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

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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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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.

Table 1
Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
C. acetobutylicum ATCC 824	Wild-type	ATCC
E. coli NEB 10-beta		NEB
Derivatives and plasmids		
pAN2	Tet^r, Φ3T I gene, p15A origin	Heap et al., 2007; Mermelstein and Papoutsakis, 1993
pMTL500E	Ap^r, Em^r, ColE1 origin, pAMβ1 origin	Oultram et al., 1988
pMTL007S-E1	Sp^r, ColE1 origin, pCB102 origin, *ermB* RAM	Heap et al., 2010
pEC750C	Cm^r, ColE1 origin, pIP404 origin	Collas, unpublished
pFC008	pMTL500E derivative with *adh ctfA ctfB* (constitutive *thl* promoter) and *adc* (natural promoter) insertions	Collas et al., 2014
pFW01	Em^r, ColE1 origin, pCB102 origin	This study
$pCas9_{con}$	pFW01 derivative with *cas9* (constitutive miniPthl promoter) insertion	This study
$pCas9_{ind}$	pFW01 derivative with *cas9* (inducible Pcm-tetO2/1 promoter) and *tetR* (miniPthl promoter) insertions	This study
pGRNA-upp	pEC750C derivative with gRNA_upp cassette insertion	This study
pGRNA-upp_{oc}	pGRNA-upp derivative with upp_{oc} template insertion	This study
pGRNA-$\Delta_{43\text{-}348}upp$	pGRNA-upp derivative with $\Delta_{43\text{-}348}upp$ template insertion	This study
pGRNA-Δ*upp*::ipa8	pGRNA-upp derivative with Δ*upp*::ipa8 template insertion	This study
pGRNA-adhE1	pEC750C derivative with gRNA_adhE1 cassette insertion	This study
pGRNA-thl	pEC750C derivative with gRNA_thl cassette insertion	This study
pGRNA-*thl-adh*	pGRNA-thl derivative with *thl-adh* template insertion	This study

[a] Tet^r, tetracycline resistant; Sp^r, spectinomycin resistant; Cm^r, chloramphenicol resistant; Em^r, 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} KH_2PO_4, 0.6 g L^{-1} K_2HPO_4, 1 g L^{-1} $MgSO_4$ $7H_2O$, 6.6 mg L^{-1} $FeSO_4$ $7H_20$, 0.1 g L^{-1} 4-aminobenzoic acid, 2.9 g L^{-1} CH_3COONH_4 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 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, $pCas9_{con}$ and $pCas9_{ind}$, 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_{oc} template caused two nucleotide substitutions within the 20-bp target sequence recognised by the gRNA, which resulted in a

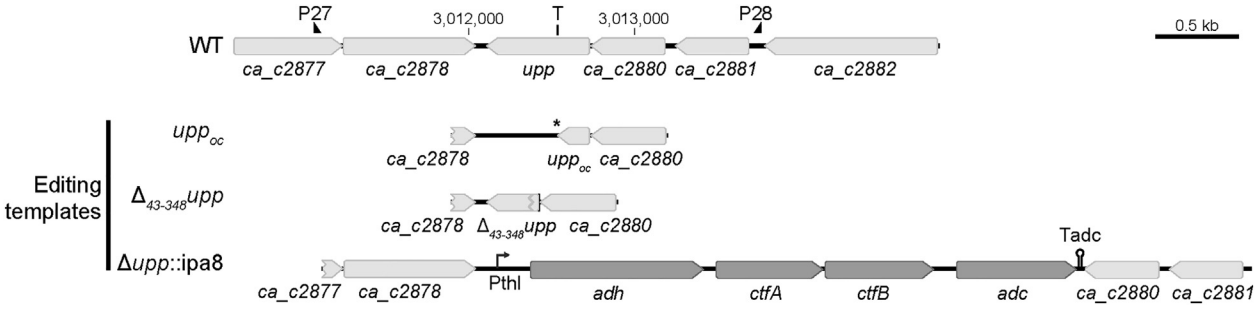

Fig. 1. Templates used to edit the *upp* locus. Genes are annotated according to the deposited sequence of *Clostridium acetobutylicum* ATCC 824 (GenBank accession number NC_003030). The wild-type (WT) sequence is numbered according to the revised version of the ATCC 824 genome sequence (Nölling et al., 2001; Ehsaan et al., 2016). The asterisk indicates a premature stop codon, while the black triangles indicate primer binding sites. T, target site of CRISPR/Cas9; PthI, *ca_c2873* promoter; Tadc, *ca_p0165* transcription terminator.

