16

Metabolic Engineering and the Synthetic Biology Toolbox for *Clostridium*

Rochelle C. Joseph[1], Susan Kelley[1], Nancy M. Kim[2], and Nicholas R. Sandoval[1]

[1] *Department of Chemical and Biomolecular Engineering, Tulane University, New Orleans, LA, USA*
[2] *Interdisciplinary BioInnovation PhD Program, Tulane University, New Orleans, LA, USA*

16.1 Introduction

The primary driver for interest in *Clostridium* is the ability for consumption of a wide variety of carbon sources into a handful of valuable/useful compounds. The well-studied ABE fermentation (acetone, butanol, and ethanol) of the type strain *Clostridium acetobutylicum* has been the subject of interest for decades, primarily due to acetone first, then butanol as a next generation biofuel. Indeed, the allied acetone production process during WWI (critical for cordite production) relied on the "Weizmann process," which fermented potato starch using *C. acetobutylicum* (or, the Weizmann strain). The second component of the ABE fermentation, butanol, has several advantages as a biofuel over the yeast-based ethanol production from corn that is currently blended in fuel. Ethanol production by yeast has millennia of human engineering behind it. Butanol production, therefore, requires engineering of *Clostridium* to achieve titers that can compete with ethanol along with bioprocess engineering [1]. Research has also turned, as it has with ethanol production, to the use of waste carbon sources, primarily lignocellulosic biomass. Here, a major challenge is the toxic compounds that are formed during hydrolysis of the biomass [2], and research has been ongoing both on improved methods of processing biomass and engineering tolerance to such compounds. Due to the wide breadth of clostridial feedstock consumption capabilities, waste carbon and energy sources now include gaseous feedstocks (such as syngas) and crude glycerol (derived from biodiesel production). The *Clostridium* genus is home to multiple industrially relevant strains. *Clostridium* species are natively capable of cellulosic and hemicellulosic biomass degradation (e.g. *C. cellulolyticum*) [3], carbon fixation (e.g. *C. carboxidivorans*, *C. ljungdahlii*) [4], advanced biofuel production (e.g. *C. acetobutylicum*, *C. beijerinckii*) [5], platform chemical production (e.g. *C. pasteurianum*) [6], and even acting as anticancer therapeutics (e.g. *C. novyi-NT*) [7]. Additionally, biotechnological development in this genus will aid research in strains of medical interest (e.g. *C. botulinum, C. tetani, C. perfringens*) [8, 9].

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While these powerful capabilities have been well known for decades, the major stumbling block has been the difficulty of working with and engineering *Clostridium* species. Members of the genus *Clostridium* are Gram+, sporulating anaerobic firmicutes, thus requiring special equipment to work with them. While compatible with plasmid manipulation, transformation is notoriously inefficient and the number of unique genetic elements available for use in *Clostridium* species (e.g. inducible promoters, terminators, antibiotic resistance markers) are far fewer than those available for *E. coli*. Thus, metabolic engineering advancements have not been made at the same level of progress compared to more tractable bacteria. However, the impetus to engineer *Clostridium* is strong, as the innate capabilities of *Clostridium* with respect to biofuel production and feedstock consumption are superior to similar traits of meticulously engineered *E. coli* strains [10]. In this chapter, we endeavor to explore the state-of-the-art methods of metabolic engineering in *Clostridium*, including a discussion on engineered genetic parts that enable such progress to occur.

From a broad perspective, in the quest of producing fuels and chemicals from metabolically engineered strains, there are two competing design paradigms. One method aims to take some phenotypic trait (e.g. cellulose consumption, butanol production) from an organism with few or no engineering tools and bestow it upon some chassis strain (e.g. *E. coli*) with the overarching rationale that strain engineering will be faster. High throughput methodologies can enable rapid construction of balanced pathways [11], synthetically designed genetic parts [12, 13], and whole-genome recoding [14]. This method often requires substantial work to achieve growth and consumption rates, productivities, and/or titers that are common to the native production strains. Not only does this require the importation of genes expressing the biochemical pathway enzymes, but also knocking out competing pathways, engineering the regeneration of cofactors, expression of helper proteins, and a synthetic regulatory structure. For example, when the butanol production pathway from *Clostridium* was imported into *E. coli*, the first attempts yielded low titers ($<1\,g\,l^{-1}$ [15]) and subsequent metabolic engineering led to titers of $15–20\,g\,l^{-1}$ in batch conditions and even up to $30\,g\,l^{-1}$ with in situ product removal [16, 17]. This is compared to *C. acetobutylicum* where the type strain produces $\sim 15\,g\,l^{-1}$ butanol in a batch reaction [18], a chemically mutagenized strain is able to produce $19\,g\,l^{-1}$ (batch) or $30\,g\,l^{-1}$ (with in situ product recovery) [16], and a rationally engineered strain in a continuous fermentation can achieve productivities of $14\,g\,l^{-1}\,h^{-1}$ [1].

The competing paradigm is to engineer strains with desired traits to improve or augment the phenotype of interest. This approach enjoys the benefit of starting with strains already endowed with the genes and cofactors necessary to produce [19–21]. However, untangling the existing regulatory structure surrounding the phenotype of interest (e.g. uncoupling sporulation from butanol production in *C. acetobutylicum*) can prove difficult.

The development of an advanced genome engineering "toolbox" in non-platform organisms brings these two models closer together. Advancements in synthetic biology methods are necessary and have brought closer the utilization of the biotechnologically important capabilities of the *Clostridium* genus. In this chapter, we briefly overview metabolic engineering strategies

applied to clostridia, and focus more on the methods for genetic engineering the *Clostridium* genus, continued challenges that hamper progress, and the application of "-omics" methods to better inform engineering approaches.

16.2 Aims of Metabolic Engineering in *Clostridium*

The primary biotechnological goal of *Clostridium* engineering is the production of biofuels and other chemicals, preferably from waste carbon streams including gaseous and lignocellulosic feedstocks [1].

Efforts common among metabolic engineering across bacteria involve the disruption or deletion of genes that encode the biochemical pathways to undesired metabolites. For the increased production of solvents, acidogenic pathways can be disrupted as shown in *C. beijerinckii* to improve solvent production from biomass hydrolysate [22]. This has been done in thermophilic strains as well, as exemplified by the disruption of *C. thermocellum*'s hydrogen, acetic acid, lactic acid, and formic acid pathways in a single quintuple deletion strain, which doubled the ethanol yield from cellulosic biomass [23]. Another theme in *Clostridium* metabolic engineering is exchanging genetic material within the genus (and other closely related organisms) to endow properties of one strain onto another. For example, the ABE fermentation of *C. acetobutylicum* was modified to produce isopropanol–butanol–ethanol (IBE) through expression of an alcohol dehydrogenase from *C. beijerinckii* NRRL B-593, which reduced accumulation of undesired acetone through conversion to isopropanol [24]. Ethanol pathway genes from *Thermoanaerobacterium saccharolyticum* improved ethanol productivity in *C. thermocellum* by twofold compared to an unmodified strain [25]. In an ambitious example, *C. thermocellum* was endowed with butanol production through the systematic installation of thermostable butanol pathway genes from multiple anaerobes [26]. When coupled with other techniques, such as directed evolution and co-culture strategies, even greater production gains can be achieved upon the successes of rational metabolic engineering.

For non-cellulolytic biofuel producers, lignocellulosic biomass must be pretreated and saccharified to make available the sugars for fermentation, which also leads to the release of compounds inhibitory to growth and production [2]. Adaptive library evolution (or, directed evolution) is a widely used method to generate and adapt strains to overcome feedstock and product toxicity. Typically, adapted strains with improved phenotypes are sequenced and notable mutations are impressed onto the parent strain to test for efficacy. This method has been used to improve the growth and productivity of many *Clostridium* strains for faster growth rate in glucose [27], butanol tolerance [16], crude corn stover hydrolysate [22], and crude glycerol (a byproduct of biodiesel) [28]. Rational strategies to mitigate inhibitory compounds have been done for furfural [29, 30] and other inhibitory compounds [31, 32], but require a priori knowledge of the mechanism of inhibition. Fermentation of hydrolysates using various *Clostridium* species has led to butanol titers up to $15\,g\,l^{-1}$ [33, 34].

