from less than a fraction of a percent up to approximately 60% (Heap et al., 2007; Shao et al., 2007), and this can make screening become a major barrier to isolating group II intron gene knockouts. Dong et al. (2012) screened approximately 900 transformants using colony PCR in order to identify one knockout colony. To overcome this inefficiency, ClosTrons have been modified to contain an optional retrotransposition-activated marker [RAM; (Heap et al., 2007)], based on antibiotic selection that is activated only upon intron insertion, but not when carried on the intron-containing plasmid (Zhong et al., 2003). With the improved RAM functionality, targeting frequency is elevated substantially, generally ranging from 20 to 100% (Heap et al., 2007). However, certain predicted insertion sites still experience inefficient targeting (with targeting frequencies of 2.5–20%) and demand relatively extensive colony screening despite the presence of a selectable RAM. Further, although the lactococcal Ll.ltrB intron represents the model bacterial group II intron for conducting gene knockouts, superior alternatives have been unearthed in recent years. Of particular relevance is the EcI5 intron, which is isolated from a virulent *E. coli* plasmid and has been shown to retrohome several-fold better than Ll.ltrB (up to 98% targeting efficiency without selection) (Zhuang et al., 2009). However, this superior intron has not yet been applied to the clostridia. On the other hand, a thermophilic cyanobacterial group II intron has recently been developed for gene targeting in thermophiles, with *C. thermocellum* acting as the model host species (Mohr et al., 2013). Thermotargetron disruption efficiencies were found to be similar to the superior EcI5 intron (67–100% targeting efficiency without selection) when assayed using six different chromosomal gene insertions (Mohr et al., 2013). Higher level integration is presumed to arise from the increased temperatures used for gene targeting (48 °C compared to 37 °C for the Ll.ltrB and EcI5 introns), which enhances DNA melting for more effective base pairing between the intron RNA and the host DNA.

Despite its proven utility within *Clostridium*, group II intron-mediated gene knockouts suffer from certain inherent limitations (Table 7). It is estimated that a 1 kb gene is expected to possess at least five predicted group II intron insertion sites. Disruption of particularly small genes becomes difficult, as a 400 bp gene, for example, gives a minimum of only two potential intron insertion sites. Sites that target the antisense strand of a coding gene are generally preferred, as sense orientation insertions can yield conditional knockouts if the LtrA intron-encoded protein remains present (Guo et al., 2000), thus reducing the number of usable insertion sites. Occasionally sites with high scores, based on the developed computer algorithm (Perutka et al., 2004), still do not result in a stable knockout mutant. Ectopic intron insertions, which occur concomitantly with the desired disruption due to sequence similarity, have also been shown to be a relatively common event in group II intron technology (Lehmann and Schmidt, 2003). Such events can be partially circumvented or avoided altogether by

Table 8
Summary of available ClosTron shuttle vectors for performing chromosomal gene knockouts in various *Clostridium* species.
Data is adapted and updated from Heap et al. (2010), among others.

Organism	Plasmid transfer method	Intron-harboring shuttle vector	Replication origin	Intron promoter[a]	Reference
C. acetobutylicum	Electrotransformation	pSY6	pIM13	*ptb*	Shao et al. (2007)
		pMTL007C-E2	pCB102	*fdx* (*C. sporogenes*)	Heap et al. (2010)
C. beijerinckii	Conjugation or electrotransformation	pMTL007S-E2	pCB102	*fdx* (*C. sporogenes*)	Heap et al. (2010)
		pWJ1	NR	*ptb* (*C. acetobutylicum*)	Xiao et al. (2012)
		pYW1	NR	*ptb*	Wang et al. (2013)
C. botulinum	Conjugation or electrotransformation	pMTL007C-E2	pCB102	*fdx* (*C. sporogenes*)	Heap et al. (2010)
		pMTL007C-E5	pCD6	*fdx* (*C. sporogenes*)	
C. butyricum	Conjugation	pMTL007	pCB102	*fac*/*fdx* (*C. pasteurianum*)	Cai et al. (2011)
		pMTL007C-E2	pCB102	*fdx* (*C. sporogenes*)	Cai et al. (2013)
C. cellulolyticum	Electrotransformation	pWH199	pAMβ1	*fdx* (*C. pasteurianum*)	Li et al. (2012)
		pSY6	pIM13	*ptb* (*C. acetobutylicum*)	Cui et al. (2012)
C. difficile	Conjugation	pMTL007C-E2	pCB102	*fdx* (*C. sporogenes*)	Kuehne et al. (2010)
		pMTL007C-E5	pCD6	*fdx* (*C. sporogenes*)	Heap et al. (2010)
C. perfringens	Electrotransformation	pJIR750ai	pIP404	*cpb2*	Chen et al. (2005)
		pMTL007C-E5	pCD6	*fdx* (*C. sporogenes*)	Heap et al. (2010)
C. phytofermentans	Conjugation	pQint3367	pAMβ1	*pfo*	Tolonen et al. (2009)
C. sordellii	Conjugation	pMTL007C-E5	pCD6	*fdx* (*C. sporogenes*)	Heap et al. (2010)
		pDLL2	pCB102	*gdh*	Carter et al. (2011)
C. sporogenes	Conjugation	pMTL007C-E2	pCB102	*fdx* (*C. sporogenes*)	Heap et al. (2010)
C. thermocellum	Electrotransformation	pHK-TT1A	pTHT15	*groEL*	Mohr et al. (2013)