silent mutation and a premature stop codon, TAA (ochre mutation), leading to the synthesis of a truncated 66-aa Upp protein. Editing using the $\Delta_{43\text{-}348}$*upp* template caused a 306-bp internal deletion within the 630-bp coding sequence of *upp*. The editing performed using the Δ*upp*::ipa8 template caused the replacement of *upp* by the operonic structure ipa8 from plasmid pFC008. 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 $pCas9_{con}$ 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 $pCas9_{ind}$ 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 $pCas9_{ind}$ 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 $pCas9_{ind}$ 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 $pCas9_{ind}$ and pGRNA-upp on plates supplemented with the inducer. In contrast, colonies containing $pCas9_{ind}$ and pGRNA-upp_{oc} were isolated on plates supplemented with aTc (Fig. 2). Colonies were also obtained when transformants containing $pCas9_{ind}$ and either pGRNA-$\Delta_{43\text{-}348}$*upp* or pGRNA-Δ*upp*::ipa8 were grown on aTc-supplemented media (data not shown). Notably, aTc-resistant $pCas9_{ind}$ colonies containing pGRNA-Δ*upp*::ipa8 were obtained at lower frequencies (10^{-4} colonies/total colonies) than those containing either pGRNA-upp_{oc} or pGRNA-$\Delta_{43\text{-}348}$*upp* (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 $pCas9_{ind}$ and either pGRNA-upp_{oc} (10 colonies tested), pGRNA-$\Delta_{43\text{-}348}$*upp* (10 colonies tested) or pGRNA-Δ*upp*::ipa8 (4 colonies tested) were able to grow on solid media supplemented with 5-FU, while colonies containing $pCas9_{ind}$ 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 on any of the editing templates used. Desired mutations in the *upp* gene were confirmed by sequencing the PCR product in all four modified strains containing pGRNA-upp_{oc} (Fig. 3A), and the expected 306-bp deletion was detected in the four colonies transformed with pGRNA-$\Delta_{43\text{-}348}$*upp* (Fig. 3B). Similarly, the replacement of *upp* by the ipa8 operon was confirmed in the four colonies containing pGRNA-Δ*upp*::ipa8 (Fig. 3C).

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 after two rounds of growth 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 $pCas9_{ind}$ 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) were 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; Ehsaan 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 variations or the replacement of the whole gene by the ipa8 operon. The two remaining mutants contained undesired modifications elsewhere in their genome;

