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

François Wasels\(^a\), Jennifer Jean-Marie\(^a\), Florent Collas\(^b\), Ana M. López-Contreras\(^b\), Nicolas Lopes Ferreira\(^a\)

\(^a\) IFP Energies nouvelles, Biotechnology Department, 1 et 4 avenue de Bois-Préau, 92852 Rueil-Malmaison, France
\(^b\) Wageningen Food and Biobased Research, Bornse Weilanden 9, 6709WG Wageningen, The Netherlands

**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 CloTron (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...
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. 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 manipulation 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 StuI/XhoI 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 pMTL0500E (primers P03 and P04), with a PCR amplification of the repH origin from pMTL0075-E1 (primers P05 and P06). A cassette consisting of the miniPtl promoter and the thr terminator separated by the Ncol and SalI restriction sites was synthesised and sub-cloned into the pEX-A2 vector (Eurofins Genomics), amplified with primers P07 and P08 and cloned into Xhol/Smal-digested pFW01. A codon-optimised cas9 gene from S. pyogenes flanked by Ncol and SalI was synthesised (Genscript, Supplementary Text) and cloned into a Ncol/SalI-digested vector, yielding pCas9con. The sequence of the Pcm-tetO2/1 promoter was synthesised (Eurofins Genomics) and cloned into Xhol/Ncol-digested pCas9con. A cassette containing the tetR gene under the control of the miniPtl promoter was cloned into the pEX-A2 vector (Eurofins Genomics), amplified using primers P07 and P08, and cloned into the Smal/Sacl-digested intermediate plasmid, yielding pCas9ind.

Three gRNA expression cassettes, composed of the miniPtl promoter and a 20-nt guiding sequence targeting either upp, adhE1 or thr 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 Xhol/SalI-digested pEX750C, yielding pGRNA-upp, pGRNA-adrE1 and pGRNA-thl, respectively.

The pGRNA-uppcon 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 EcoRI/SalI-digested pGRNA-upp vector. The pGRNA-Δ43-348upp 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 EcoRI/SalI-digested pGRNA-upp vector. The Δupp:ipa8 editing template was constructed using a Gibson assembly in the BamHI/EcoRI-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 EcoRI and BamHI and ligated to obtain pGRNA-Δupp:ipa8.

To construct the thr-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 thr 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 SalI and EcoRI, 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 thiampenicol, supplemented with anhydrotetracycline (aTc) at concentrations ranging from 0 to 500 ng mL⁻¹. Isolated colonies were selected for subsequent analyses.
F. Wasels et al.

Journal of Microbiological Methods 140 (2017) 5–11

Table 1

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. acetobutylicum</em> ATCC 824</td>
<td>Wild-type</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>E. coli</em> K-12</td>
<td></td>
<td>NEB</td>
</tr>
<tr>
<td><strong>Derivatives and plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAN2</td>
<td>Tet', Φ3T I gene, p15A origin</td>
<td>Heap, et al., 2007; Mermelstein and Papoutsakis, 1993</td>
</tr>
<tr>
<td>pMTL500E</td>
<td>Ap', ColE1 origin, pAM1 origin</td>
<td>Outram, et al., 1988</td>
</tr>
<tr>
<td>pMTL007S-E1</td>
<td>Sp', ColE1 origin, pCR102 origin, ermB RAM</td>
<td>Heap, et al., 2010</td>
</tr>
<tr>
<td>pEC750C</td>
<td>Cm', ColE1 origin, pIP404 origin</td>
<td>Collas, unpublished</td>
</tr>
<tr>
<td>pFG008</td>
<td>pMTL500E derivative with adh ctfA ctfB (constitutive thl promoter) and adc (natural promoter) insertions</td>
<td>Gollas et al., 2014</td>
</tr>
<tr>
<td>pFW01</td>
<td>Em', ColE1 origin, pCB102 origin</td>
<td>This study</td>
</tr>
<tr>
<td>pCas9con</td>
<td>pFW01 derivative with cas9 (inducible Pcm-tetO2/1 promoter) and reF (miniPthl promoter) insertions</td>
<td>This study</td>
</tr>
<tr>
<td>pGRNA-upp</td>
<td>pEC750C derivative with gRNA-upp cassette insertion</td>
<td>This study</td>
</tr>
<tr>
<td>pGRNA-upp_adhE1</td>
<td>pGRNA-upp derivative with uppE template insertion</td>
<td>This study</td>
</tr>
<tr>
<td>pGRNA-upp_adhE1</td>
<td>pGRNA-upp derivative with ΔuppE template insertion</td>
<td>This study</td>
</tr>
<tr>
<td>pGRNA-thl</td>
<td>pEC750C derivative with gRNA-thl cassette insertion</td>
<td>This study</td>
</tr>
<tr>
<td>pGRNA-thl-adh</td>
<td>pGRNA-thl derivative with thl-adh template insertion</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Tet', tetracycline resistant; Sp', spectinomycin resistant; Cm', chloramphenicol resistant; Em', erythromycin resistant.

