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Adaptation and application of a two-plasmid inducible CRISPR-Cas9 system in *Clostridium beijerinckii*

Mamou Diallo^{a,*}, Rémi Hocq^{b,1}, Florent Collas^a, Gwladys Chartier^b, François Wasels^b,
Hani Surya Wijaya^a, Marc W.T. Werten^a, Emil J.H. Wolbert^a, Servé W.M. Kengen^c,
John van der Oost^c, Nicolas Lopes Ferreira^{b,*}, Ana M. López-Contreras^a

^a Bioconversion Group, Wageningen Food and Biobased Research, Bornse Weilanden 9, 6708WG Wageningen, the Netherlands

^b IFP Energies nouvelles, Biotechnology Department, 1 et 4 avenue de Bois-Préau, 92852 Rueil-Malmaison, France

^c Laboratory of Microbiology, Wageningen University, Stippeneng 4, 6708 WE Wageningen, the Netherlands

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ABSTRACT

Recent developments in CRISPR technologies have opened new possibilities for improving genome editing tools dedicated to the *Clostridium* genus. In this study we adapted a two-plasmid tool based on this technology to enable scarless modification of the genome of two reference strains of *Clostridium beijerinckii* producing an Acetone/Butanol/Ethanol (ABE) or an Isopropanol/Butanol/Ethanol (IBE) mix of solvents. In the NCIMB 8052 ABE-producing strain, inactivation of the SpoIIIE sporulation factor encoding gene resulted in sporulation-deficient mutants, and this phenotype was reverted by complementing the mutant strain with a functional *spoIIIE* gene. Furthermore, the fungal cellulase-encoding *celA* gene was inserted into the *C. beijerinckii* NCIMB 8052 chromosome, resulting in mutants with endoglucanase activity. A similar two-plasmid approach was next used to edit the genome of the natural IBE-producing strain *C. beijerinckii* DSM 6423, which has never been genetically engineered before. Firstly, the *catB* gene conferring thiamphenicol resistance was deleted to make this strain compatible with our dual-plasmid editing system. As a proof of concept, our dual-plasmid system was then used in *C. beijerinckii* DSM 6423 $\Delta catB$ to remove the endogenous pNF2 plasmid, which led to a sharp increase of transformation efficiencies.

1. Introduction

Solventogenic *Clostridia* are mesophilic, rod shaped gram positive, anaerobic and sporogenic bacteria belonging to the *Firmicutes* phylum. Model species such as *Clostridium acetobutylicum* and *Clostridium beijerinckii* are able to produce a wide range of valuable chemicals among which acetone/isopropanol, butanol and ethanol during the A/IBE fermentation [1–5]. Moreover, *Clostridium* species have been reported to produce other chemicals such as acetoin, 2,3-butanediol [2,6], or 1,3-propanediol [7–9]. However, the A/IBE fermentations suffer from the low end-products final concentrations mainly related to their toxicity [10].

Over the last decades, many efforts were made to improve the solvent tolerance and productivity of solventogenic *Clostridia* by genetic

engineering. Forward genetics (i.e. random mutagenesis) have been mostly used to study molecular mechanisms in *Clostridia* [11,12], because targeted mutagenesis has long been impeded by the lack of efficient genetic tools for the *Clostridium* genus. Since the adaptation of the TargeTron technology [13] to the *Clostridia* genus in 2007 and the creation of a genetic tool called ClosTron [14,15]), targeted approaches have been greatly facilitated. This technology allows gene disruption through the precise insertion of a mobile group II intron into the genome. However, polar effects on flanking genes have been reported, making the subsequent analysis of mutant phenotypes difficult [16]. Moreover, considering that this method does not enable large genetic insertions, other tools were created to alleviate these bottlenecks. In particular, strategies using counter-selection markers have been developed to select rare allelic exchange events [17–19]. In 2013, Al-

Abbreviations: ABE, Acetone Butanol Ethanol; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas, CRISPR-associated protein; Cas9n, Cas9 nickase; DAE, Double Allelic Exchange; DSB, Double-Strand Break; gDNA, genomic DNA; gRNA, guide RNA; HR, Homologous Recombination; NHEJ, Non-Homologous End Joining; PAM, Protospacer Adjacent Motif; PCR, Polymerase Chain Reaction; SAE, Simple Allelic Exchange

* Corresponding authors.