To avoid the necessity of pretreating biomass prior to fermentation, consolidated bioprocessing combines the saccharification and fermentation steps using

cellulolytic organisms. Cellulolytic *Clostridium* species express cellulosomes to generate sugar monomers from cellulose and cellobiose. Work has been done to improve or bestow biofuel production on cellulolytic organisms, particularly in thermophiles [25, 35], while less work has been done to express a functional cellulosome on solventogens [36, 37]. Ethanol formation at titers of $>20\,g\,l^{-1}$ and yields of >70% have been achieved in *C. thermocellum* through a sequential genetic engineering step eliminating competing pathways followed by adaptive library evolution [38, 39]. Adaptive evolution has also been applied to species amenable to consolidated bioprocessing. *C. thermocellum* was adapted to butanol in a chemostat where the resultant strain was tolerant to $15\,g\,l^{-1}$ butanol (improved from $5\,g\,l^{-1}$ tolerance). A mutation in the *adhE* gene was found to be causative, and the mechanism of tolerance was the elimination of reverse flux of added butanol, altering internal redox balances [40]. Systems biology coupled with metabolic modeling can identify key targets of mutation for metabolic engineers [41]. For example, in *C. thermocellum*, five reactions were identified as the least thermodynamically favorable for generating ethanol from cellobiose [42].

Even with highly productive strains, carbon escape, the loss of carbon to carbon dioxide as sugars are incorporated into the central metabolism as acetyl-CoA, limits the viability of biofuel production. The gaseous carbon consumption capability of *C. ljungdahlii* and *C. carboxidivorans* has been posited to be a solution to reassimilate gaseous C1 compounds through the Wood–Ljungdahl pathway (WLP). Attempts have been made through either endowing solventogenic pathways into C1 fermentors (e.g. acetone production in *C. ljungdahlii*) [4] or the WLP into solventogens [43, 44]. Both types of attempts have proven difficult to achieve high titers of the desired product due to the difficulty of either engineering acetogenic *Clostridium* or generating a functional WLP in solventogens. Syntrophic co-culture strategies may achieve the desired outcome through allowing cells to exchange metabolites akin to naturally occurring microbiota [45, 46]. Interestingly, direct contact between cells has been observed, indicating that transfer of metabolites does not go through an intermediate culture broth stage, but rather is exchanged directly from cell to cell, resulting in yields impossible if fermented in a monoculture [45]. Similar co-culture strategies have been employed for the consumption of cellulosic biomass to allow for both five- and six-carbon sugar consumption [47]. Strategies involving many strains or even entire microbiomes have been explored to exploit *Clostridium* strains such as *C. kluyveri* that can perform chain elongation from short-chain fatty acids to medium- or long-chain fatty acids through the addition of alcohols [48, 49]. Such co-culture strategies will still require genomic engineering and optimization in order to achieve maximal productivity.

16.3 *Genomic Editing* in *Clostridium*

Clostridium genome editing has evolved from the initial reports of single-crossover insertions of plasmids in the 1990s to now include intron- and

transposon-based disruption methods as well as plasmid-based double-crossover insertion methods coupled with counterselection markers, including CRISPR-based cleavage.

16.3.1 ClosTron

Targeted gene disruption can be achieved using Group II Mobile introns. The ClosTron system is a *Clostridium*-optimized version of the TargetTron method [50, 51]. While use of the TargeTron/ClosTron systems necessarily leaves a genetic scar, these methods avoid the cloning and transformation of large homologous arms most often required for successful editing when using other targeted gene knockout methods.

ClosTron works by expression of a ribonucleoprotein complex comprising the intron-encoded protein (IEP, LtrA based on the *Lactococcus lactis* Ll.LtrB intron) and the corresponding RNA in a lariat conformation. This RNA contains the genetic material to be inserted as well as the targeting regions, two exon binding sequences of 5 and 9 nt lengths providing a total of 14 bases of specificity for RNA-DNA interaction (akin to a crRNA in CRISPR systems). This specificity is coupled with the IEP-DNA interactions over two ranges of 17 and 11 nt lengths, where 7 and 1 bases contribute the majority of the binding strength, respectively (akin to a PAM site in a CRISPR system) [52]. This relative complexity in identifying optimal integration loci is mitigated by a free online tool to design the requisite cloning primers [53]. The ClosTron system is minimally dependent on the host, as the IEP performs the maturation, endonuclease, and reverse transcriptase activities necessary for the chromosomal insertion. The method for selecting successful insertion events depends on the inclusion of the retrotransposition-activated selection marker (RAM) [50]. This RAM, most commonly an antibiotic resistance gene, is inactivated by a Group I intron that self-catalytically spliced out of the mRNA only in the event of a chromosomal insertion event, preventing expression of the resistance marker from the plasmid (Figure 16.1a). Inclusion of flanking FRT sites permits the removal of the selection marker through additional steps.

The Targetron/ClosTron system has been used to perform gene targeting in both Gram-positive and Gram-negative bacteria [54] including several *Clostridium* spp.: *C. perfringens* [55, 56], *C. acetobutylicum* [57–60], *C. beijerinckii* [53, 61], *C. pasteurianum* [62], and *C. botulinum* [63, 64]. ClosTron has also been used in the closely related species *Clostridioides difficile* (*née Clostridium difficile*) [65–68]. While integration of genetic material is possible with ClosTron, the size of the cargo cannot be very large and efficiency falls rapidly with increasing amounts of cargo DNA [53].

16.3.2 Transposon-Based Random Mutagenesis

Quasi-random insertional mutagenesis in *Clostridium* spp. has been achieved through transposon-based methods. The Tn916/Tn1545 family of transposons (called conjugative transposons) were among the first elements available for random gene insertions in *Clostridium* and contain the conjugation,

Color Fig: **16.1**

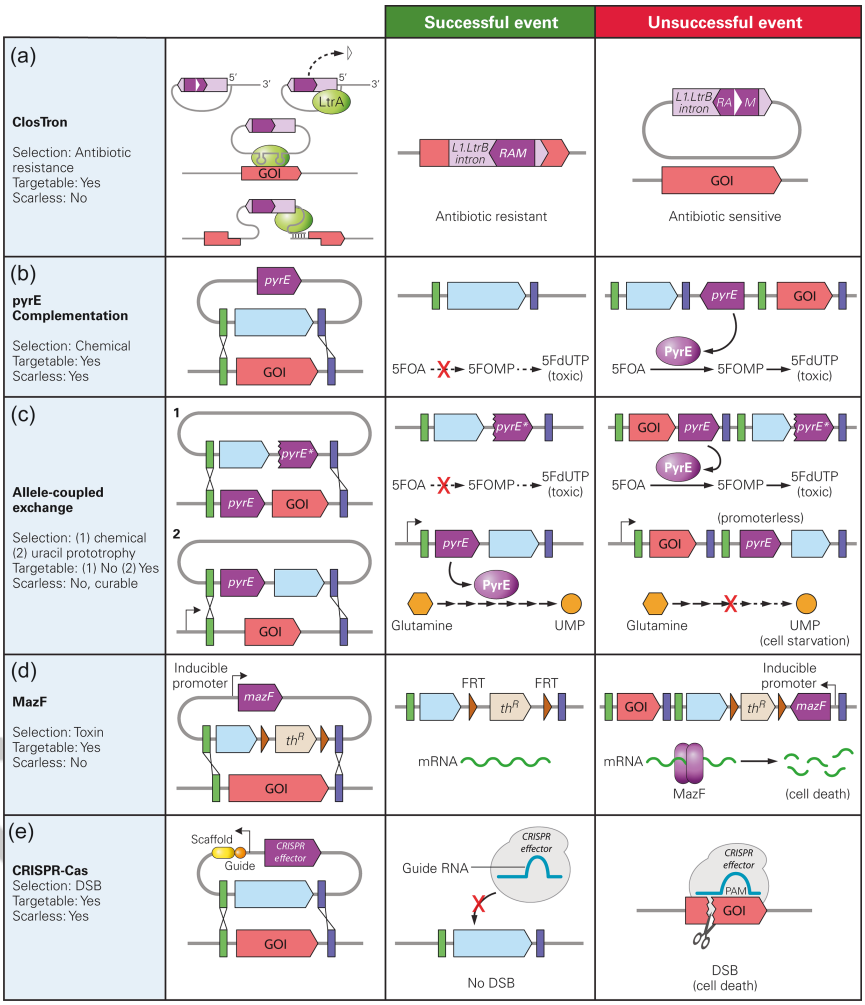

Figure 16.1 Counterselection markers used in *Clostridium* spp. and their mechanisms of selection. The native gene of interest (GOI) is represented in red, desired insert in blue, and the counterselection marker gene in dark purple. The green and blue bars represent regions of homology between the chromosome and donor plasmid. (a) ClosTron: RAM disrupted by a Group I intron (white triangle) is only active after the L1.LtrB intron is inserted into the chromosome; (b) *pyrE* complementation: PyrE catalyzes conversion of 5-fluoroorotic acid (5FOA) to 5-fluororotidine monophosphate (5FOMP) producing toxic fluorodeoxyuridine monophosphate (FdUTP); (c) allele-coupled exchange: (i) double-crossover event at the *pyrE* locus results in truncated version of pyrE for counterselection with same mechanism as B, (ii) successful homologous recombination inserts a promoter-less copy of the *pyrE* gene directly downstream of a native constitutive promoter, allowing production of uracil 5′ monophosphate (UMP). Note: must be performed on pyrE-deficient strain; (d) MazF protein degrades mRNA at 5′-ACA-3′ sequences; (e) Cas9: successful homologous recombination gRNA-targeted double stranded break resulting in cell death.

