Synthetic Biology Tools for Genome and Transcriptome Engineering of Solventogenic *Clostridium*

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Strains of *Clostridium* genus are used for production of various value-added products including fuels and chemicals. Development of any commercially viable production process requires a combination of both strain and fermentation process development strategies. The strain development in *Clostridium* sp. could be achieved by random mutagenesis, and targeted gene alteration methods. However, strain improvement in *Clostridium* sp. by targeted gene alteration method was challenging due to the lack of efficient tools for genome and transcriptome engineering in this organism. Recently, various synthetic biology tools have been developed to facilitate the strain engineering of solventogenic *Clostridium*. In this review, we consolidated the recent advancements in toolbox development for genome and transcriptome engineering in solventogenic *Clostridium*. Here we reviewed the genome-engineering tools employing mobile group II intron, *pyrE* alleles exchange, and CRISPR/Cas9 with their application for strain development of *Clostridium* sp. Next, transcriptome engineering tools such as untranslated region (UTR) engineering and synthetic sRNA techniques were also discussed in context of *Clostridium* strain engineering. Application of any of these discussed techniques will facilitate the metabolic engineering of clostridia for development of improved strains with respect to requisite functional attributes. This might lead to the development of an economically viable butanol production process with improved titer, yield and productivity.

Keywords: *Clostridium*, synthetic biology, mobile intron, CRISPR, Cas, synthetic sRNA, UTR

INTRODUCTION

Strain improvement for production of fuels or any biobased industrial product could be achieved by employing any of the following two strategies: (i) heterologous expression of metabolic pathway genes in a non-native producers, and (ii) improvement of native producers (Arora et al., 2019; Banerjee et al., 2019; Choi et al., 2019). However, achieving titer values in heterologous host matching to those being produced by native organisms, it requires a significant effort with high chances of failure. Therefore, the strategy of improving native strains with necessary genes of the desired pathway and cofactor regeneration capability is preferred (Park et al., 2018; Rhie et al., 2019).

However, this strategy of strain improvement in *Clostridium* sp. has been limited by the availability of appropriate genome engineering tools.

Clostridium genus comprises many industrially important strains for biorefinery applications such as cellulosic and hemicellulosic biomass degradation, carbon fixation, advanced biofuel and platform chemical production and as anti-cancer therapeutics (Jang et al., 2012; Malaviya et al., 2012; Liu J. et al., 2015; Jones et al., 2016; Staedtke et al., 2016; Noh et al., 2018; Woo et al., 2018; Xin et al., 2018; Strecker et al., 2019a). The full potential of *Clostridium* genus for biorefinery applications could only be realized by advancement in the synthetic biology toolkits for strain improvement. During the last decade, tremendous progresses have been made in the development of genome engineering toolkit for strain engineering of *Clostridium* species. Development of genetic tools in *Clostridium* have been well reviewed by various research groups (Pyne et al., 2014; Liu Y. J. et al., 2015; Minton et al., 2016; Moon et al., 2016; Joseph et al., 2018; Kuehne et al., 2019; McAllister and Sorg, 2019; Wen et al., 2019b,c). Most of these reports are focused on couple of tools with an explanation in depth.

In this work, we have reviewed overall recent toolbox for genome and transcriptome engineering in solventogenic *Clostridium*, which could be used to develop improved clostridia strains, for production of sustainable and commercially viable industrial scale products. Brief features of the synthetic toolbox are summarized in **Table 1**. Consolidated information in this review dealing with strain improvement tools for *Clostridium* will aid the scientific and industrial sector to select the appropriate tools for strain improvement.

MOBILE GROUP II INTRON BASED GENE-KNOCKOUT

Mobile group II intron technology is also known as "ClosTron" when applied in context of *Clostridium* genus. In this method a gene is disrupted by inserting the mobile intron into a target locus in the chromosome by a process termed as retrohoming, making this technology a convenient, efficient and specific method of gene disruption (Heap et al., 2007, 2010; Shao et al., 2007; Jang et al., 2012, 2014; Mohr et al., 2013; Liu Y. J. et al., 2015). Among various mobile group II introns, *Ll.LtrB* and *TeI3c/4c* have been extensively used for gene knockout in the solventogenic *Clostridium*. *Ll.LtrB* intron includes intron RNA domain and open reading frame (ORF) domain. Intron RNA domain contains splicing sites consisting of exon binding sites (EBS) 1, EBS 2, and δ (**Figure 1A**). The ORF domain contains genes encoding reverse transcriptase (RTase), maturase, and endonuclease (**Figure 1A**). *TeI3c/4c* intron has been employed to develop genome engineering tool for thermophilic *Clostridium thermocellum*, since the intron could be melted down at high temperatures (Mohr et al., 2013).

