Journal of Microbiological Methods 140 (2017) 5–11

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A two-plasmid inducible CRISPR/Cas9 genome editing tool for *Clostridium acetobutylicum*

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ARTICLE INFO

Keywords:
Clostridium acetobutylicum
CRISPR/Cas9
Genome engineering
Metabolic engineering

ABSTRACT

CRISPR/Cas-based genetic engineering has revolutionised molecular biology in both eukaryotes and prokaryotes. Several tools dedicated to the genomic transformation of the *Clostridium* genus of Gram-positive bacteria have been described in the literature; however, the integration of large DNA fragments still remains relatively limited. In this study, a CRISPR/Cas9 genome editing tool using a two-plasmid strategy was developed for the solventogenic strain *Clostridium acetobutylicum* ATCC 824. Codon-optimised *cas9* from *Streptococcus pyogenes* was placed under the control of an anhydrotetracycline-inducible promoter on one plasmid, while the gRNA expression cassettes and editing templates were located on a second plasmid. Through the sequential introduction of these vectors into the cell, we achieved highly accurate genome modifications, including nucleotide substitution, gene deletion and cassette insertion up to 3.6 kb. To demonstrate its potential, this genome editing tool was used to generate a marker-free mutant of ATCC 824 that produced an isopropanol-butanol-ethanol mixture. Whole-genome sequencing confirmed that no off-target modifications were present in the mutants. Such a tool is a prerequisite for efficient metabolic engineering in this solventogenic strain and provides an alternative editing strategy that might be applicable to other *Clostridium* strains.

1. Introduction

Clostridium acetobutylicum is a Gram-positive, spore-forming, anaerobic bacterium that utilises a wide range of different carbon sources (including C6 and C5 sugars and glycerol) to produce a mixture of acetone-butanol-ethanol and other compounds of interest. In recent years, several metabolic engineering tools have been developed to improve or modify the industrial capacities of this bacterium, mainly based on group II-intron retargeting or homologous recombination (HR) techniques. Group II-intron retargeting techniques such as Targetron (Shao et al., 2007) or ClosTron (Heap et al., 2007; Heap et al., 2010) are rapid but only allow insertional mutagenesis, which may result in undesired polar effects. In contrast, HR-based techniques allow a more precise modification of the genome but are time consuming and not always efficient (Heap et al., 2012).

More recently, genome editing strategies based on CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated proteins) have been developed, enabling genome editing through the creation of double-strand breaks (DSBs) in the DNA (Jinek et al., 2012; Cong et al., 2013). Several groups have worked on the adaptation of this genome editing tool to the *Clostridium* genus (Wang et al., 2015; Xu et al., 2015; Huang et al., 2016; Li et al., 2016; Pyne et al., 2016; Wang et al., 2016); however, with the exception of Huang et al. (2016), who adapted CRISPR/Cas9 to *Clostridium ljungdahlii*, all groups reported difficulties when generating mutants that constitutively expressed the heterologous native Cas9 nuclease from *Streptococcus pyogenes*. These challenges may arise from the low transformation and HR frequencies observed when manipulating *Clostridium*, or from the supposed absence of non-homologous end joining (NHEJ) mechanisms in these organisms (Xu et al., 2015). Several strategies were successfully adopted to circumvent this problem.

The Cas9 nickase (Cas9n) catalyses a single-strand nick in the DNA (Jinek et al., 2012), protecting organisms from the potential detrimental effects of DSBs while inducing HR events (Metzger et al., 2011). The use of Cas9n allowed the generation of one-step deletions in the genome of several *Clostridium* species, including *C. acetobutylicum* ATCC

Abbreviations: aTc, anhydrotetracycline; CRISPR, Clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated proteins; Cas9n, Cas9 nickase; DSB, Double-strand break; gDNA, genomic DNA; gRNA, guide RNA; HR, Homologous recombination; NHEJ, Non-homologous end joining; SOE, splicing by overhang extension; 5-FU, 5-fluorouracil

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http://dx.doi.org/10.1016/j.mimet.2017.06.010
Received 27 April 2017; Received in revised form 9 June 2017; Accepted 9 June 2017
Available online 10 June 2017

824 (Xu et al., 2015; Li et al., 2016). Cas9n is a less powerful selection tool than Cas9 however, and sometimes the isolation of correctly edited cells requires serial transfers in fresh medium (Xu et al., 2015). Fragments of up to 1.72 kb have been inserted using this technique, but the integration of larger fragments remains unsuccessful (Xu et al., 2015). Until now, only short deletions of 20 bp were reported in *C. acetobutylicum* ATCC 824, with editing efficiencies ranging from 6.7% to 100% depending on the target and on the number of colonies analysed (Li et al., 2016).