recombination, regulation, and accessory functions required for chromosomal disruption. Unlike ClosTron, which requires specific targeting for disruption, transposon-based mutagenesis is commonly used to perform loss-of-function screens. In use, expression of Tn916 for mutagenesis is often plasmid-based [69], but has been demonstrated from the chromosome of hosts such as *E. coli* and *Bacillus subtilis* [70]. The use of Tn916-like transposons is limited by size, a tendency to insert in multiple copies or specific "hot spots," and deletions at the insertion site [71–73]. Non-conjugative transposon systems, such as Mu phage and EZ-Tn*5*-based transposon systems, have addressed several of these concerns and have been successfully employed in *Clostridium* [56, 74, 75], but are limited by a preferential insertion into *rRNA* genes [74, 75].

Mariner-transposable *Himar1*-based systems have also been developed for random mutagenesis in *Clostridium* [76–79]. The *Himar1* transposable element (a transposase gene flanked by short inverted terminal repeat sequences) was originally discovered in the horn fly and has been shown to insert quasi-randomly into the genomes of several bacteria species, including members of the *Clostridium* genus. Successful *Himar1*-based events have been recorded in *C. acetobutylicum* [78], *C. perfringens* [77], *C. sporogenes* [80], and *C. ljungdahlii* [81]. *Himar1* transposase binds and cuts the element in its ITR region, which, in turn, is inserted at a TA dinucleotide target site. This has been a valuable tool for mutagenesis in AT-rich *Clostridium* as it inserts on average at one copy per cell. When coupled with next-generation deep sequencing, this method, termed TraDIS, can yield large genotype-to-phenotype data, as exemplified through identification of gene essentiality in *C. difficile* [82].

16.3.3 Counterselection Markers

Counterselection markers enable the isolation of double-crossover homologous recombination events in *Clostridium* spp.

Another method for targeted gene disruption in *Clostridium* method for targeted gene disruption in *Clostridium* is performed via plasmid-based, double-crossover recombination events. This way differs from the abovementioned intron and transposon-based methods in that here the site-specific targeting is achieved through two large (>500 nt usually) regions of homology flanking the cargo DNA. Additionally, this method relies entirely on the host's native recombination machinery as no heterologous protein expression is required. The term "double crossover" refers to the two separate recombination events that must occur for successful editing; the first integrates the entire plasmid into the genome while the second excises the vector backbone from the chromosome.

Counterselection markers, genetic elements that result in cell death when present, are useful for selecting chromosomal insertions that do not contain the vector backbone (i.e. single-crossover events). Few successful double-crossover mutations were isolated prior to the development of *Clostridium*-specific counterselection marker systems [83, 84]. Previously described homologous recombination mutations were often single-crossover integrations, which are by their nature unstable [85–90].

One counterselection method involves deactivating an easily screenable gene on the chromosome first and then complementing the mutant strain with a heterologous version of that gene as a counterselective marker. Uracil auxotrophic mutants, formed by disrupting *pyrE*, *pyrF*, or *upp* genes, require uracil supplementation for growth and are resistant to the antimetabolites 5-fluoroorotic acid (5-FOA) or 5-fluorouracil (5-FU). Double-crossover events in these mutants can easily be isolated by including a functional copy of the disrupted gene on the backbone of the donor DNA plasmid as successful events are marked by cells that no longer require uracil supplementation or demonstrate a resistance to 5-FOA or 5-FU [91–93] (Figure 16.1b).

Similarly, in *C. perfringens*, disruption of the *galKT* operon produces mutants unable to produce the enzymes involved in galactose metabolism. GalK catalyzes the production of galactose-1-phosphate (Gal-1-P) from galactose, and GalT catalyzes its consumption. The accumulation of Gal-1-P is believed to inhibit cell growth by causing intracellular stress and inducing stress-responsive genes [94, 95]. By including only the *galK* gene and not the *galT gene* on the integration vector and plating mutant cells on galactose-supplemented plates, unedited cells do not grow due to an accumulation of Gal-1-P while mutants that undergo a double-crossover event can be isolated [96].

Allelic Coupled Exchange (ACE) couples a counterselection marker gene to a desired double-crossover event. This has been demonstrated in two ways. One method exploits the 5-FOA resistance conferred by a disrupted *pyrE* or *pyrF* gene. This method does not require the cells to be auxotrophic for uracil prior to recombination, nor does it rely on a heterologous version of the gene as a counterselection marker. ACE technology employs asymmetric homology arms to direct the order in which crossover events occur. The longer arm, homologous to a 1200 bp region immediately downstream of the *pyrE* of *pyrF* directs the first-crossover event in which the entire plasmid is incorporated into the genome. The second-crossover event excises the plasmid backbone and is directed by the shorter arm, which is homologous to a 300 bp internal region of the *pyrE* or *pyrF* gene. This second recombination replaces the wildtype *pyrE* gene with a truncated form, thereby producing a mutant that can be screened based on 5-FOA resistance [93]. Alternatively, a promoter-less heterologous *pyrE* gene or antibiotic marker can be inserted in the integration vector with the regions of homology such that a successful double-crossover event places the silent gene directly downstream of a constitutive promoter [93]. However, unlike previous methods, which relied heavily on ClosTron technology to first produce auxotrophic mutants, *pyrE* mutants can be created utilizing ACE technology while the use of an antibiotic marker circumvents the need for a requisite mutant strain [93, 97] (Figure 16.1c). ACE has been proven to be applicable over a range of *Clostridium* species, having been used for gene editing in *C. acetobutylicum* [98–100] and *C. sporogenes* [78, 93], as well as *C. difficile* [9, 93].

Several heterologous genes have been used for counterselection. The cytosine deaminase gene (*codA*) from *E. coli* can be used for counterselection based on the ability of the CodA protein to catalyze the conversion of 5-fluorocytosine (5-FC), an innocuous pyrimidine analog, to 5-FU [101]. *codA* can only be used for counterselection in strains with a functional *upp* gene but no native *codA*

gene [98]. However, a bioinformatics survey suggests several *Clostridium* species contain *codA* homologs, restricting the applicability of *codA* among the genus [102].

In order to genetically manipulate *C. saccarobutylicum*, a markerless deletion system was developed using *codBA* operon utilizing the *codA* gene and a *codB* gene that encodes a cytosine permease. A suicide vector containg *catP* gene for thiamphenicol resistance and the *codBA* gene fuses DNA segments around the deletion target [103]. This led to further markerless deletion and insertion techniques to edit both *C. acetobutylicum* and *C. saccharobuylicum* [104].

Restriction-less, markerless counterselection markers have also been used to continuously produce *n*-butanol with glucose as a sole carbon source by deleting *ldhA*, *ctfAB*, *adc*, *ptb*, *buk*, and *adhE2* genes. This optimized the acetyl-CoA conversion to *n*-butanol. By creating a stable process to produce *n*-butanol, the Weizmann process can be used to ferment and scale up production of *n*-butanol [1].

Toxin-antitoxin systems are another useful source of counterselection markers. The *E. coli*-based *mazF* is an mRNA interferase, coded along with *mazE* in an operon. Under regular cell conditions, *mazE* binds to and inhibits *mazF* activity. During cellular stress, *mazE* is degraded, allowing *mazF* to bind mRNA, degrading them at 5′-ACA′3′ sequences, thereby arresting cell growth. *mazF*, coupled with an antibiotic-resistant marker flanked by FRT sites, can be used as a counterselection marker in plasmid based-homologous recombination. *mazF* is placed on the gene disruption plasmid under the control of an inducible *lac* promoter. A double-crossover event can be isolated in cells able to grow on lactose-supplemented plates [102] (Figure 16.1d). The use of *mazF* requires no prior mutation for successful screening, is independent of the availability of *Clostridium* genetic parts, and has been shown to function across *Clostridium* species [28, 80, 102]. Flp-frt recombination has also been used to eliminate the backbone of a donor plasmid following a single-crossover event in *C. acetobutylicum*, allowing for the use of an antibiotic gene as a marker for the crossover event after the donor plasmid had been cured [105].