Moreover, *Ll.LtrB* intron has further been modified to include a retrotransposition-activated selection marker (RAM) (Zhong et al., 2003). RAM consists of a selection marker and is inserted into the intron. A group I intron is inserted into the

marker to inactivate the marker itself. Inserted group I intron is self catalytically spliced out of mRNA in an orientation dependent manner, so that a functional marker gene can only be expressed after successful chromosomal insertion occurs (Joseph et al., 2018).

At the first stage of the clostridia gene knockout using *Ll.LtrB* intron, single gene knockouts mutant, such as *spo0A, pta, ack, ptb, buk, hbd, hydA* and *argA* variants have been constructed across the *Clostridium* genus, including *C. acetobutylicum*, *C. beijerinckii*, *C. botulinum*, and *C. difficile* (Heap et al., 2010; Dingle et al., 2011; Jang et al., 2012; Baban et al., 2013; Honicke et al., 2014; Lawson and Rainey, 2016; Liu et al., 2016). In 2012, a new method for second gene deletion was reported which could overcome the necessity of removing the plasmid used for the first gene deletion and resulted in the construction of various *C. acetobutylicum* strains, including *pta/buk, pta/ctfB, ptb/buk,* and triple mutant *pta/buk/ctfB* strains (Jang et al., 2012). In this technique, two genes encoding the erythromycin and chloramphenicol resistance enzymes were used as mutant selection marker and the concept of plasmid incompatibility was employed (Jang et al., 2012). In 2014, the same group reported the fourth and fifth gene deletion process for the construction of mutants *pta/buk/ctfB/adhE1* and *pta/buk/ctfB/adhE1/hydA* of *C. acetobutylicum* (Jang et al., 2014).

Curing and off-target manipulation remained one of the major limitations of mobile group II intron technology (Wen et al., 2019c). Curing efficiency of the plasmid containing mobile intron was enhanced by cloning *pyrF* (orotidine 5-phosphate decarboxylase) to ClosTron plasmid. The *pyrF* encodes essential enzyme of pyrimidine biosynthesis which can use 5-fluoroorotic acid (FOA) as a substrate and converts it to toxic compound and is widely used as counter selection marker (Sato et al., 2005; Tripathi et al., 2010; Heap et al., 2012). Once FOA gets converted to toxic compound by *pyrF* in the ClosTron plasmid, only cured strain could survive in the FOA added media. The cured strain can be rapidly selected by *pyrF*-based screening system, even on one plate (Cui et al., 2014).

Another problem with ClosTron is that it accidently affects and manipulates the off-target genome and cause unexpected genotypes and phenotypes (Heap et al., 2012). To overcome this, a highly regulated ClosTron system has been developed by inducing L-arabinose inducer (ARAi) to reduce off-target possibility (Zhang J. et al., 2015). To verify the impact of inducible ClosTron using ARAi system, pSY6-mspI (Cui et al., 2012) and pGZ-pyrF-cipC (Cui et al., 2014) were modified by introducing ARAi system in *C. cellulolyticum* H10 ΔpyrF strain. Surprisingly, it was found that the off-target manipulation frequency was decreased to 0 by inducible ClosTron ARAi system (Zhang J. et al., 2015).

GENOME EDITING USING *PYRE* ALLELES

Recently, allele coupled exchange (ACE) method has been developed which facilitates the insertion of complex heterologous DNA of varying size into the host genome (Ng et al., 2013;

TABLE 1 | Summary of synthetic biology tools and strategies applied for genome and transcriptome engineering of solventogenic *Clostridium*.