E-mail addresses: mamou.diallo@wur.nl (M. Diallo), nicolas.lopes-ferreira@ifpen.fr (N.L. Ferreira).

¹ Mamou Diallo and Rémi Hocq contributed equally to this work.

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Table 1
Strains and plasmids used in this study.

Strains and plasmids	Relevant information	Reference or source
Clostridia		
<i>Clostridium beijerinckii</i> NCIMB 8052	ABE producer, sensitive to erythromycin (25–50 µg/mL) and spectinomycin (650 µg/mL)	[46]
<i>Clostridium beijerinckii</i> DSM 6423	IBE producer, <i>adh</i> gene, sensitive to erythromycin (25–50 µg/mL), resistant to thiamphenicol (15 µg/mL)	[3]
<i>Clostridium difficile</i> str. 630	Gene <i>xytR</i> and promoter of the gene <i>xytB</i>	[29]
Other microbial strains		
<i>E. coli</i> XL1-blue	endA1 <i>gyrA96</i> (nalR) <i>thi-1</i> <i>recA1</i> <i>relA1</i> <i>lac</i> <i>glnV44</i> F'[::Tn10 <i>proAB</i> + <i>lacIq</i> Δ(<i>lacZ</i>)M15] <i>hsdR17</i> (rK- mK +)	Agilent
<i>E. coli</i> NEB 10-beta	Δ(<i>ara-leu</i>) 7697 <i>araD139</i> <i>fhuA</i> Δ <i>lacX74</i> <i>galK16</i> <i>galE15</i> e14-φ80 <i>dlacZ</i> ΔM15 <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>nupG</i> <i>rpsL</i> (Str ^R) <i>rph</i> <i>spoT1</i> Δ(<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>)	NEB
<i>E. coli</i> INV110	F' { <i>tra</i> Δ36 <i>proAB</i> <i>lacI^q</i> <i>lac</i> ΔM15} <i>rpsL</i> (Str ^R) <i>thr</i> <i>leu</i> <i>endA</i> <i>thi-1</i> <i>lacY</i> <i>galK</i> <i>galT</i> <i>ara</i> <i>tonA</i> <i>tsx</i> <i>dam</i> <i>dcm</i> <i>supE44</i> Δ(<i>lac</i> - <i>proAB</i>) Δ(<i>mcr</i> C <i>mrr</i>)102::Tn10(Tet ^R)	Invitrogen
<i>S. cerevisiae</i> W303	MATa/MATα { <i>leu2-3,112</i> <i>trp1-1</i> <i>can1-100</i> <i>ura3-1</i> <i>ade2-1</i> <i>his3-11,15</i> } [<i>phi</i> +]	ATCC 200060
C. beijerinckii NCIMB 8052 genome editing plasmids		
pMTL500E	Ap ^R ; Em ^R ; <i>colE1</i> ; pAMβ1	[35]
pSEC500E	Ap ^R ; Em ^R ; <i>colE1</i> ; pAMβ1; 2µ; URA3	This study
pYC100S	Sp ^R ; <i>colE1</i> ; pCB102; 2µ; URA3	This study
pWUR100S (pS)	Sp ^R ; <i>colE1</i> ; pCB102	This study
pE_X_cas9	pSEC500E derivative with <i>cas9</i> (inducible <i>xytB</i> promoter) insertion	This study
pS_X ^R KR	pWUR100S derivative with <i>Kan^R</i> ; chimera reprogrammable gRNA (inducible <i>xytB</i> promoter) and MCS	This study
pS_X ^R _celA_S1	pSX ^R KR derivative with cassette “ <i>celA</i> ” and gRNA <i>celA_S1</i> (PAM sequence located 36 bp downstream of the <i>hbd</i> gene (<i>cbei_0325</i>) stop codon) insertion	This study
pS_X ^R ThlPcelA_S1	pS_X ^R _celA_S1 derivative with the <i>thl</i> promoter from <i>cbei_0411</i>	This study
pS_X ^R _Δ <i>spoIIE</i> _S1	pSX ^R KR derivative with cassette “Δ <i>spoIIE</i> ” and gRNA <i>spoIIE_S1</i> (PAM located 571 bp downstream the start codon of <i>spoIIE</i>) insertion	This study
pWUR3	pMTL500E derivative with <i>celA</i> (constitutive promoter <i>egfA</i> and its signal peptide) insertion	[41]
SpoIIE complementation plasmid		
pSpoIIE	pWUR100S derivative with the <i>spoIIE</i> (<i>cbei_0097</i>)	This study
C. beijerinckii DSM 6423 genome editing plasmids		
pFW01	Em ^R ; <i>colE1</i> ; pCB102	Wasels et al. (2017)
pCas9 _{ind}	pFW01 derivative with <i>cas9</i> (inducible Pcm-tetO2/1 promoter) and <i>tetR</i> (miniPthl promoter) insertions	Wasels et al. (2017)
pEC750C	Tm ^R ; <i>colE1</i> ; pIP404	Wasels et al. (2017)
pCas9 _{ind} -Δ <i>catB</i> _S1	pCas9 _{ind} derivative with cassette “Δ <i>catB</i> ” and gRNA <i>catB_S1</i> (PAM sequence located 76 bp upstream of the <i>catB</i> gene (<i>CIBE_3859</i>) stop codon (inducible Pcm-2tetO1 promoter)) insertion	This study
pGRNA-pNF2_S1	pEC750C derivative with gRNA pNF2_S1 (PAM sequence located 285 bp downstream of the <i>CIBE_p20002</i> gene start/ stop codon (inducible Pcm-2tetO1 promoter)) insertion	This study