16.3.4 CRISPR-Based Editing in *Clostridium*

CRISPR systems have revolutionized gene editing in several non-model organisms including *Clostridium* species. Natively found in prokaryotes [106, 107] and some few bacteriophages [108], these systems have been repurposed for a variety of gene manipulation applications in a wide range of organisms [109–111]. In *Clostridium* and other bacterial species, CRISPR systems, most commonly CRISPR/Cas9, have been used as a counterselection tool based on their ability to implement a double stranded break (DSB) to a targeted DNA region. *Clostridium* spp. lack or have inefficient nonhomologous end-joining (NHEJ) systems, therefore a chromosomal DSB results in cell death [112, 113]. Studies in *E. coli* have shown that DSBs can enhance homologous recombination in bacteria, whereby homology directed repair (HDR) occurs after a break has been induced [114], but studies in various *Clostridium* species suggest that HDR efficiency is too low to select for successful HDR events [115–117]. Counterselection, in the context of plasmid-based double-crossover editing, is achieved by the targeting of the

CRISPR/Cas system to the wildtype sequence to eliminate non-edited members of the population. The use of CRISPR/Cas9 can enable scarless edits and represents a major advancement in *Clostridium* genomic editing.

The Type II CRISPR system native to the *Streptococcus pyogenes* bacterium was the first exploited for genomic engineering based on its minimality [118] with other CRISPR systems having been discovered and repurposed since [119]. The CRISPR/Cas9 system consists of a single effector protein (Cas9), which can bind to and implement a DSB to a targeted DNA system, when co-expressed with a single guide RNA (sgRNA, a fusion of the individual crRNA and trRNAs found in the native system) targeting a 20 bp region immediately adjacent to the protospacer adjacent motif (PAM, NGG for *Spy*Cas9). While the CRISPR/Cas9 system has been used as a counterselection tool in several *Clostridium* species (Table 16.1) [105, 117, 120, 136], its use has been hindered by several factors. First, the limited number of characterized genetic parts for *Clostridium* poses a challenge. A dearth of well-characterized, tightly regulated, promoters can lead to simultaneous constitutive expression of both the Cas9 protein and sgRNA, resulting in very few transformed colonies even in the presence of a homologous repair template, as Cas9 activity induces cell death before recombination can occur [105, 117, 120, 136]. One way this can be addressed is through a two-plasmid system, where the donor template and sgRNA are introduced separately from the Cas9 protein, allowing time for recombination prior to counterselection [105, 115, 143]. Not only has this method facilitated the isolation of recombinants at high efficiencies, but it avoids the transformation of very large plasmids, which reduce transformation efficiency. However, it requires two separate transformation events [121]. Successful recombinants have been isolated at a rate of up to 100% [121] with commonly observed efficiencies of greater than 50% (Table 16.1).

Although inducible promoters have been employed for Cas9 expression [105, 121] their use has not always mitigated cell death as basal expression from leaky promoters can still be lethal. In response, methods for more stringent control of Cas9 expression have been developed. RiboCas employs the use of a theophylline-induced riboswitch for tight control of Cas9 expression. It has facilitated genomic deletions of up to 2.4 kbp across four *Clostridium* species at 100% efficiency and was used in *C. sporogenes* to facilitate the insertion of a 2.9 kbp fragment at efficiencies over 90% (Table 16.1) [8]. Anti-CRISPR proteins, proteins which are able to post-translationally inhibit the nuclease activity of CRISPR effector proteins, also reduce unwanted Cas9 activity [151]. The antiCas protein AcrIIA4 has been coupled with a dual-plasmid CRISPR-Cas9 system in *C. acetobutylicum* to limit undesired activity of the Cas9 protein, resulting in significantly higher transformation rates and 100% gene editing efficiency [122].

Cas9 nickase (Cas9n) systems exploit CRISPR gene editing while circumventing the lethality associated with the co-expression of a guide RNA with Cas9. This method utilizes Cas9n, a Cas9 with one of its nuclease sites mutated, so a nick results in lieu of a DSB. Implementing a single nick into the genome via Cas9n allows homologous recombination without the lethal effects of a Cas9-mediated DSB, thus permitting a mixed population of edited and unedited strains to coexist. CRISPRn has been used to implement gene deletions and

Table 16.1 CRISPR-based gene and gene repression in *Clostridium* spp.

Species	CRISPR effector	Number of spacers	Desired edit (efficiency, maximum deletion/insertion, HA length)	Notes	References
C. acetobutylicum	*Spy*Cas9	1	Deletion (100%, 66 bp, 500–1000 bp); replacement (100%, 306 bp, 1000 bp); DNM (100%, N/A, 664 bp)		[120–122]
	*Spy*Cas9n	1	Deletion (7–100%, 20 bp, NR)	1	[116]
C. autoethanogenum	*Spy*Cas9	1	Deletion (>50%, NR, NR)		[105]
	*Spy*Cas9	1	Deletion (0–100%, 2379 bp, 400–1000 bp); insertion (75–87%, 1614 bp, 1000 bp); SNM (>99%, N/A, 1000 bp)		[115, 117]
C. beijerinckii	*Spy*Cas9n	1	Deletion (0–100%, 1149 bp, 150–1200 bp)	2	[116, 123, 124]
	*Spy*Cas9n-Apobec1-UGI	1	Base editing (11–100%)		[125]
	*As*Cas12a	1	Deletion (100%, 1021 bp, 500 bp)		[126]
C. botulinum	*Spy*Cas9	1	Deletion (100%, 2400 bp, NR)		[8]
C. cellulolyticum	*Spy*Cas9n	1	Deletion (100%, 23 bp, 200–1000 bp), insertion (95–100%, 1720 bp, 100–1000 bp)	3	[127–129]
C. chauvoei	*Spy*Cas9		Deletion (100%, 948 bp, 1000 bp)		[130]
	*Spy*Cas9	1	Deletion (20–100%, 2400 bp, 1000 bp); insertion (80%, >400 bp, NR)		[8, 131–133]
C. difficile	*As*Cas12a	1	Deletion (37.5–100%, 46.7 kbp, 500–1000 bp)		[134]
		2	Deletion (25–58.3%, 46.7 kbp, 500–1000 bp)	4	[134]
	Cas3[b)]	1	Deletion (100%, 258 bp, 1200 bp)		[135]
C. Ijungdahlii	*Spy*Cas9	1	Deletion (50–100%, 2600 bp, NR); insertion (100%, 8500 bp, N/A)	5, 6	[136, 137]
	d*Spy*Cas9-Deaminiase fusion	1	Base editing (12.5–100%)		[138]
	*Fn*Cas12a	1	Deletion (100%, 2600 bp, 1000–2500 bp)		[139]

(*continued*)

Table 16.1 (Continued)

Species	CRISPR effector	Number of spacers	Desired edit (efficiency, maximum deletion/insertion, HA length)	Notes	References
	*Spy*Cas9	1	Deletion (100%, 2400 bp, 1000 bp)		[8, 140]
C. pasteurianum	*Spy*Cas9n	1	Deletion (100%, 1200 bp, 1000 bp)		[141]
	Cas3[b)]	1	Deletion (100%, 762 bp, NR)		[140]
C. saccharoperbutyl-acetonicum	*Spy*Cas9	1	Deletion (>75%, NR, 1000 bp); insertion (NR, 2560 bp, 1000 bp)	7	[142–144]
	Cas3[b)]	1	SNP (20%, N/A, NR); deletion (100%, NR, 760 bp); insertion (35–60%, 5000 bp, 950 bp)		[145]
C. sporogenes	*Spy*Cas9	1	Deletion (100%, 2400 bp, NR); insertion (>90%, 2900 bp, NR)		[8]
C. thermocellum	*Geo*Cas9n		Deletion (0.21–91%, NR, 50–1000 bp)	8, 9, 10	[146]
	Cas3[b)]	1	Deletion (14–70%, NR, 50–1000 bp)	9, 10	[146]
	*Spy*Cas9	1	Deletion (25%, 816 bp, 500 bp)		[147]
C. tyrobutyricum	*As*Cas12a	1	Deletion (12.5%, 816 bp, 500 bp)		[147]
	Cas3[b)]	1	Deletion (100%, NR, 300–1000 bp); replacement (100%, NR, 500 bp)	11	[148]
	Cas3[b)]	2	Deletion (100%, NR, 300 bp)	4, 5	[148]
Species	CRISPR effector	Target strand (level of repression)	Method of detection		References
C. acetobutylicum	d*Spy*Cas9	Template (20%); nontemplate (45–90%)	Fluorescence of targeted reporter AFP; RT-qPCR, GC analysis of metabolites		[116, 120]
C. beijerinckii	d*Spy*Cas9	Target strand not reported (65–95%)	RT-qPCR, GC analysis of metabolites; enzyme activity assay, measure protein concentration		[116, 149]