2.4. Whole-genome sequencing

Genomic DNA was sequenced on an Illumina MiSeq sequencer with a 2 × 250 paired-end sequencing kit. Reads were mapped against the corrected genome and pSOL sequences of *C. acetobutylicum* ATCC 824 (Nölling et al., 2001; Ehsaan et al., 2016) using Geneious 9.1.3 (Kearse et al., 2012).

2.5. Fermentation assays

Fermentation assays were performed in modified Gapes medium (Gapes et al., 1996) containing 2.5 g L⁻¹ yeast extract, 1 g L⁻¹ KH₂PO₄, 0.6 g L⁻¹ K₂HPO₄, 1 g L⁻¹ MgSO₄, 7H₂O, 6.6 mg L⁻¹ FeSO₄ 7H₂O, 0.1 g L⁻¹ 4-aminobenzoic acid, 2.9 g L⁻¹ CH₃COONH₄ and 60 g L⁻¹ glucose. The initial pH of the medium was 6.2. Fresh colonies were used to inoculate 10 mL Gapes media. After 24 h of growth in an anaerobic chamber at 35 °C without agitation, 3 mL of these precultures were used to inoculate 10 mL Gapes media. After 24 h of growth in an anaerobic chamber at 35 °C without agitation, 3 mL of these precultures were inoculated into a 100 mL flask containing 30 mL Gapes liquid medium. The flasks were sealed and incubated at 35 °C with agitation at 150 rpm for 72 h. The concentrations of the solvents produced were determined by gas chromatography. The fermentation samples were centrifuged at 15,000 g for 5 min and the supernatant was diluted with an internal standard (0.5 g L⁻¹ propanol) to enable quantification. The solvents were separated on a Porabond-Q column (25 m length, 0.32 mm internal diameter, 0.5 μm film thickness; Agilent Technologies) equipped with a flame ionisation detector. Helium was used as carrier gas, at a flow rate of 1.6 mL min⁻¹.

3. Results

Previous work performed using *Clostridium* bacteria showed that the use of a plasmid containing cas9 under the control of a constitutive promoter dramatically reduced the likelihood of obtaining transformants. In the present study, two cas9-expression plasmids, pCas9con and pCas9 advant, were constructed using the shuttle vector pFW01, in which cas9 was placed under the control of the constitutive miniPthl promoter (Dong et al., 2012) or the inducible Pcm-tetO2/1 promoter (Dong et al., 2012), respectively.

To evaluate the robustness of this newly developed CRISPR/Cas9 tool (patent: FR15/59846; Fig. S3), the upp gene (ca_c2879, coding for a phosphoribosyl transferase) was targeted. The deletion of this gene enables *C. acetobutylicum* ATCC 824 to grow on solid media containing 5-FU (Croux et al., 2016), making its inactivation easily detectable. A gRNA expression cassette consisting of a 20-nt sequence targeting upp fused with the chimeric gRNA sequence (Mali et al., 2013) under the control of the miniPthl promoter was cloned into the pEC750C shuttle vector. Three different templates were designed to repair the resulting DSB and concomitantly inactivate upp (Fig. 1). The editing performed using the upp template caused two nucleotide substitutions within the 20-bp target sequence recognised by the gRNA, which resulted in a
silent mutation and a premature stop codon, TAA (ochre mutation), leading to the synthesis of a truncated 66-aa Upp protein. Editing using the Δ43-348upp template caused a 306-bp internal deletion within the 630-bp coding sequence of upp. The editing performed using the Δupp-p:ipa8 template caused the replacement of upp by the operonic structure ipa8 from plasmid pPC008. This operon contains genes from *C. acetobutylicum* ATCC 824 involved in the assimilation of acids (ctfA, ctfB) and the production of acetone (adc), as well as the adh gene from *C. beijerinckii* DSM 6423 encoding a secondary alcohol dehydrogenase able to convert acetone into isopropanol. Similar genetic operons have previously been overexpressed in *C. acetobutylicum* ATCC 824, allowing the strain to efficiently produce an isopropanol-butanol-ethanol mixture (Collas et al., 2012; Lee et al., 2012).