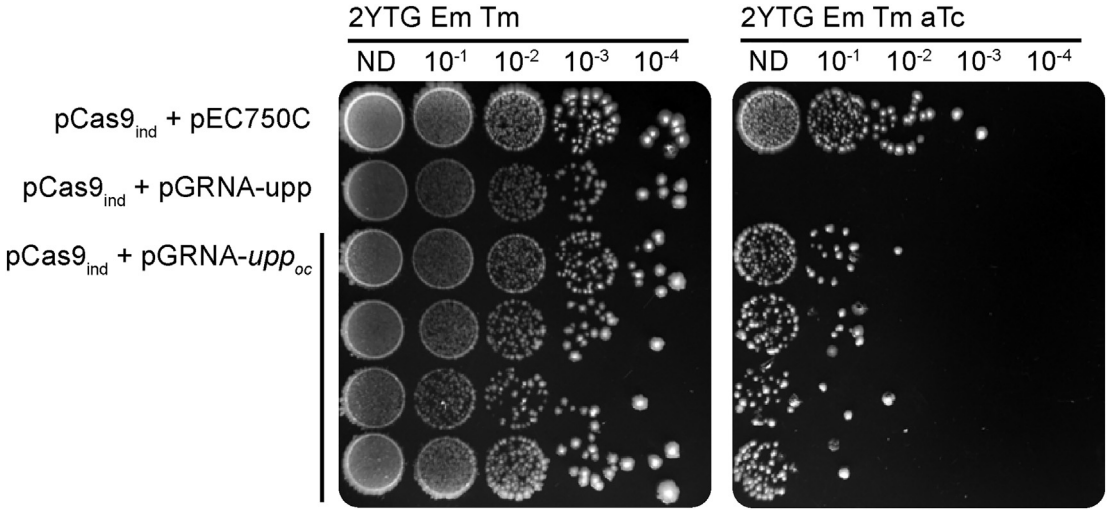

Fig. 2. Selection of *Clostridium acetobutylicum* cells edited with the upp_{oc} template on aTc-containing plates. Independent transformants containing $pCas9_{ind}$ and either pEC750C, pGRNA-upp or pGRNA-upp_{oc} were suspended in liquid 2YTG, serially diluted and spotted on plates containing the appropriate 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.