C. cellulovorans	d*Spy*Cas9	Target strand not reported (95%)	RT-qPCR, GC analysis of metabolites	[150]
C. ljundahlii	d*Fn*Cas12a	Template (88–99%)	RT-qPCR, GC analysis of metabolites	[139]

1) Lower efficiencies with ptb promoter vs. thl.
2) HA of 500 bp or less resulted in less than 50% efficiency.
3) Short homology arms did not significantly affect efficiency. Attempts to insert fragments of 3000 and 6000 bp were unsuccessful using HA of 1000 bp.
4) Duplexed CRISPR array used to target multiple crRNAs to a single gene for increased editing efficiency AND to target multiple genes simultaneously.
5) Subculturing required for maximum efficiency.
6) 8.5 kbp inserted by coupling CRISPR/Cas9 system with phage serine integrase system.
7) Also used for curing of 136 kbp megaplasmid [144].
8) Attempts to utilize wt *Geo*Cas9 yielded no transformed colonies.
9) Spacer of approximately 30 bp optimal. PAM Site = TCR where R is adenine or guanine.
10) Coupling CRISPR system with heterogeneous recombineering machinery greatly increased recombination efficiency.
11) Low editing efficiencies observed with HA of 50 bp even when recombineering machinery is expressed.

All mentions of Cas9 refer to *Streptococcus pyogenes*-derived Cas9. Reported editing efficiencies are the fraction of successful mutants of total colonies screened.
Abbreviations: DNM, dinucleotide modification; NR, not reported; SNM, single nucleotide modification.
Cas3 is the effector protein in the native *C. pasteurianum* type I-B CRISPR system.
a) Targeted region downstream of Ccel_3198 gene.
b) Endogenous system.

insertions in *C. acetobutylicum* [113], *C. beijerinckii* [116], *C. cellulolyticum* [128], *C. pasteurianum* [140] with up to 100% efficiency.

While the most common, *Spy*Cas9 use on *Clostridium* genomes (~30% GC content) has been limited by relative unavailability of the G-rich PAM site as well as the inability to easily target multiple genes simultaneously. To address these challenges, Type V-A systems have been used in *Clostridium* species. Like the Type II CRISPR systems, the Type V-A only requires a single CRISPR effector protein, Cas12a, co-expressed with a CRISPR RNA (crRNA) to implement a DSB. Cas12a proteins recognize T-rich PAM sites and are capable of self-processing CRISPR arrays into individual crRNAs, thereby easily allowing for multiple targets simultaneously (i.e. multiplexing) [139, 152]. Various Cas12a proteins have been employed for counterselection in *Clostridium* species. *As*Cas12a (from *Acidaminococcus* sp.), which recognizes a TTTV PAM site, has been the most used Cas12a protein in *Clostridium*. *As*Cas12a-mediated editing has been demonstrated in *C. beijerinckii*, *C. difficile*, and *C. tyrobutyricum* [126, 134, 147]. In *C. difficile*, a CRISPR array was used to target *As*Cas12a to multiple regions simultaneously. Multiplexed CRISPR activity is able to accommodate both the editing of multiple genes concurrently and the targeting of a single gene in multiple regions for increased editing efficiency [134]. A second Cas12a protein, *Fn*Cas12a, native to *Francisella novicida*, has been used for counterselection in *C. ljundahlii* with 100% observed efficiency [139] (Table 16.1). In that study, *Fn*Cas12a was selected as it was least toxic to cells from a group of four Cas12a proteins. This study suggests the level of toxicity of heterogeneous Cas effector proteins may be specific to species.

Type I-B CRISPR systems are endogenous to several *Clostridium* species and can be programmed via expression of synthetic CRISPR arrays for gene targeting in their native species. The repurposing of endogenous CRISPR systems to facilitate gene editing circumvents the need to transform large plasmid constructs, as only a synthetic CRISPR array and donor DNA is required for successful activity. Utilization of native systems has especially been beneficial in species whose primary method of plasmid uptake is via conjugation such as *C. difficile* [153] and *C. tyrobutyricum* where conjugation efficiencies have impeded CRISPR-based editing progress. *C. tyrobutyricum*'s native CRISPR system, which recognizes a TCR PAM site, has been used for gene editing with 100% efficiencies. The utility of this method is exemplified by the replacement of the *cat1*, which previously could not be deleted, with *adhE*, which mostly eliminated butyrate production in *C. tyrobutyricum*, a known hyper-butyrate producer, and converted it into a hyper-butanol producing strain [148]. *Spy*Cas9 and *As*Cas12a systems have successfully been employed in *C. tyrobutyricum* due to increasing conjugation efficiencies [147], but with lower success rates. Endogenous CRISPR systems have also been employed in *C. difficile* [153], *C. pasteurianum* [140], and *C. thermocellum* [146]. One major challenge in using the endogenous CRISPR system is the identification of the corresponding PAM sequence, which may require bioinformatic approaches along with wet lab experiments to identify functional motifs.

Thermophillic *Clostridium*, of general interest for their cellulose consumption capabilities, cannot use the well-characterized CRISPR systems that have been

successfully employed in mesophilic strains. In *C. thermocellum*, the use of both its native Type I-B CRISPR system, as well as a Cas9 variant from *Geobacillus stearothermophilus* (*Geo*Cas9), has been performed [146]. Editing efficiencies of up to 70 and 94% were achieved using the endogenous Type I-B CRISPR system and the *Geo*Cas9 respectively.

In general, longer regions of homology on the donor plasmid have been shown to increase efficiency. In *C. cellulolyticum,* donor template arm lengths greater than 0.2 kbp had an efficiency of more than 95% when compared with smaller arms, which were only 55% efficient in a CRISPRn system [127]. A similar study using Cas9 in *C. acetobutylicum* demonstrated an increased efficiency when homology arm lengths of 1 kbp were used as opposed to 500 bp arm lengths [120]. In *C. tyrobutyricum*, homology arms of 300 bp were used with its endogenous CRISPR system, compromising the rate of recombination for smaller plasmid size and increased transformation efficiency. In this case, maximal efficiency was achieved via subculturing, which has also been shown to enhance the edited population across all CRISPR systems [136, 139, 146, 148].

CRISPR systems have been further repurposed for the regulation of gene expression. In catalytically dead effector proteins, the nuclease active sites are mutated such that the proteins retain their ability to bind to DNA but are unable to perform endonuclease activity. In *Clostridium* species, these proteins, most commonly, d*Spy*Cas9, have been used for targeted gene repression, whereby, transcription of a gene is sterically hindered by the CRISPR effector protein bound to it. CRISPR interferance (CRISPRi) has enabled simple, tunable, reversible gene knockdown at a transcriptional level through the co-expression of the catalytically dead effector protein and sgRNAs, designed with the aid of bioinformatics tools. This method is reversible, with no permanent change in the genome [154]. Additionally, CRISPRi activity can be modulated not only through controlling the expression of effector protein [155], but also by its relative position to the promoter and gene start site [156], allowing tight control of gene expression.

*dSpy*Cas9 has allowed gene knockdowns in *C. acetobutylicum* [120], *C. beijerinckii* [116, 149], in *C. cellulovorans* [150], and *C. difficile* [8, 131, 132] and has been used to silence both native [116, 149, 150] and heterologous genes [120]. d*Fn*Cas12a has been used to regulate gene expression in *C. ljundahlii* [138].Gene repression of up to 97% was achieved with dSpyCas9 and 99% using d*Fn*Cas12a, although the effectiveness of CRISPRi varies among species with similar configurations [116], as well as among genes targeted within a single species (Table 16.1). The tunability of dCas9 has yet to be fully explored in *Clostridium*, as it has in other bacterial systems [155, 156]. Control of effector protein expression is important as constant expression is needed to supress genes. One study showed activity of the knockdown target unintentionally increased over the time of fermentation, which was attributed to the variable strength of the commonly used thiolase promoter used [149].