Sequential transformation experiments were performed to introduce the cas9-expression plasmids (pFW01-derivatives) followed by the gRNA-expression plasmids (pEC750C-derivatives) into *C. acetobutylicum* ATCC 824. Cells containing the empty pFW01 were successfully transformed with all of the pEC750C derivatives. As anticipated, no transformants were obtained that contained the pCas9ind in combination with any pEC750C derivative harbouring the gRNA expression cassette, whether or not the editing template was included. This result suggests that Cas9 is functional and that the low HR frequency does not allow edited cells to be obtained when constitutively expressing the nuclease gene. Cells containing pCas9ind were successfully transformed with all pEC750C derivatives at frequencies similar to those of cells containing pFW01, indicating that expression of cas9 is correctly repressed in the absence of the inducer, aTc. Moreover, cells containing pCas9ind transformed with pEC750C derivatives harbouring the gRNA expression cassette did not survive on plates containing aTc, highlighting the stringent control of the inducing system. The results of the transformation experiments are summarised in Supplementary Table S3.

Transformants containing pCas9ind and pEC750C derivatives obtained on plates without the inducer were further investigated, and induction of cas9 expression was performed by spot-plating the cells on 2YTG solid media supplemented with erythromycin, thiamphenicol and increasing concentrations of aTc (Fig. 2). Overall, a toxic effect was observed for the inducer, with aTc reducing the viability of cells, including in the transformants containing the empty pEC750C. No growth was observed for transformants containing pCas9ind and pGRNA-upp on plates supplemented with the inducer. In contrast, colonies containing pCas9ind and pGRNA-upp were isolated on plates supplemented with aTc (Fig. 2). Colonies were also obtained when transformants containing pCas9ind and either pGRNA-Δ43-348upp or pGRNA-Δupp::ipa8 were grown on aTc-supplemented media (data not shown). Notably, aTc-resistant pCas9ind colonies containing pGRNA-Δupp::ipa8 were obtained at lower frequencies (10−4 colonies/total colonies) than those containing either pGRNA-uppΔ or pGRNA-Δ43-348upp (10−2–10−3 colonies/total colonies; Fig. 2).

Isolated colonies were selected from aTc-supplemented plates and streaked onto 2YTG solid media, either with or without 5-FU. All colonies containing pCas9ind and either pGRNA-uppΔ (10 colonies tested), pGRNA-Δ43-348upp (10 colonies tested) or pGRNA-Δupp::ipa8 (4 colonies tested) were able to grow on solid media supplemented with 5-FU, while colonies containing pCas9ind and pEC750C were not (data not shown). In parallel, the gDNA of four colonies for each transformation was extracted from cells that were not exposed to 5-FU. The upp locus was analysed by PCR using primers P27 and P28 that do not hybridize to the strain to efficiently produce an isopropanol-butanol-ethanol mixture (Collas et al., 2012; Lee et al., 2012).

To determine whether the gRNA-expression plasmid could be efficiently removed to permit the introduction of another pEC750C-derived plasmid targeting other genes, two colonies of each type were streaked on 2YTG supplemented with erythromycin. Erythromycin-resistant and thiamphenicol-susceptible colonies were easily isolated (at least 50% of the colonies tested) were able to grow on these plates), which could be used for further rounds of modification through the introduction of new targeting plasmids. An additional round of growth on 2YTG without any antibiotics was performed to curate pCas9ind in 100% of the tested colonies.

To validate the efficiency and detect putative off-target events that could have been caused by the use of this tool, the genome of six independent mutants (two of each type) was sequenced. In all of them, the same modifications were detected when compared to the re-sequenced ATCC 824 wild-type strain (Nölling et al., 2001; Ebasa et al., 2016); six single nucleotide variations, one single nucleotide deletion and one four-nucleotide insertion. Of the six mutants analysed, four contained only the desired modifications to the upp gene, i.e. either the 306-bp internal deletion, two single nucleotide variants or the replacement of the whole gene by the ipa8 operon. The two remaining mutants contained undesired modifications elsewhere in their genome.

---

Fig. 2. Selection of *Clostridium acetobutylicum* cells edited with the uppΔ template on aTc-containing plates. Independent transformants containing pCas9ind and either pEC750C, pGRNA- upp or pGRNA-uppΔ were suspended in liquid 2YTG, serially diluted and spotted on plates containing the appropriate antibiotics only (left plate), or supplemented with 500 ng mL−1 aTc, the inducer of cas9 expression (right plate). ND, not diluted; Em, erythromycin; Tm, thiamphenicol; aTc, anhydrotetracycline.
however, these mutations appeared to be unrelated to the activity of CRISPR/Cas9, since none of them were associated with sequences that would have been targeted by the gRNA. All the modifications detected in the mutants are listed in Supplementary Table S4.

To investigate the fermentation profiles of the CRISPR/Cas9-modified strains, the solvent production of the two sequenced Δupp::ipa8 mutants and the two sequenced Δ43-348upp mutants were compared to the wild-type ATCC 824 strains, using glucose as a carbon source. After 72 h of fermentation, the wild-type strain and the Δ43-348upp mutants yielded 4.32 ± 0.03 and 3.62 ± 0.08 g L⁻¹ acetone, respectively, and no isopropanol was detected in the medium (Fig. 4). The two Δupp::ipa8 mutants produced 4.45 ± 0.34 g L⁻¹ of isopropanol and a residual 0.21 ± 0.08 g L⁻¹ of acetone, confirming that the adh gene from the ipa8 operon was correctly expressed and functional.