NR: not reported.

[a] Unless stated otherwise, the promoter controlling intron and IEP transcription is native to the host organism.

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optimal intron design and employing an algorithm designed to scan the genome for prospective unwanted insertion sites (TargeTronics, LLC; http://www.targetrons.com/). Still, current clostridial group II intron knockout protocols advocate the use of both gene complementation and Southern blot analyses to rule out the presence of multiple intron insertions in isolated mutants (Cooksley et al., 2012), greatly increasing the labor and time for obtaining desired knockout mutants. An additional consideration for group II intron technology is curing knockout mutant cells of the intron donor plasmid, which has proven to be a challenge for some clostridial host–vector combinations [e.g., (Li et al., 2012)]. Plasmid curing in *Clostridium* is commonly achieved by subculturing in non-selective growth medium for 4–5 days (Al-Hinai et al., 2012; Li et al., 2012) or 5–7 successive transfers (Cui et al., 2012; Tolonen et al., 2009), thus further increasing the time needed to obtain an intron insertional mutant. This shortcoming has been alleviated in *C. cellulolyticum* by expressing a selectable *pyrF* gene from the ClosTron donor plasmid (Cui et al., 2014). The resulting construct allows one-step plasmid curing by *pyrF* negative selection in the presence of 5-fluoroorotic acid (FOA). A caveat of this technique is that the host *pyrF* gene must be inactivated to permit plasmid-based selection. Alternatively, plasmid curing has been greatly enhanced in *C. acetobutylicum* by expressing an inducible asRNA possessing homology to mRNA from the pIM13 replication protein, *repL* (Al-Hinai et al., 2012). Lastly, the ClosTron system has been investigated for its capacity to insert heterologous DNA into bacterial genomes, since cargo DNA can be inserted into the Ll.ltrB intron and delivered during intron retrohoming. Targeting efficiency was found to decrease markedly with cargo sequences greater than 1 kb (Heap et al., 2010), impeding the usefulness of this strategy, especially for introns with a selectable RAM.

Based on the extensive applicability with a broad host range, group II intron technology has served as a powerful tool for clostridial genetic and metabolic engineering. Nonetheless, certain applications, particularly for exogenous DNA delivery, demand more sophisticated chromosomal engineering tools for use in the clostridia.

Advanced and anticipated genome editing technologies

Development of advanced, genome editing technologies allowing precise manipulation of clostridial chromosomes, encompassing chromosomal deletions, replacements, point mutations, and insertions, has yet to be realized due to inefficient host homologous recombination and inefficient transformation. Indeed, the isolation of targeted chromosomal integration mutants remain a laborious task and, to date, only a few homologous-recombination-based mutant strains have been isolated. Such methods have employed both replicative and non-replicative vectors for chromosomal integration using the natural recombination systems of the host organisms (Brown et al., 1994; Green et al., 1996). Unstable single crossover events tend to dominate, however, leading to unwanted insertion of the entire integrative vector into the host chromosome (Coleman and Hudson, 1995; Green et al., 1996; Nair et al., 1999). Such barriers have been partially alleviated in recent years by utilizing negative selection to select for double crossover integration events. Numerous counter-selection markers have been exploited for this purpose in a range of clostridia, including *mazF* from *B. subtilis* (Al-Hinai et al., 2012) and *codA* from *E. coli* (Cartman et al., 2012), in addition to native clostridial *pyrE*, *pyrF*, *galK*, *tdk*, and *hpt* genes (Argyros et al., 2011; Heap et al., 2007, 2012; Nariya et al., 2011; Tripathi et al., 2010). Nevertheless, many native clostridial markers are limited in their use, as the chromosomal copy must first be inactivated prior to the introduction of plasmid-encoded counter-selection (Cartman et al., 2012).