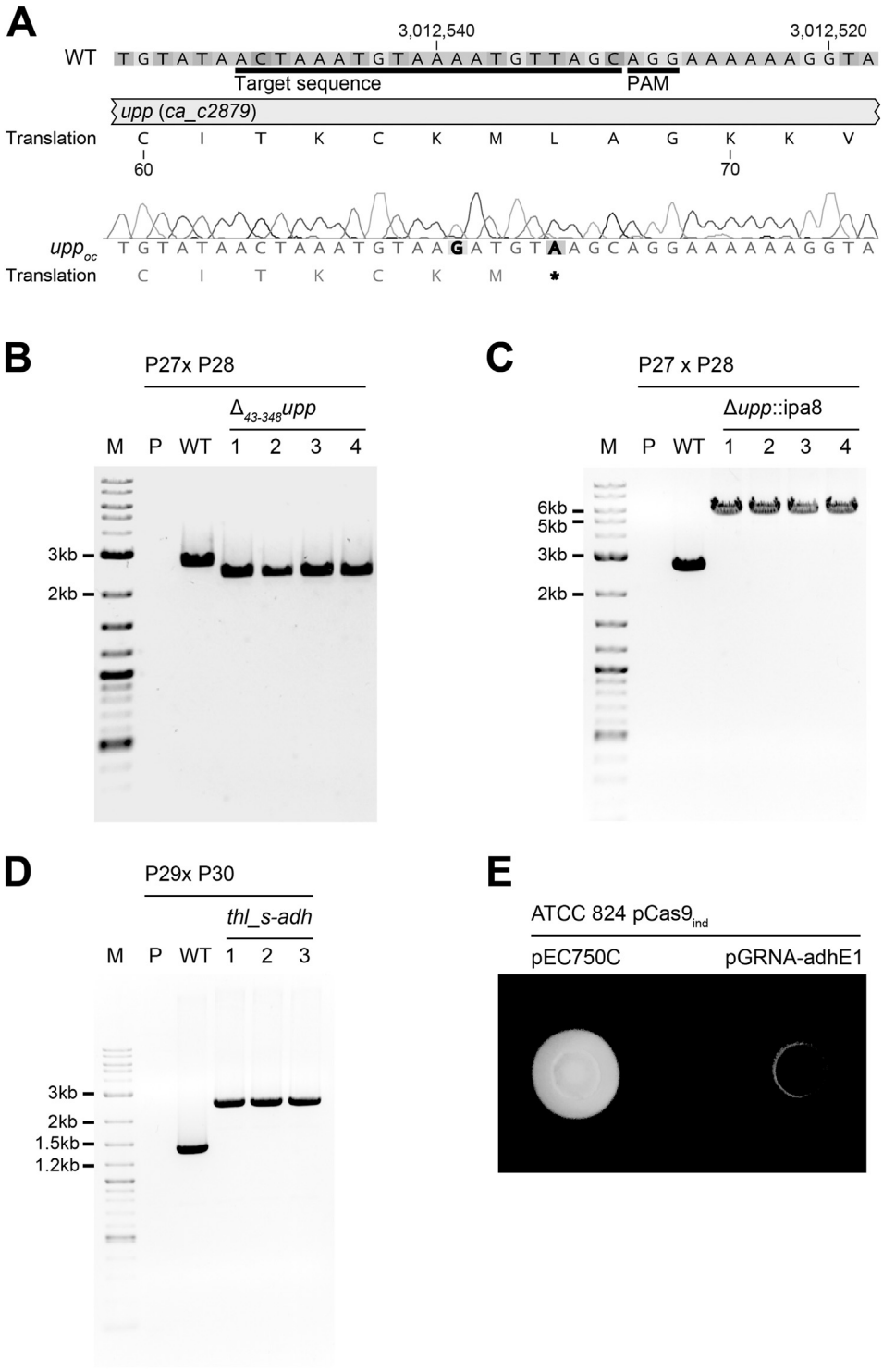

Fig. 3. Editing of the *Clostridium acetobutylicum* ATCC 824 genome. (A) Sequencing of the *upp* gene in cells edited with the upp_{oc} template. The genome sequence is numbered according to the revised version of the genome sequence of ATCC 824 (Nölling et al., 2001; Ehsaan et al., 2016). The corresponding translation is indicated above the nucleotide sequence. The asterisk indicates a stop codon, while modifications are indicated in bold. The same result was obtained for the four colonies tested. PAM, Protospacer adjacent motif. (B) PCR amplification of the *upp* locus in cells edited with the pGRNA-$\Delta_{43\text{-}348}upp$ template. Amplification yields a 2720 bp product in the WT strain and a 2414 bp product in correctly edited cells. Lane M, 2-log DNA ladder (0.1 to 10 kb; New England Biolabs [NEB]); Lane P, amplification on pGRNA-$\Delta_{43\text{-}348}upp$. (C) PCR amplification of the *upp* locus in cells edited with the Δ*upp*::ipa8 template. Amplification yields a 2720 bp product in the WT strain and a 5705 bp product in correctly edited cells. Lane M, 2-log DNA ladder (0.1 to 10 kb; NEB); Lane P, amplification on pGRNA-Δ*upp*::ipa8. (D) PCR amplification performed with primers P29 and P30 of the *thl* locus in cells edited with the *thl-adh* template. Amplification yields a 1406 bp product in the WT strain and a 2477 bp product in correctly edited cells. Lane M, 2-log DNA ladder (0.1 to 10 kb; NEB); Lane P, amplification on pGRNA-*thl-adh*. (E) Representative amylolytic activity of *C. acetobutylicum* ATCC 824 $pCas9_{ind}$ transformed with pEC750C or pGRNA-adhE1 on a glucose + starch plate.

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 Δ*upp*::ipa8 mutants and the two sequenced $\Delta_{43\text{-}348}upp$ 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 $\Delta_{43\text{-}348}upp$ 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 Δ*upp*::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

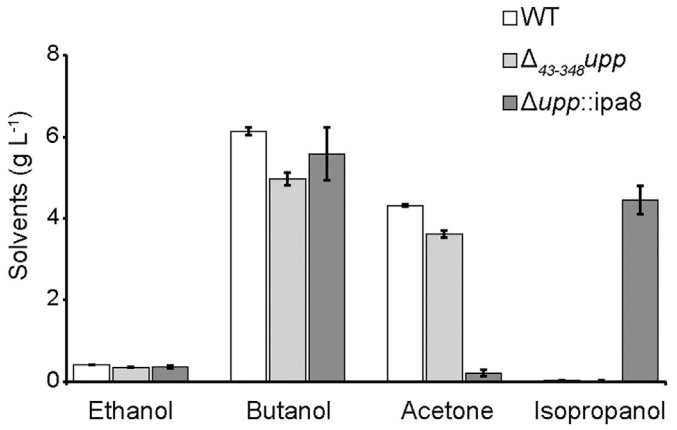

Fig. 4. Final solvent concentrations in flask fermentations of the *Clostridium acetobutylicum* ATCC 824 wild-type, $\Delta_{43\text{-}348}upp$ and Δ*upp*::ipa8 mutant strains. All results shown are average values ± standard deviations from duplicate experiments over 72 h. Results for $\Delta_{43\text{-}348}upp$ and Δ*upp*::ipa8 strains are mean values for two biologically independent mutants.