Categories	Tools and strategy	Brief description	Selection guide	References
Genome engineering	Mobile group II Intron	• Site-directed disruptions based on retrohoming of mobile group II introns • Insertion of intron into target site • Plasmid based • Ribonucleoprotein complex formation • Retrotransposition-activated selection marker (RAM) to help in selection	• Knockout • Knockdown	Chen et al., 2005, 2007; Shao et al., 2007; Heap et al., 2007; Baban et al., 2013; Jang et al., 2012, 2014; Pyne et al., 2014; Liu Y. J. et al., 2015; Liu et al., 2016; Xu et al., 2015; Meaney et al., 2015, 2016; Lawson et al., 2016
	pyrE allele exchange	• Works on the principle of deactivating an easily screenable gene (*pyrE*) • Complementing the mutant strain with a heterologous version of *pyrE* gene as a counter selective marker	• Knockout • Insertion • Exchange	Tripathi et al., 2010; Heap et al., 2012; Ng et al., 2013; Bankar et al., 2015; Zhang N. et al., 2015; Croux et al., 2016; Ehsaan et al., 2016a;
	CRISPR/Cas	• RNA-guided target specific DNA cleavage system • Originated from bacterial adaptive immune system • Needs single guide RNA (sgRNA), Cas endonuclease, and homologous arms for recombination	• Knockout • Knockdown	Xu et al., 2015, 2017; Nagaraju et al., 2016; Bruder et al., 2016; Li et al., 2016; Pyne et al., 2016; Wang Y. et al., 2017, Wang et al., 2018
	Phage serine integrase-mediated genome engineering	• Use two heterologous phage attachment/integration systems • Dual Integrase Cassette Exchange (DICE) strategy • Needs CRISPR/Cas9 assistance	• Knockout • Insertion	Huang et al., 2019
Transcriptome engineering	Synthetic regulatory RNA (sRNA)	• Knockdown tool based on synthetically designed sRNA • Complementarily binds to target mRNAs and block translation	• Knockdown • Overexpression (by repressor knockdown)	Cho and Lee, 2017
	Untranslated regions (UTR) engineering	• UTR modulation • Better mRNA stability by addition of small stem loop structure in the 5'-UTR	• Knockdown • Overexpression (by repressor knockdown)	Lee et al., 2016
	CRISPRi	• Knockdown tool using catalytically inactivated effector dCas9 proteins	• Knockdown • Overexpression (by repressor knockdown)	Bruder et al., 2016; Li et al., 2016; Wang et al., 2016b; Wen et al., 2017; Woolston et al., 2018; Muh et al., 2019

Zhang N. et al., 2015; Ehsaan et al., 2016a; Minton et al., 2016). In ACE, a counter selection marker is coupled to a desired double crossover event (**Figure 1B**). The counter selection marker entitles the isolation of double cross over through homologous recombination. The *pyrE* and *codA* genes are the most frequently used selectable marker in ACE Technology. The gene *codA* encodes for the enzyme cytosine deaminase while, *pyrE* encodes orotate phosphoribosyl transferase, which is a key enzyme required in the *de novo* pathway for pyrimidine biosynthesis.

In clostridia genome editing, *pyrE* allele has been primarily employed. Mutant and wild type *pyrE* allele confers resistance and sensitivity to FOA, respectively. The advantages of *pyrE* allele based recombination includes: (i) rapid insertion of heterologous DNA, (ii) double crossovers which forms the stable integration, (iii) allows large insert size, and (iv) has higher efficiency as compared to simple ClosTron and random mutagenesis (Ng et al., 2013; Ehsaan et al., 2016b; Minton et al., 2016).