Gene expression knockdowns have also been accomplished on the translational level in *Clostridium* through antisense RNA (asRNA) technology. The asRNA knockdown method involves targeting an mRNA transcript using its asRNA. This method has been used to investigate the function of genes in various *Clostridium*

species [128, 157–160], including essential genes where a genetic knockout is unfeasible. asRNA technology has also been used to manipulate gene expression affecting solvent titers [161, 162]. asRNAs are tunable and reversible, and have been effective in silencing, achieving as high as 90% repression levels of certain genes. However, despite their lengths (>100 bp) asRNAs have been shown to be promiscuous, binding especially to transcripts with a high homology to the target sequence [157]. Consequently, asRNA technology requires large constructs for efficient gene repression while CRISPRi provides repression specificity, using a 20 nt sgRNA choice.

Several other applications of catalytically dead CRISPR effector proteins have been recognized based on their very ability to selectively bind to specified DNA sequences. Base editors, deaminases which indiscriminatingly install cytosine-to-thymine or adenine to guanine point mutations to DNA, have been tethered to dCas9 (as well as Cas9n) to implement targeted point mutations in several organisms [163–168]. The fusion of the base editor to dCas9 limits its activity to the region in which the dCas9 protein is targeted. In *Clostridium*, CRISPR-mediated base editing has been performed in *C. ljundahlii* and *C. beijericnkii* with editing efficiencies of between 11 and 100% [138, 169]. While CRISPR-based gene activation and imaging tools [170–173] have been developed in other bacterial species, the application of these tools has yet to be realized in *Clostridium* species.

Application of CRISPR tools overall is still limited in *Clostridium* due to low plasmid transformation efficiencies and a lack of characterized recombineering and NHEJ tools. While the use of native CRISPR systems circumvents the need to express heterologous Cas effector proteins, the applicability of these systems may be limited to strains with functional CRISPR/Cas machinery and by unknown PAM sequences. Recombineering, through lambda red technology, has facilitated gene engineering in *E. coli* via the use of linear DNA repair templates, a process that skips the cloning steps required in plasmid-based homologous recombination methods. Coupled with CRISPR, this technology enables multiplexed ssDNA recombineering events with efficiencies allowing large libraries ($>10^5$ members) to be constructed in parallel [174]. However, the lack of ssDNA recombineering machinery functional in *Clostridium* hinders the development of comparable *Clostridium*-based technologies. Although a RecT protein from *C. perfringens* demonstrated recombineering activity in *C. acetobutylicum*, the results obtained were not comparable to routine recombineering events in *E. coli* [175]. Similarly, the expression of Ku and LigD genes from *Mycobacterium tuberculosis* enabled NHEJ in *E. coli* following the implementation of a DSB via Cas9 [176]. Although NHEJ-related genes (*ku, DNA ligase,* and *ligD*) are found on the *C. cellulolyticum,* they are not highly expressed and NHEJ events have not been observed in the species after a Cas9 DSB [127]. Heterologous expression of such genes may enable NHEJ in *Clostridium*.

16.4 Genetic Parts in *Clostridium*

Rational engineering of solventogenic clostridia relies on having access to a "toolbox" of well-characterized biological parts including promoters, ribosomal

binding sites (RBS), origin of replication (ORI), and terminators for further advancement of metabolic engineering efforts in *Clostridium*. To enhance productivity and yield of desired products, the "toolbox" should enable assemblies of individual or grouped genes to be well-regulated [177].

16.4.1 Promoters

Promoters play a critical role in controlling gene expression. Precise control of gene expression allows balancing gene expression of metabolic pathways and constructing compatible genetic circuits. Therefore, expanding the availability of well-characterized promoters is essential in proper construction of genetic circuits for *Clostridium*. Several *Clostridium* regulatory promoters and transcription factors have been identified through systems biology and bioinformatic analysis. Transcriptional analysis data of differential carbohydrate utilization in the presence of glucose in *C. acetobutylicum* [178] were used to develop new sugar-based inducible promoter systems for the carbon source of interest [179]. Sugar-based inducers are simultaneously utilized as carbon sources in *Clostridium* hosts, and thus depleted over time and are complicated by carbon catabolite repression [180].

As in *E. coli*, lactose, and the unconsumable isopropyl β-D-1-thiogalactopyranosidethe (IPTG)-responsive promoters are the most widely used inducible systems in *Clostridium* (Table 16.2). Pfac system, engineered with the *E. coli* LacI regulator and *lacO*, is responsive to 1 mM IPTG in *C. acetobutylicum*, *C. botulinum*, *C. sporogenes*, and *C. difficile* [51, 188]. Sugar-based inducers are utilized as carbon sources in *Clostridium* hosts and complicated by carbon catabolite repression.

Tetracycline/Anhydrotetracycline (aTc)-inducible systems regulated by TetR from *S. aureus* [158], *C. autoethanogenum* [186], and *E. coli* [181] are commonly used systems in *Clostridium*, however, can lead to inducer toxicity at high concentrations [180].

Use of *Clostridium* promoters is complicated by the limited supply of native promoters originating from a few strains that are not always transferrable across hosts within the same genus. Commonly used constitutive promoters such as the *ptb* (phosphotransbutyrylase) and *thl* (thiolase) from *C. acetobutylicum* and *C. pasteurianum* have shown varied promoter activity in different strains and stages of growth [189, 190]. Furthermore, high basal expression is often observed across *clostridial* inducible promoters such as the ARAi system [80], lactose-inducible promoters using the transcriptional regulator BgaR [182], xylose-inducible promoter-repressor system [179, 187, 191], as well as many others (Table 16.2) when used in organisms other than the base strain, thus, affecting the dynamic range.

Optimization strategies to further fine-tune the biosensor performance to respond with the appropriate sensitivity and signal output can be achieved by altering the number, location, and sequence of TF binding sites. Through the addition of two *tetO1* operator sequences surrounding the −35 and −10 boxes of a native constitutive promoter, the creation of a tetracycline/aTc-inducible promoter, Pcm-2*tetO1*, reduced basal expression and increased binding of TetR. This resulted in a 313-fold induction compared to a 41-fold induction performed

Table 16.2 Inducible promoters in *Clostridium*.

Inducer	Transcription factor *Source*	Promoter *Source*	Species tested	Dynamic range	Reporter gene	References
L-Arabinose	AraR *C. acetobutylicum*	P_{ptk} *C. acetobutylicum*	*C. cellulolyticum*	>800-fold (Imaging)	*gusA* (MUG) *PpFbFPm*	[80]
		P1341–2	*C. acetobutylicum*	60-fold	*mCherryOpt*	[179]
		C. acetobutylicum P1343	*C. beijerinckii*	32-fold		
			C. acetobutylicum	21-fold		
		C. acetobutylicum	*C. beijerinckii*	27-fold		
aTc	TetR *E. coli*	Pcm-2tetO1	*C. acetobutylicum*	313-fold	*gusA* (X-Gluc)	[181]
		P_{cm}/tetO1 *C. acetobutylicum*/*E. coli*	*C. ljungdahlii*	28-fold		
		Pcm-tetO2/1	*C. acetobutylicum*	120-fold		
Fructose	FruR *C. acetobutylicum*	P0231 *C. acetobutylicum*	*C. acetobutylicum*	2-fold	*mCherryOpt*	[179]
				<2-fold	*lacZ* (X-gal)	
		P0234 *C. acetobutylicum*	*C. acetobutylicum*	4-fold	*mCherryOpt*	
			C. beijerinckii	2-fold		
Lactose	BgaR *C. perfringens*	P_{bgaL} *C. perfringens*	*C. perfringens*	80-fold (Imaging)	*gusA* (ONPG) *yfp*-tagged *pilB*	[182]
			C. acetobutylicum	10-fold	*gusA*	[102]
			C. ljungdahlii	8-fold	*gusA*	[183]
	$LacI^Q$ *E. coli*	P_{fac} system	*C. acetobutylicum*	10-fold	*Ll.ltrB*	[51]
		P_{fdx}/lacO *C. pasteurianum*/*E. coli*	*C. sporogenes*	100-fold	*Ll.ltrB*	
Lamin-aribiose	GlyR3 *C. thermocellum*	P_{celC}/celO *C. thermocellum*	*C. thermocellum*	40-fold	*spo0A*	[184]