To illustrate the versatility of the tool, two other modifications were performed in the genome of C. acetobutylicum ATCC 824. Firstly, a gRNA expression cassette was designed to target the end of the thl gene (ca_c2873). An editing template was constructed that allows both the modification of the target sequence and the insertion of the adh gene from C. beijerinckii DSM 6423. The gene was inserted between the end of the thl coding sequence and its transcriptional terminator, so that both genes are present in an operon structure under the control of the
Results for \( \Delta \) shown are average values ± standard deviations from duplicate experiments over 72 h. Several groups have reported that they no longer contained the megaplasmid (data not shown).

The absence of selection markers in the genome of the modified strains currently being studied (Heap et al., 2009; Poehlein et al., 2017).

Since \( C. acetobutylicum \) ATCC 824 does not possess any endogenous CRISPR/Cas machinery, Cas9 from \( S. pyogenes \) was utilised. A codon-adapted cas9 gene was synthesised in order to maximise the selection of correctly edited cells. As a consequence, and as already observed by other authors (Xu et al., 2015; Li et al., 2016; Pyne et al., 2016; Wang et al., 2016), cas9 constitutive expression did not result in any transformants being obtained, because of the low transformation efficiency and low HR frequencies in \( C. acetobutylicum \). This problem was solved by placing the gene under the control of the Pcm-tetO2/1 inducible promoter (Dong et al., 2012), and the induction of cas9 expression yielded edited cells in which HR events had occurred. These initial results indicated that, as expected, editing frequencies are related to the type of modification attempted, with large insertions being obtained at lower frequencies than small deletions or nucleotide substitutions. As an example, colonies edited with either the upp::ipa8 template (causing a 2-nt substitution and a 306-bp deletion, respectively) were obtained at higher frequencies than cells edited with the upp::ipa template, which causes a 3.6-kb insertion. The strategy was highly efficient, with correct modifications observed in 100% of cells tested, an improvement upon the editing frequencies using Cas9 previously reported in \( C. acetobutylicum \) ATCC 824 (Li et al., 2016). As previously reported (Xu et al., 2015), no NHEJ mechanism was involved in repair of the DSBs, since all the analysed mutants contained the exact sequences of the editing templates used, rather than containing the errors prominent in NHEJ. Moreover, no off-target modification was detected and, among the six mutants sequenced, four contained only the desired modifications, while the two others contained supplementary mutations unrelated to the strategy used.

Overall, this method enables the rapid introduction of marker-free genomic modification of any type, from the substitution of a few nucleotides to large deletions or insertions. Once cells containing \( \text{Cas9}_{\text{宪}} \) and \( \text{pGRNA-adrE1} \) were introduced into bacterial cells, which is particularly challenging for \( \text{Clostridium} \) strains because of their low transformation efficiencies and low HR frequencies that are inherent to \( \text{Clostridium} \) bacteria (Wang et al., 2015; Xu et al., 2015; Huang et al., 2016; Li et al., 2016; Pyne et al., 2016; Wang et al., 2016). Most of these strategies are based on the use of a single plasmid that harbours all the new possibilities for synthetic biology approaches in industrial biotechnology involving \( \text{Clostridium} \) solventogenic strains. As an initial example, the adh gene from \( C. beijerincki \) DSM 6423 was inserted into the genome of \( \text{ATCC 824} \), either alone or in combination with other genes involved in acid re-assimilation and acetone production, placed under the control of a strong constitutive promoter. To our knowledge, this is the first report of a \( C. acetobutylicum \) strain able to produce an isopropanol-butanol-ethanol mixture without any selection marker.

Future work will focus on the optimisation of this tool through the determination of the maximum fragment length that can be inserted or deleted, and of the minimal size required for the homology arms. One hint was provided by Xu et al. (2015), who reported a high editing efficiency using homology arms of at least 0.2 kb with their \( \text{Cas9}_{\text{宪}} \) system in \( \text{C. cellulolyticum} \). Moreover, this two-plasmid inducible CRISPR/Cas9 genome editing tool will be employed in other strains of the \( \text{Clostridium} \) genus in the future, to demonstrate its varied possibilities.
Funding

Work at Wageningen Food and Biobased Research was supported by European Union’s Horizon 2020 Macrofuels project (contract number 654010).

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgements

This work has benefited from the High-Throughput Sequencing Platform facilities and expertise of the Institute for Integrative Biology of the Cell (I2BC), France.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2017.06.010.

References