The *pyrE* cassettes consists of two arms, i.e., right homology arm (RHA) and left homology arm (LHA) with the internal region comprising of *pyrE* gene (**Figure 1B**). A plasmid is constructed with a selectable marker (antibiotic resistance gene), origin of replication and a sequence containing ∼300-bp homologous to *pyrE* gene and a longer sequence of ∼1,200-bp homologous to the adjacent region of 3′ end of *pyrE*. Once the *pyrE* based pseudo-suicide plasmid is delivered into *Clostridium* cells, single crossover is formed through homologous recombination. Subsequently, the single crossover mutant is inoculated into the media containing FOA and uracil (Heap et al., 2012). Metabolization of FOA kills the single crossover cells carrying the active *pyrE* gene. Inactivation of *pyrE* happens only if double recombination had occurred on both 1200-bp long sequence and 300-bp short sequence and the FOA does not affect the cells obtained by such double crossovers (Ng et al., 2013). The final double crossovers are formed by ACE of shorter left homology arm of 300-bp by the second single crossover, which
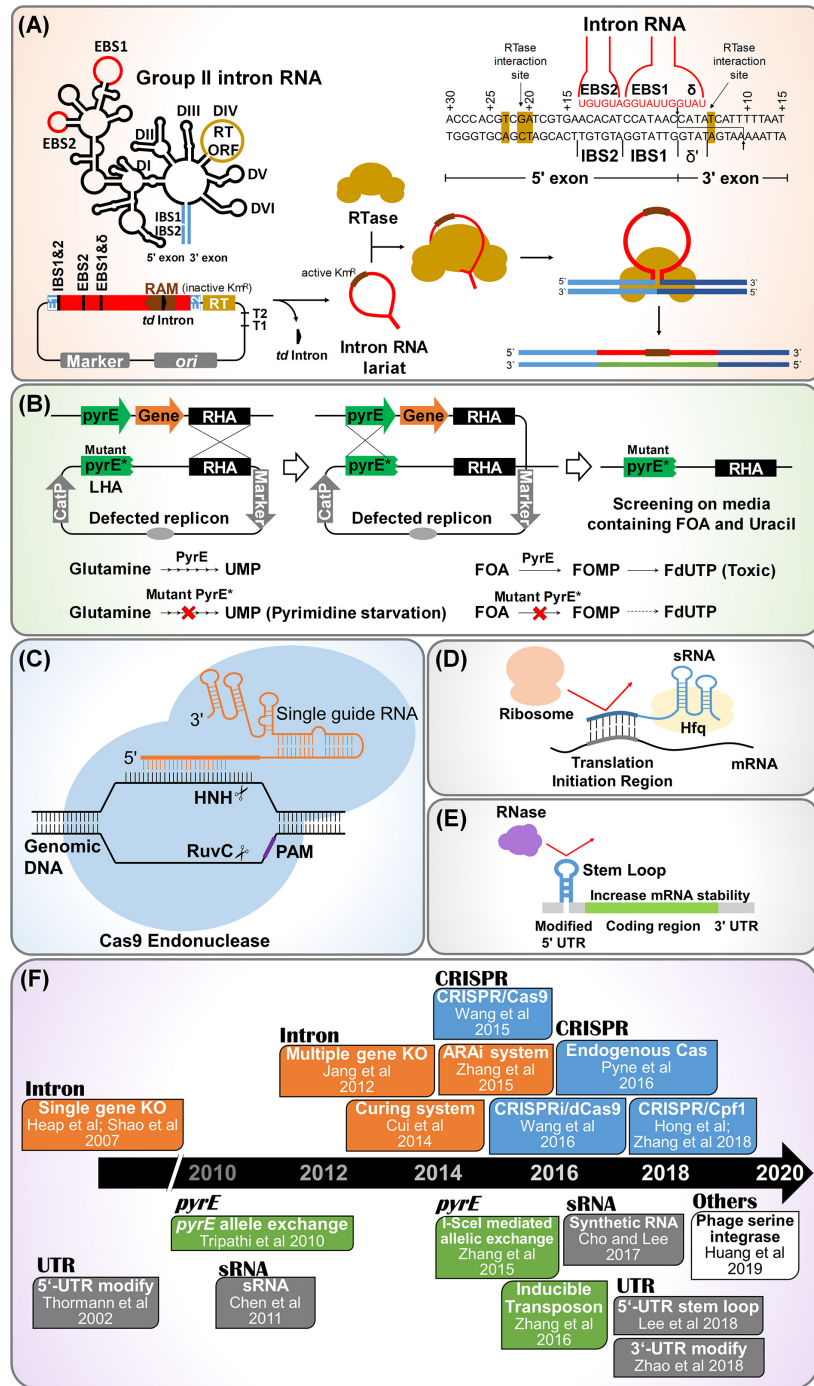
FIGURE 1 | Synthetic biology tools developed for genome and transcriptome engineering of solventogenic *Clostridium*. **(A)** Mobile group II intron-based genome engineering. Also known as ClosTron in context of *Clostridium* sp. In this technology, site directed gene disruption is achieved by insertion of the mobile group II intron into the target locus of chromosome. Abbreviations: RAM, retrotranscription-activated marker (typically kanamycin resistant marker containing self-splicing group I intron, phage T4 *td* intron); RTase, reverse transcriptase; EBS, exon binding site; IBS, intron binding site. **(B)** *pyr*E based allele exchange technology for genome engineering. Here, *pyr*E encoding orotate phosphoribosyl transferase is used as counter selection marker to ensure double crossover event. The *pyr*E-mutant (PyrE*) and wild type (PyrE) are resistant and sensitive to 5-fluoroorotic acid (FOA), respectively. Abbreviation: RHA, right homology arm. **(C)** CRISPR/Cas system for genome engineering. This needs single guide RNA containing crRNA and tracrRNA, Cas endonuclease, and homologous arm for recombination. Abbreviation: PAM, protospacer-adjacent motif. **(D)** Synthetic regulatory RNA (sRNA) based knockdown strategy. sRNA are having regulatory role in gene expression, mediated by chaperon Hfq. sRNA binds to complimentary mRNA sequences, prohibiting ribosome clamping at ribosome binding site located in translation initiation region. **(E)** 5′-UTR engineering for regulation of gene expression. The insertion of a small stem loop structure in the 5′-UTR increases the mRNA stability by blocking RNase, resulting in a high gene expression. **(F)** Timeline of notable events in the development of synthetic biology tools for genome and transcriptome engineering of solventogenic *Clostridium*.