Maltose	CcpA V302N *C. acetobutylicum*	P0532–3 *C. acetobutylicum*	*C. acetobutylicum*	11-fold	*mCherryOpt*	[179]
Sucrose	LicT *C. acetobutylicum*	P0423–5 *C. acetobutylicum*	*C. acetobutylicum*	21-fold	*mCherryOpt*	[179]
Tetra-cycline	TetR *S. aureus*	P_{xyl}/tetO *B. subtilis/S. aureus*	*C. difficile*	200-fold	*gusA*	[158]
			C. acetobutylicum	4.5-fold	*phiLOV2.1*	[185]
			C. sordellii	5.6-fold		
	TetR	tet3nO P_{thl}/tetO	*C. autoethanogenum*	NR	*catP*	[186]
		tet3nO (optimized) $P_{thl\text{-}IPL12}$/tetO	*C. autoethanogenum*	9-fold higher than tet3no		[105]
Xylan	XylR *C. cellulolyticum*	P1133 *C. cellulolyticum*	*C. cellulolyticum*	10-fold	*PpFbFP*	[113]
Xylose	XylR *S. xylosus*	P_{xylA}/xylO *S. xylosus*	*C. acetobutylicum*	17-fold	*gusA*	[187]
	XylR *C. difficile*	P_{xylB}/xylO *C. difficile*	*C. difficile*	100-fold	*mCherryOpt*	[180]
	XylR *C. acetobutylicum*	P2610–12 *C. acetobutylicum*	*C. acetobutylicum*	3-fold	*mCherryOpt*	[179]
				60-fold	*lacZ* (X-gal)	
		P3451–2 *C. acetobutylicum*	*C. acetobutylicum*	300-fold	*mCherryOpt*	
				60-fold	*lacZ* (X-gal)	
			C. beijerinckii	20-fold	*mCherryOpt*	
		P1133 *C. cellulolyticum*	*C. acetobutylicum*	5-fold	*mCherryOpt*	
			C. beijerinckii	2-fold	*mCherryOpt*	

by the one operator version, Pcm-*tetO1* [181]. In another TetR-based inducible system, the engineered tet3no promoter [186] was further optimized through randomizing in the spacer between the −35 and −10 boxes [105]. The basal expression of the improved tet3no promoter had ninefold higher dynamic range than the original tet3no promoter, thus, improving CRISPR/Cas9-mediated gene deletion by 50% in *C. autoethanogenum* under the control of the optimized promoter.

Artificial promoter libraries with varying promoter activities are often utilized to identify useful promoters to obtain the desired phenotype. Mutagenesis of the flanking regions surrounding the −35 and −10 consensus sequences of the *thl* promoter yielded a range of promoter strengths covering two orders of magnitude for use in *C. acetobutylicum* and *C. ljungdahlii* [190]. Generation of a library displaying a wide distribution of promoter strengths requires the proper mutation stringency for the promoter of interest to ensure there is a range of active promoters. Over-mutating the sequence space can lead to complete loss of promoter activity in the intended organism of study, while limiting the number of randomized bases can reduce the range of various promoter activities [192]. Controlling the mutation frequency of the thiolase promoter generated a library displaying a distribution of promoter strengths over a 260-fold range [192].

16.4.2 Reporters

Reporter genes act as an observable proxy for some unobservable process. Helpfully, they can be used to provide measurement of gene expression for screening and characterization of promoters. An ideal reporter has high sensitivity and specificity, a large dynamic range of detection, and low endogenous levels of the reporter.

16.4.2.1 Enzyme-Based Reporters

Chloramphenicol acetyltransferase (CAT) reporter, encoded by *catP*, has been extensively used to measure promoter activity in the *Clostridium* genus [193, 194]. When expressed by an active promoter, the CAT enzyme catalyzes an acetyl group from exogenously added substrate, acetyl-CoA, to the antibiotic chloramphenicol (Cm), which then can be used as an antibiotic section marker. Addition of compound 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) is then used to quantify the concentration of the thiol groups of the acetylated Cm under the absorbance at 412 nm [195]. However, its use is limited to strains naturally resistant to Cm, such as *C. acetobutylicum* and *C. beijerinckii* [195]. Yet, a *catP-lacZ* dual reporter system was implemented in *C. acetobutylicum* to rapidly screen and characterize the thiolase promoter library [190]. The initial screen would select and partition for promoters with various CAT activity on agar with varying Cm concentrations, ranging from 100 to 1200 μg ml^{-1} chloramphenicol. Then the *lacZ* gene is subsequently used as a colorimetric reporter to accurately measure promoter activities.

Commonly used reporters to study quantitative and qualitative gene expression in *Clostridium* are the *β*-galactosidase enzymes, encoded by *lacZ* from *Thermoanaerobacterium thermosulfurigens* EM [189, 196, 197]

and the β-glucuronidase enzyme, encoded by *gusA* (or *uidA*) from *E. coli* [80, 181, 183, 198]. Both *lacZ* and *gusA* reporters are versatile in their detection platform depending on the substrate. Measurement of β-galactosidase activity as a colorimetric reporter is done by adding substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-GAL) in agar plates [179, 197]. Substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) can be used for colorimetric and spectrophotometric assays [182]. β-Glucuronidase reporters utilize analogous substrates to β-galactosidase reporters: 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) for colorimetric [181, 199], and *p*-nitrophenyl-β-D-glucuronide (PNPG) for spectrophotometric detection. For fluorescent assays, 4-methylumbelliferyl-β-D-galactopyransoside (MUG) for fluorescent assays is used in both β-galactosidase and β-glucuronidase assays [80, 187]. The *gusA* gene is smaller and more stable compared to *lacZ*, and has been reported to have lower background when used as a fusion reporter in *Clostridium* [198]. Other enzymatic reporters not as commonly used but available in *Clostridium* include the β-1,4-endoglucanase gene (*eglA*) isolated from *C. saccharobutylicum* [200], thermostable lipase (*tilA*) gene from *Pseudomonas fluorescens* [201], AmyE^{opt} comprising of a codon-optimized amylase gene from *C. acetobutylicum* and secreting PPEP-1 signal sequence [202], and the alkaline phosphatase gene (*phoZ*) isolated from *Enterococcus faecalis* [203, 204].

16.4.2.2 Bioluminescent Reporters

Bioluminescent reporters are useful as they can amplify the signal from genes expressed at low levels. Despite the need for oxygen (generally undesired for anaerobic *Clostridium*), luciferase reporters (*luc* and *lucB* from firefly *Photinus pyralis*) in *Clostridium* spp. can be used with the addition of ATP and washing cells with a neutral buffer [81, 197, 205]. This luciferase has also been applied in developing and optimizing clostridia-based cell-free systems [206]. In *C. difficile*, a luciferase fusion with secreting protein PPEP-1 (sLuc^{opt}) enabled low-background expression anaerobically with measurement in an aerobic environment, albeit with a delay in signal [202]. Similarly, bacterial luciferases (*luxAB*) of the lux system from *Vibrio fischeri* provide a luminescent output in the presence of flavin mononucleotide (FMN) and long-chain aldehyde substrate in *C. perfringens* [81, 194]. A major limitation with the use of bioluminescence is that while they can be expressed in anaerobic bacteria, transcription activity is not directly correlated with the output [207].

16.4.2.3 Fluorescent Reporters

Fluorescent reporters such as green fluorescent protein (GFP) and its variants allow real-time measurements of gene expression at a single cell level without the need of substrates. *Clostridium* species can express GFP and its variants under anaerobic conditions; however, the intrinsic chromophore of the fluorescent protein requires exposure to molecular oxygen to fluoresce [208]. In addition, the protein complex responsible for cell division quickly disassembles when O_2 disrupts energy metabolism pathway in *Clostridium*.

While an anaerobic reporter would be more suitable, the fluorescence intensity of GFP-based reporters is often more favorable, however, in more

oxygen-tolerant clostridia such as *C. perfringens* [182, 209]. A codon-optimized enhanced GFP (*Cp*EGFP) surpassed the codon-optimized version of anaerobic fluorescent protein, *Bs*FbFP, after exposed to air for 20 minutes [209]. To quantitatively measure sporulation events, however, it was required to resuspend the bacterial culture in PBS to reduce background fluorescence caused by media constituents. When using codon-optimized cyan fluorescent protein (CFPopt) [210] and mCherryOpt [208], fixation of *C. difficile* cells was necessary to prevent cell lyses upon exposure to an aerobic environment and to preserve the fluorescent protein structure. This process known as "aerobic fluorescence recovery" allows the chromophore to mature in fixed cells.

Due to an intrinsic green and blue autofluorescence in *Clostridium* cells, yellow fluorescent protein (YFP) [182] and mCherry are better alternates to GFP and CFP in *Clostridium*. Complete fluorescence with low autofluorescence background was observed after being exposed to oxygen for two hours in *C. difficile* with the use of mCherry [208]. Furthermore, mCherry is smaller in size than other GFP variants.