thl promoter. Plasmids pGRNA-thl and pGRNA-*thl-adh* were introduced into cells containing $pCas9_{ind}$. The *cas9* induction was then performed as described above. All three colonies obtained from the aTc-supplemented plates were found to be correctly edited, as shown by a PCR performed on their gDNA (Fig. 3D). Secondly, a gRNA expression cassette targeting the *adhE1* gene located on the pSOL megaplasmid was designed and cloned into pEC750C to yield pGRNA-adhE1. The viability of cells containing $pCas9_{ind}$ and pGRNA-adhE1 was not reduced by the induction of *cas9* expression on aTc-supplemented plates (data not shown). The loss of the megaplasmid in edited colonies was confirmed by the absence of amylase activity on a plate supplemented with starch and coloured using an iodine solution (Fig. 3E), as described previously (Sabathé et al., 2002). Moreover, PCR analyses of three colonies confirmed that they no longer contained the megaplasmid (data not shown). Similarly, the introduction of pGRNA-adhE1 in ATCC 824 containing $pCas9_{con}$ yielded transformants that did not contain the pSOL megaplasmid (data not shown).

4. Discussion

The use of CRISPR/Cas-based genome editing tools is revolutionising genetics, allowing efficient genome editing in a broad range of organisms including bacteria of the *Clostridium* genus, which are known to be challenging to engineer. Several groups have reported the adaptation of this tool using strategies compatible with the low transformation efficiencies and low HR frequencies that are inherent to *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 elements needed, i.e. the 4.1-kb *cas9* gene, the gRNA-expression cassette and the editing template, in addition to the replication origins for *E. coli* and *Clostridium* and antibiotic resistance gene(s). Assembling all of these elements yields relatively large plasmids that are difficult to introduce into bacterial cells, which is particularly challenging for *Clostridium* strains because of their low transformation efficiency. For this reason, and because HR frequencies are also low in *Clostridium* strains, a constitutive CRISPR/Cas9 system is not well suited to *Clostridium* genomic editing.

In this study, a two-plasmid inducible CRISPR/Cas9 genome editing tool was successfully developed. To our knowledge, the concomitant use of two plasmids has not been reported previously in *Clostridium* species, probably because of the lack of compatible replication origins and selection markers available. Recently, however, the increasing number of sequenced *Clostridium* genomes has enabled the identification of new extra-chromosomal elements, as well as compatible replicons for most of the *Clostridium* 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_{oc} or the $\Delta_{43\text{-}348}upp$ templates (causing a 2-nt substitution and a 306-bp deletion, respectively) were obtained at higher frequencies than cells edited with the Δ*upp*::ipa8 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 Cas9n 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 $pCas9_{ind}$ are transformed with the targeting plasmid harbouring the gRNA expression cassette, one round of growth on aTc-supplemented plates is sufficient to obtain correctly edited cells, illustrating the high efficiency of this strategy. Moreover, in cases requiring multiple genetic modifications, an intermediate mutant harbouring only $pCas9_{ind}$ can be easily obtained after a few rounds of growth on appropriate plates for the curation of the targeting plasmid, making it ready for a new round of modification (data not shown).

It is not possible to modify any non-essential genetic mobile element using this technique, as the Cas9 action results in the simple loss of the element. A CRISPR/Cas9 strategy is therefore not applicable for the modification of non-selectable extra-chromosomal elements, such as the non-essential pSOL from *C. acetobutylicum* ATCC 824 (Cornillot et al., 1997).

The absence of selection markers in the genome of the modified strains and the potential to perform sequential modifications suggest new possibilities for synthetic biology approaches in industrial biotechnology involving *Clostridium* solventogenic strains. As an initial example, the *adh* gene from *C. beijerinckii* DSM 6423 was inserted into the genome of 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 Cas9n system in *C. cellulolyticum*. Moreover, this two-plasmid inducible CRISPR/Cas9 genome editing tool will be employed in other strains of the *Clostridium* genus in the future, to demonstrate its varied possibilities.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

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