also leads to the excision of the plasmid (Minton et al., 2016). This technology has been found to be applicable for many species of *Clostridium* genus (Heap et al., 2012).

Butanol yield in *C. pasteurianum* has been reported to be improved by application of *pyrE* based genome editing toolkit. For this, deletion mutations were created in three genes of *C. pasteurianum*: hydrogenase (*hydA*), redox response regulator (*rex*), and glycerol dehydratase (*dhaBCE*), using plasmid pMTL-KS01. This resulted in increased availability of NADPH in cell due to depletion of 1,3-propanediol synthesis, which eventually contributed to improved butanol production (Schwarz et al., 2017). Similarly, successful expression of cellulosomal subunits in *C. acetobutylicum* has also been achieved using this method (Kovacs et al., 2013). Few other *Clostridium* species modified using ACE technology includes *C. acetobutylicum*, *C. sporogenes*, and *C. difficile* (Heap et al., 2012; Ng et al., 2013; Bankar et al., 2015; Zhang J. et al., 2015; Ehsaan et al., 2016b; Minton et al., 2016; Willson et al., 2016).

CRISPR/CAS BASED CLOSTRIDIA GENOME ENGINEERING

Clustered regulatory interspaced short palindromic repeats (CRISPR) have been developed as one of the most advanced genetic engineering tools along with CRISPR-associated (Cas) protein (Doudna and Charpentier, 2014). As bacterial genome manipulation tool, CRISPR/Cas system needs single guide RNA (sgRNA), Cas endonuclease, and homologous arms for recombination (Jiang et al., 2013). The *Streptococcus pyogenes* type II CRISPR was the first CRISPR system which was exploited for genome engineering applications. Cas9 endonuclease is the basis of CRISPR based genome editing system. Cas9 recognize the protospacer adjacent motif (PAM) site (5′-NGG-3′ in *S. pyogenes*) and cleave at the 3′ end of the target gene (Mojica et al., 2009; Garneau et al., 2010; Jinek et al., 2012) (**Figure 1C**).