Another genome editing strategy was to exploit the endogenous CRISPR/Cas machinery found in some *Clostridium* strains (Pyne et al., 2016). Observing that heterologous expression of *cas9* was toxic in *Clostridium pasteurianum* ATCC 6013 even in the absence of associated guide RNA (gRNA), Pyne et al. exploited its native CRISPR/Cas6 machinery. Introducing only a synthetic CRISPR array and an editing template, they reduced the size of the plasmid used and quadrupled the efficiency of the transformation. Pyne et al. also generated a 750-bp deletion in the genome with a 100% editing efficiency in the transformants, using either heterologous Cas9 or endogenous Cas6. The transformation efficiency remained low however, and CRISPR/Cas systems are not naturally present in all strains of *Clostridium*, including *C. acetobutylicum* ATCC 824. Furthermore, only deletions have been reported using this method; no other modifications, such as nucleotide substitutions or insertions, were tested.

A third strategy was to place *cas9* under the control of a lactose-inducible promoter, enabling its expression to be activated in a larger bacterial population to enhance the selection of low-frequency HR events (Wang et al., 2016). The researchers generated a 1.5-kb deletion, a 1.6-kb gene insertion and single-nucleotide modifications within the genome of *C. beijerinckii* (Wang et al., 2016). A disadvantage of this strategy is that the template length is limited by the cargo capacity of the plasmid, which already harbours the 4.1-kb *cas9* gene.

Here, we developed a two-plasmid inducible CRISPR/Cas9 strategy for *C. acetobutylicum* ATCC 824. This strategy enabled several chromosomal modifications, including the integration of a larger fragment than previously reported. Furthermore, it was also successfully applied to remove the 192-kbp pSOL megaplasmid.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Relevant characteristics of the bacterial strains and plasmids used in this study are listed in Table 1. *C. acetobutylicum* was grown anaerobically at 35 °C in liquid 2YTG medium (16 g L^{-1} Bacto tryptone, 10 g L^{-1} yeast extract, 4 g L^{-1} NaCl and 5 g L^{-1} glucose) or solid 2YTG with 1.5% agar supplemented with 20 μg mL^{-1} erythromycin and/or 20 μg mL^{-1} thiamphenicol if necessary. Solid media was supplemented with 50 μg mL^{-1} 5-fluorouracil (5-FU) where specified. *Escherichia coli* was grown aerobically at 37 °C and 200 rpm in liquid LB medium or solid LB with 1.5% agar supplemented with erythromycin (500 μg mL^{-1} for solid media and 100 μg mL^{-1} for liquid media), chloramphenicol (25 μg mL^{-1} for solid media and 12.5 μg mL^{-1} for liquid media), or tetracycline (20 μg mL^{-1}) if necessary.

2.2. DNA manipulation, plasmid construction and transformation

All enzymes for DNA modification as well as a Gibson Assembly kit were purchased from New England Biolabs (NEB). Q5 High-Fidelity Polymerase (NEB) was used for all PCR amplifications, except for colony PCRs, which were performed using DreamTaq DNA polymerase (Thermo Fisher Scientific). Genomic DNA (gDNA) was extracted from *C. acetobutylicum* using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). Plasmids and PCR product purification kits were purchased from Qiagen. All primers used in this study are listed in Supplementary Table S1.