Ap, ampicillin; Em, erythromycin; Sp, spectinomycin; Tm, thiamphenicol; Kan, kanamycin; Tet, tetracycline.

Hinai et al. described a markerless genome editing strategy using the MazF toxin from *Escherichia coli* as a counter selection marker [17]. The methodology enabled the deletion of several genes and the insertion of a 3.6 kb DNA fragment leaving a FRT scar in the modified chromosome. Ehsaan et al. developed the ACE system (Allele Coupled Exchange) using the *pyrE* gene, encoding the pyrimidine biosynthesis enzyme “orotate phosphoribosyltransferase”, as a positive and negative selection marker [18,20]. However, these methods are time consuming as they require several selection and subculturing steps. The cells in which the plasmid has been integrated into the genome via a single crossover event are selected and then subcultured in selective media to further allow the allelic exchange. This lengthens the engineering process and favors the occurrence of spontaneous mutations.

CRISPR-Cas9 technology for genetic modification of eukaryotic and prokaryotic cells provides an alternative to these genome editing methods [21]. Recently, several publications reported the use of CRISPR-Cas9 tools for scarless genome editing in *Clostridium cellulolyticum* [22], *C. acetobutylicum* [23] and *C. beijerinckii* [23–26]. Most groups reported difficulties when the native Cas9 nuclease from *S. pyogenes* was constitutively expressed. Indeed, the double strand break (DSB) generated by Cas9 is reported to be lethal because of the absence of non-homologous end-joining (NHEJ) mechanisms in *Clostridia* [22,27]. Therefore, the Cas9 nuclease was often replaced by a Cas9 nickase [23,25] in which the RuvC domain was mutated (D10A) [28]. This methodology enabled the insertion of DNA fragments up to 1.7 kb after serial transfers [22]. Alternatively, Wang et al. [24] overcame the DSB lethality by placing the *cas9* gene under the control of a lactose inducible promoter [29] enabling deletions, insertions or single-nucleotide modifications (SNM) within the genome of *C. beijerinckii*. All

the systems described above are based on a “single vector strategy”. Such strategies are however limited due to the length of the *cas9* gene (4.1 kb), which restricts the cargo capacity of the transformation vectors and therefore limits the size of potential genome insertions.

Approaches using two separate plasmids for the sequential introduction of the *cas9* gene and the gRNA sequence into a targeted strain have been recently described in the solventogenic *C. acetobutylicum* [30] and in the acetogen *C. autoethanogenum* [31]. In the case of *C. acetobutylicum*, the system enabled precise genome editing with an efficiency of 100%. Indeed, the system enabled several modifications ranging from a single base pair substitution to the genomic insertion of a 3.6 kb DNA fragment. In the case of *C. autoethanogenum*, the system enabled a partial deletion of an alcohol dehydrogenase gene and the deletion of a 2,3-butanediol dehydrogenase gene (1 kb) with an efficiency of 50%. Although being powerful, the two-plasmid editing strategy requires two functional selection markers, which might not always be available for some strains.