Various strains of *Clostridium* genus have been manipulated using the CRISPR/Cas9 system including *C. acetobutyricum* (Bruder et al., 2016; Li et al., 2016; Wasels et al., 2017), *C. beijerinkii* (Wang et al., 2016b), *C. autoethanogenum* (Nagaraju et al., 2016), *C. difficile* (McAllister et al., 2017; Wang et al., 2018), *C. cellulolyticum* (Xu et al., 2015, 2017), *C. pasteurianum* (Pyne et al., 2016), *C. ljungdahlii* (Huang et al., 2016), and *C. saccharoperbutylacetonicum* (Wang S. et al., 2017). However, the expression of Cas9 becomes detrimental for bacteria, including clostridia, in terms of the toxicity it causes. The mutation of 10th amino acid (aspartic acid to alanine) in Cas9 inactivates it's RuvC-like nuclease domain resulting in formation of Cas9 nickase (Cas9n), which can cleave only single-strand of DNA (Jinek et al., 2012; Nishimasu et al., 2014; Swarts and Jinek, 2018; Li et al., 2019). Cas9n have advantage in terms of overcoming the toxicity caused by expression of Cas9. Introduction of highly regulated inducible promoter for Cas9 expression is another strategy to circumvent the associated toxicity (Wang et al., 2016a; McAllister et al., 2017; Wasels et al., 2017). Nevertheless, the CRISPR/Cas9n system is still being used

for clostridia genome editing (Wang et al., 2016b; Wang S. et al., 2017; Wang Y. et al., 2017; Wang et al., 2018; McAllister et al., 2017; Wasels et al., 2017).

Moreover, modified CRISPR systems like CRISPR interference (CRISPRi) and dCas9 has also been developed to knockdown of the essential genes required for host survival (Jinek et al., 2012; Qi et al., 2013; Peters et al., 2016; Zheng et al., 2019). The dCas9 has two silenced catalytic domains (D10A and H840A; RuvC-like and HNH domains, respectively) which remains bound and block the target DNA instead of cleavage. CRISPRi/dCas9 system has also been applied to develop several mutant strains of *Clostridium* sp. (Bruder et al., 2016; Wang et al., 2016a,b; Wen et al., 2017; Woolston et al., 2018; Muh et al., 2019).

Similar to Cas9, the Cpf1 from *Acidaminococcus* sp. is another protein that is used for PAM recognition in CRISPR based system. While Cas9 recognizes G-rich PAM site, the PAM recognition site for Cpf1 is T-rich (5′-TTTN-3′) (Swarts and Jinek, 2018) making it best suited for application in AT-rich organisms like *Clostridium* sp. (Zetsche et al., 2015; Yamano et al., 2016). Single CRISPR/Cpf1 system plasmid can make multiple mutants in a single application (Zetsche et al., 2017; Hong et al., 2018; Zhang et al., 2018a). CRISPR/Cpf1 system has been applied in *C. ljungdahlii*, *C. difficile*, and *C. beijerinckii* (Hong et al., 2018; Zhang et al., 2018a; Zhao et al., 2019).

Additionally, endogenous CRISPR systems have been developed in *C. pasteurianum* and *C. tyrobutyricum* to overcome the toxic effect associated with Cas9 and Cpf1 endonucleases (Pyne et al., 2016; Zhang et al., 2018b). The endogenous CRISPR system uses endonuclease encoded by the genome and can contain multiple pre-crRNAs under one promoter, facilitating multiple genome modification using a single plasmid (Luo et al., 2014; Makarova et al., 2015; Pyne et al., 2016; Zhang et al., 2018b).

SYNTHETIC SRNA AND UNTRANSLATED REGION ENGINEERING AS POTENTIAL DOMAINS FOR *CLOSTRIDIUM* STRAIN IMPROVEMENT

Prokaryotic small RNAs (sRNA) are short strands of ribonucleotides (about 50–500 nucleotides) which have a regulatory role in maintaining the cellular processes (Gottesman, 2004). Based on the existence of natural sRNA, synthetic small RNAs are produced to alter the gene expression of the organisms. Many such naturally occurring sRNAs have been detected and analyzed in *Clostridium* sp. (Chen et al., 2011), which leads to the development of synthetic sRNA (Na et al., 2013).