The pFW01 plasmid was constructed by a *Stu*I/*Xho*I ligation of the amplicon obtained using a splicing by overhang extension (SOE) PCR (Higuchi et al., 1988) of the *ermB* gene (primers P01 and P02) and the *rep* origin from pMTL500E (primers P03 and P04), with a PCR amplification of the *repH* origin from pMTL007S-E1 (primers P05 and P06). A cassette consisting of the miniPthl promoter and the *thl* terminator separated by the *Nco*I and *Sal*I restriction sites was synthesised and subcloned into the pEX-A2 vector (Eurofins Genomics), amplified with primers P07 and P08 and cloned into *Xho*I/*Sma*I-double-digested pFW01. A codon-optimised *cas9* gene from *S. pyogenes* flanked by *Nco*I and *Sal*I was synthesised (Genscript, Supplementary Text) and cloned into a *Nco*I/*Sal*I-double-digested vector, yielding $pCas9_{con}$. The sequence of the Pcm-tetO2/1 promoter was synthesised (Eurofins Genomics) and cloned into *Xho*I/*Nco*I-double-digested $pCas9_{con}$. A cassette containing the *tetR* gene under the control of the miniPthl promoter was cloned into the pEX-A2 vector (Eurofins Genomics), amplified using primers P07 and P08, and cloned into the *Sma*I/*Sac*I-double-digested intermediate plasmid, yielding $pCas9_{ind}$.

Three gRNA expression cassettes, composed of the miniPthl promoter and a 20-nt guiding sequence targeting either *upp*, *adhE1* or *thl* fused with the chimeric gRNA sequence (Jinek et al., 2012), were cloned into the pEX-A2 vector (Eurofins Genomics), amplified with primers P07 and P08, and cloned into the *Xho*I/*Sal*I-double-digested pEC750C, yielding pGRNA-upp, pGRNA-adhE1 and pGRNA-thl, respectively.

The pGRNA-upp_{oc} plasmid was constructed by cloning the 1306-bp SOE PCR product, obtained using primer pairs P09/P10 and P11/P12 on ATCC 824 gDNA, into the *Eco*RI/*Sal*I-double-digested pGRNA-upp. The pGRNA-$\Delta_{43\text{-}348}upp$ plasmid was constructed by cloning the 1-kb SOE PCR product, obtained using primer pairs P09/P13 and P11/P14 on ATCC 824 gDNA, into the *Eco*RI/*Sal*I-double-digested pGRNA-upp. The Δ*upp*::ipa8 editing template was constructed using a Gibson assembly in the *Bam*HI/*Eco*RI-double-digested pUC19, inserting the 1-kb *upp* flanking regions amplified with primer pairs P15/P16 and P17/P18 from ATCC 824 gDNA and the PCR product amplified with primers P19/P20 from plasmid pFC008. The resulting template and pGRNA-upp were digested with *Eco*RI and *Bam*HI and ligated to obtain pGRNA-Δ*upp*::ipa8.

To construct the *thl-adh* editing template, the *adh* gene was amplified from pFC008 (Collas et al., 2014) with primers P21 and P22, then assembled to the regions flanking the gRNA target site in the ATCC 824 genome using a Gibson assembly in pUC19. A 651-bp fragment corresponding to the end of *thl* was optimised to alter the target site (Eurofins Genomics, Supplementary Text) and amplified with primers P23 and P24. The 600 bp located downstream of *adh* was amplified from gDNA of ATCC 824 using primers P25 and P26. The resulting template and pGRNA-thl were digested with *Sal*I and *Eco*RI, then ligated to obtain pGRNA-*thl-adh*.

A diagram summarizing plasmid construction is shown in Fig. S1 and S2. The lengths of all homology arms used for the construction of editing templates are reported in the Supplementary Table S2.

Plasmids were transformed into chemically competent NEB 10-beta competent *E. coli* cells containing pAN2 (Heap et al., 2007) for DNA methylation. The vectors were then isolated and electroporated into *C. acetobutylicum* ATCC 824 as previously described (Mermelstein and Papoutsakis, 1993).

2.3. Isolation of mutants

Independent transformants containing their respective plasmids were resuspended in liquid 2YTG, and serial dilutions were spotted onto 2YTG solid media containing erythromycin and thiamphenicol, supplemented with anhydrotetracycline (aTc) at concentrations ranging from 0 to 500 ng mL^{-1}. Isolated colonies were selected for subsequent analyses.