To develop more powerful targeted homologous-recombination-based genetic tools, Heap et al. (2012) conceived Allele-Coupled Exchange (ACE) for effective selection of double crossover mutants without the use of plasmid-encoded counter-selection. Here, the desired double-crossover recombination event is coupled to either expression or silencing of a selectable marker. This approach was demonstrated by recombination at the *pyrE* locus of *C. acetobutylicum* through positive selection on growth medium lacking uracil and negative selection by supplementing the growth medium with FOA. By carefully controlling the sequence of integration events using the length of flanking homology regions, single crossover integration of the donor plasmid was selected by *pyrE* positive selection, whereas the double crossover event, resulting in truncation of *pyrE* and plasmid excision, could be subsequently selected via FOA supplementation. The same approach was also used to fuse a promoterless, vector-borne copy of *pyrE* with a strong, native chromosomal promoter, demonstrating the utility of ACE for genome-wide recombination at any desired locus. This method has been employed for the construction of elaborate synthetic chromosomal operons expressing functional cellulosome elements in *C. acetobutylicum* (Kovacs et al., 2013).

ACE, together with similar methods developed by Cartman et al. (2012) and Al-Hinai et al. (2012), exemplifies the current state of the art in clostridial genome editing and proves indispensable for the genetic engineering of a range of medical and industrial clostridia. However, these methods are still considered laborious (Table 7). The path to simpler and more efficient genome editing techniques will be achieved by closely mimicking the pioneering genetic work done in *E. coli* and *B. subtilis*. The benchmark for performing highly precise and efficient genome editing in *E. coli* over the past decade has been phage-mediated recombinogenic engineering, or recombineering (Zhang et al., 1998). Rather than relying on natural host recombination, which is inefficient in *E. coli*, or the use of tedious counter-selection markers, recombineering utilizes efficient phage recombination systems comprised of an endonuclease gene paired with its cognate recombinase, either *recET* from the *E. coli* Rac prophage (Zhang et al., 1998) or *exo* and *bet* genes from phage lambda (Murphy and Campellone, 2003). These gene products can be employed for recombination of linear PCR cassettes (Datsenko and Wanner, 2000). Additionally, the recombinase (*recT* or *bet*) can be expressed alone for recombination of short single-stranded oligonucleotides possessing homology to host chromosomes (Ellis et al., 2001). It is expected that clostridial recombineering systems are on the verge of development, as a report detailing site-specific recombination of short synthetic nucleotides appeared recently (Dong et al., 2014). Rather than employing the proven *E. coli* recombineering machinery, which function poorly in Gram-positive hosts (Datta et al., 2008), this method relies on a *recT* homolog from *C. perfringens*. With these proof-of-principle results, it is expected that more practical phage recombination-based demonstrations will emerge in the near future.

Concluding remarks

Since the arrival of recombinant DNA technology in the 1980s, the genus *Clostridium* has faced an asymmetry, whereby available genetic methodologies do not permit full realization of the biotechnological potential harnessed by this important genus. While *C. acetobutylicum* remains the major clostridial workhorse for genetic tool development, other species, including those with more promising industrial potential and many important medical and pathogenic clostridia, have been hampered by an overall lack of available genetic engineering tools and techniques. Given these deficiencies, the genus would be vastly improved by rounding out the genetic toolkits available for individual clostridial species. Moreover, it is expected that forthcoming genetic technologies, which will allow precise and efficient recombination of linear DNA cassettes and short oligonucleotides, will not experience widespread deployment within the genus without prior development of the fundamental genetic methods discussed herein. We anticipate that our review of current clostridial genetic methodologies will serve as a useful guide to further advance the progression of untapped, immature, and intractable clostridial species, and propel the genus into the modern age of synthetic biology and precise genome editing.

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