The sRNA mediated gene expression usually results in repression of the gene which complements the sRNA nucleotide sequence, mediated by a protein called Hfq (De Lay et al., 2013). Hfq is the chaperone mediated protein which stabilizes the sRNA-mRNA binding. The translation process is prevented by sRNA binding to ribosome binding site (RBS) or by masking the access to the start codon (Na et al., 2013; Yoo et al., 2013). Recently, Cho and Lee (2017) have reported the development of synthetic small

regulatory RNA (sRNA) system for controlled gene expression in *C. acetobutylicum*, consisting of a target recognition site, MicC scaffold, and an RNA chaperon Hfq (**Figure 1D**). In this study, *C. acetobutylicum* Hfq was found to be ineffective in binding with *Escherichia coli* MicC scaffold-based synthetic sRNA, however Hfq from *E. coli* itself resulted in much enhanced knockdown efficiency. This *E. coli* MicC-Hfq sRNA system was used to knockdown *adhE1* gene expression resulting in 40% reduction in butanol production. Further, this synthetic sRNA system was used to knockdown the *pta* gene expression in PJC4BK strain, resulting in PJC4BK (pPta-Hfq[Eco]) strain with improvement of butanol titer from 14.9 to 16.9 g/l (Cho and Lee, 2017).

Untranslated regions (UTRs) are non-coding regions in the mRNA helps to regulate the gene expression. UTRs are present on both the ends of the mRNA (5′-UTR and 3′-UTR) (**Figure 1E**). There are sufficient reports to confirm that the 5′-UTR in *C. acetobutylicum* has the regulatory effect on the secondary structure of enzyme *adhE1*, which is involved in solvent production (Thormann et al., 2002; Scotcher et al., 2003). Lee et al. (2016) has recently found that the presence of a single stranded short 5′-UTR in the solventogenic *C. acetobutylicum* leads to decreased gene expression (**Figure 1E**). The insertion of a small stem loop structure in the 5′-UTR was found to increase the mRNA stability and gene expression by 4.6 folds, without any modification in the promoter or RBS (Lee et al., 2016). On the other hand, the 3′-UTR mostly harbors the terminator sequence for transcription process in mRNA (Richard and Manley, 2009). sRNA sequence containing the codons that regulates the post transcriptional and translation machinery is also attached to 3′-UTR. Most importantly 3′-UTR confer stability to the mRNA (Zhao et al., 2018). Although, there are very limited studies related to 3′-UTR regions in *Clostridium*, the presence of transcripts with long 3′-UTR is confirmed in *Clostridium* (Ralston and Papoutsakis, 2018). Although several RNAseq studies were reported in the *Clostridium*, only few studies show the data related to regulation of mRNA based on 5′- and 3′-UTRs, leaderless transcripts and non-coding RNA (Soutourina et al., 2013; Wilson et al., 2013; Sedlar et al., 2018). Further research in RNAseq and proteomics will explore the complex regulations that control mRNA stability and degradation, which will be more useful to construction synthetic toolkit.

In conclusion, many *Clostridium* sp. have potential to be utilized at industrial scale to produce value added chemicals, including butanol as fossil fuel substitute. Up to date, their true potential was underexploited due to challenges in strain improvement and unavailability of genome and transcriptome editing tools for this genus. Nevertheless, during the last decade, synthetic biology toolkits for *Clostridium* sp. have been expanded rapidly (**Figure 1F**). Furthermore, a recent advancement, such as phage serine integrase mediated site-specific genome engineering technique for *C. ljungdahlii* could be extended to other *Clostridium* species (Huang et al., 2019). The synthetic biology techniques that have been applied in other microorganisms may also be adopted to solventogenic clostridia in the near future: CRISPR associated site-specific insertion of transposons and base editing techniques (Ronda et al., 2015; Zhang et al., 2016; Lim and Choi, 2019; Strecker et al., 2019b). Utilization of improved clostridia strains could be a starting point for development of an industrial scale, commercially viable bio-based fuel and chemical production using *Clostridium* sp. using a consolidated bioprocessing concept (Wen et al., 2019a). Furthermore, these synthetic biology tools could be applied to another biotechnology fields such as degradation of plastics, such as polyethylene terephthalate and polyethylene.

AUTHOR CONTRIBUTIONS

Y-SJ and AM conceived the project. All authors analyzed the literature, compiled data, planned content, wrote the manuscript, read, and approved the final manuscript.

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