16.4.2.4 FbFP-Based Fluorescent Reporters

FMN-based fluorescent proteins (FbFPs) are an emerging class of fluorescent reporters for anaerobes [211–213]. These fluorescent reporters utilize a FMN as a chromophore cofactor, thus independent of oxygen. Due to its smaller size (~13 kDa), FbFPs have relatively faster turnover rates and are most often fused with the protein of interest without disruption of its native function in *C. difficile* [185].

Several versions of FbFP exist each with its own attributes: (i) *Pp*FbFP, based on a sensory box protein from *Pseudomonas putida*, (ii) *Bs*FbFP, based on the N-terminal LOV domain of YTVA protein from *B. subtilis*, (iii) *Ec*FbFP, a codon-optimized version of *Bs*FbFP, (iv) *i*LOV, created from the LOV2 domain of the blue light photoreceptor phototropin from *Arabidopsis thaliana* [214], and photostable version of *i*LOV (v) *phi*LOV2.1 [211]. *i*LOV and *Ec*FbFP have been engineered to retain 60–70% fluorescence in a pH range of 4–11, while *Pp*FbFP loses fluorescence at pH 11 [214]. While *i*LOV outperforms other versions in fluorescence at elevated temperatures over *Pp*FbFP and *Ec*FbFP, all three FbFPs have a higher thermal stability (50 °C) over GFP. Although *phi*LOV2.1 outperformed other FbFPs, GFP still outshines *phi*LOV2.1 [185].

Several *Clostridium* species have utilized FbFPs, including *Ec*FbFP, as fluorescent reporters: *Pp*FbFP (commercial name Evoglow-CK^{XN}-Pp1) in *C. cellulolyticum* [80, 113], *C. ljungdahlii* [215], and *C. pasteurianum* [120]; *Bs*FbFP (commercial name Evoglow-CK^{XN}-Bs2) in *C. ljungdahii* [215], *C. tyrobutyricum* [216], and *C. acetobutylicum* [217]; *Ec*FbFP in *C. beijerinckii* [213]; and *phi*LOV2.1, in *C. difficile*, *C. acetobutylicum*, and *C. sordellii* [185].

Performance of FbFP can vary across *Clostridium* hosts and readout platforms. Due to a lower quantum yield (brightness) compared to GFP, the intrinsic green autofluorescence of *Clostridium* cells lowers the fluorescence signal-to-noise ratio [185, 192]. While GFP-based reporters have a diverse palette of colors, FbFP is limited to one. A few strategies are suggested when using FbFP in *Clostridium*. Eliminating yeast and beef extract from flavin-based media such as

reinforced clostridial medium (RCM) and PETC (ATCC medium 1754) lowers fluorescent backgrounds [215]. However, removal of yeast and beef extract from the media significantly affected the growth rates of *C. ljungdahlii*, thus affecting proper quantitative analysis between protein expression and growth phase. For fluorescence microscopy, a mitigating method is to take cells expressing the fluorescent reporter and fluorescent negative cells separately through a series of different excitation wavelengths, then identify the excitation wavelength with the widest spread of the signal between the fluorescent positive and negative cells. The overall fluorescence of *phi*LOV2.1 reporter in *C. difficile* at excitation wavelengths at 450 and 470 nm were 2.1-fold and 1.7-fold higher than the non-fluorescent cell, and improved the overall noise-to-signal fluorescence [185].

16.4.2.5 FAST, HaloTag, and SNAP-Tag Fluorescent Reporters

The versatility of the fluorescence-activating and absorption-shifting tag (FAST) protein is a promising reporter for *Clostridium*. The FAST protein is expressed within the cell under a control of a promoter, and only fluoresces when bound to an exogenously added fluorogenic ligand under either aerobic or anaerobic conditions [218]. Depending on the fluorogen analog, the fluorogen–FAST complex produces various fluorescence colors: a green-yellow fluorescence is emitted using 4-hydroxy-3-methylbenzylidene-rhodanine (HMBR) upon blue light excitation.

A major limitation of metabolic engineering in *Clostridium* has been the low transformation efficiency of plasmid DNA inside the cell. Plasmid transformation was first described in *C. acetobutylicum* using 1 µg of DNA in an in vivo methylation using *B. subtilis* phage ϕ3T I methyltransferase in *E. coli* [219]. Methods to transform plasmid DNA using common techniques (e.g. electroporation) require strain-by-strain development [220]. One major obstacle is the restriction-modification systems present in each strain. Genomic DNA methylation analysis (using PacBio SMRT sequencing) can be performed to identify methylation motifs to avoid including these sequences in the plasmid of interest [28]. Additionally, bioinformatic analysis can be used to clone methylation genes in *E. coli* to protect plasmid DNA prior to transformation.

16.4.3 Terminators

At the 3′ end of an expression construct, terminators are often overlooked genetic elements in the clostridial toolbox despite their role in gene expression stability. Non-native terminators, including bidirectional *E. coli* terminator BB1_B1010 from iGEM Parts Registry, have demonstrated a 36% increase in *catP* reporter expression over the native *adc* terminator [221].

Commonly used terminators are obtained from genes *adc* (acetoacetate decarboxylase) from *C. acetobutylicum* and *fdx* (ferrodoxin) from *C. pasteurianum* [76, 78, 158, 189, 191], as well as the in silico identified CD0164 terminator of *C. difficile* 630 *fdx* gene [9, 222]. With limited selection of terminators, *E. coli rrnB T1* and *B. subtilis tyrS* tRNA terminators were found to be efficient terminators for use in *C. acetobutylicum* [188].

Availability of bioinformatic tools such as TransTermHP [223, 224], RNAMotif [223], and WebGeSTer [225] have enabled identification and prediction of *Clostridium* terminators. Expansion of clostridial terminators can further enhance the synthetic biology toolbox.

16.4.4 5′-UTRs and Riboswitches

Components within the noncoding regions of RNAs are emerging as important regulatory elements to control gene expression in *Clostridium*. Careful design of the 5′ untranslated regions (5′-UTR) of the mRNA can increase mRNA stability and gene expression of *adhE1* and *adhE2* in *C. acetobutylicum* and *C. beijerinckii* [221].

New inducible systems in *Clostridium* have been engineered using components known as riboswitches that are found within the 5′-UTRs. Riboswitches can regulate gene expression in a ligand-dependent manner, without the need of a protein regulator. Riboswitches consists of an aptamer domain where the ligand binds, and the gene expression platform that directly controls gene expression. Upon ligand binding, a conformational change of the riboswitch either exposes or blocks the RBS from ribosome binding, and thereby regulates translation of the expression platform. For example, a natural purine-responsive riboswitch was utilized in tuning expression of *adhE* to control the production of ethanol in thermophile *C. thermocellum* [226]. Other uses have included a theophylline-responsive riboswitch to tune Cas9 expression in *C. sporogenes* [8], a nickel or cobalt ion riboswitch to tune Cas9 expression in *C. scindens* [227], and an engineered glycine-responsive riboswitch from *C. pasteurianum* that helped increased production of 5-aminolevulinic acid by 11% in *E. coli* [228]. While only a few have been implemented as an alternative to inducible promoters for use in *Clostridium*, a growing number of riboswitches have been identified in the *Clostridium* genus, opening up possibilities for continued development [229–232].

Author Contributions

RJ, SK, NK, and NS prepared and edited the manuscript; RJ prepared Figure 16.1 and Table 16.1; NK prepared Table 16.2.

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Abstract

The *Clostridium* genus contains a diverse range of Gram-positive, sporulating, obligate anaerobes that have been of historical biotechnological interest due to their acetone–butanol–ethanol (ABE) solvent production. Within the last few decades, interest has grown in the *Clostridium* spp. capable of consuming a wider variety of feedstocks, which include gaseous and renewable biomass sources. Additionally, attenuated pathogens have been of interest as potential therapeutics. The fruition of the genus's great promise has been limited by the slow progress in genetic engineering and synthetic biology methods and tools, relative to workhorse organisms such as *Escherichia coli*. Recent advances in these areas, not least of which include CRISPR-based tools, renew the promise of metabolic engineering for a broad range of feedstock consumption and production of chemicals. In this chapter, we describe the current state of engineering in the *Clostridium* genus by describing efforts and continued challenges in directed evolution, the use of systems biology for greater understanding, methods for performing genomic editing, and the expanding library of genetic parts. These new capabilities and tools have expanded the number of species that are able to be engineered for biotechnological purposes, increased the throughput of genetic studies, and expanded the range of products made from *Clostridium*.

Keywords

Clostridium; genomic engineering; synthetic biology; anaerobic reporters; inducible promoters; CRISPR