In this study, we adapted our recently-described dual-plasmid CRISPR-Cas9 genetic tool [30] to edit *C. beijerinckii* strains. In the ABE model strain *C. beijerinckii* NCIMB 8052, the *cas9* gene and the gRNA expression cassette were placed on two distinct plasmids. Both *cas9* and gRNA were placed under the control of a xylose inducible promoter [32] to enable a tight control of nuclease activity. The homologous DNA repair template, required for allelic exchange, was placed on the same plasmid as the gRNA expression cassette. The lethal effect of DSB was taken as a strong asset to use the CRISPR system as a counter-selection against unedited cells. This system enabled the scarless deletion of 2.379 kb within the *spoIIE* gene (*cbei_0097*) as well as the insertion of the *celA* gene from *Neocallimastix patriciarum* in *C. beijerinckii* NCIMB

8052 with 100% and 75% efficiency, respectively.

The applicability of this two-plasmid approach was also investigated in the IBE producing *C. beijerinckii* DSM 6423 strain, for which successful genetic modification have never been reported so far. Being only able to use the erythromycin resistance gene as a selection marker, a preliminary strategy involving a single-plasmid CRISPR-Cas9 tool was undertaken to inactivate the CIBE_3859 gene (*catB*), encoding a putative amphenicol acetyltransferase. The $\Delta catB$ mutant was sensitive to thiamphenicol and therefore compatible with a two-plasmid editing approach, which was subsequently used to delete the endogenous pNF2 plasmid as a proof of concept. Interestingly, the removal of this mobile genetic element resulted in a drastically increased transformation efficiency, paving the way to further genome editing of this natural isopropanol producer.

2. Materials and methods

2.1. Strains and culture condition

Relevant characteristics of the bacterial strains and plasmids used in this study are listed in Table 1. *C. beijerinckii* NCIMB 8052 and DSM 6423 are laboratory strains. The *spoIIE* mutant was stored as vegetative cells in mCGM with 20% glycerol at -80°C . All the other strains were kept as spore suspension. Except for fermentation assays, liquid cultures of *C. beijerinckii* NCIMB 8052 and related mutants were grown in mCGM containing per liter: yeast extract, 5 g; KH_2PO_4 , 0.75 g; K_2HPO_4 , 0.75 g; asparagine- H_2O , 2 g; $(\text{NH}_4)_2\text{SO}_4$, 2 g; cysteine, 0.5 g; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.4 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.01 g; $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.01 g; glucose, 10 g. These microorganisms were grown and selected on mCGM agar containing per liter: yeast extract, 1 g; tryptone, 2 g; KH_2PO_4 , 0.5 g; K_2HPO_4 , 1 g; $(\text{NH}_4)_2\text{SO}_4$, 2 g; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.1 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.01 g; $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.015 g; CaCl_2 , 0.01 g; CoCl_2 , 0.002 g; CaCl_2 , 0.002 g; glucose, 50 g and agar 12 g. *C. beijerinckii* DSM 6423 and related mutants were cultivated in liquid 2YTG (per liter: tryptone, 16 g; yeast extract, 10 g; NaCl, 5 g; glucose, 20 g, pH 5.2) and grown on solid 2YTG (same as above, with neutral pH, 15 g/L agar and 5 g/L glucose). *Clostridium* mutants were selected in culture medium containing 50 $\mu\text{g}/\text{mL}$ of erythromycin (Duchefa), 650 $\mu\text{g}/\text{mL}$ spectinomycin (Duchefa) and/or 15 $\mu\text{g}/\text{mL}$ thiamphenicol (Sigma-Aldrich), as needed. Liquid media were made anaerobic by flushing with nitrogen gas or by incubation for at least 4 h in an anaerobic chamber. Cultivation was performed at 37°C , without shaking, and anaerobically in (i) an anaerobic chamber or (ii) in glass serum vials as described above. *E. coli* and *S. cerevisiae* strains were stored as vegetative cells in 15% glycerol solution at -80°C . *E. coli* was grown on LB medium, supplemented with 100 $\mu\text{g}/\text{mL}$ of ampicillin (Duchefa), 100 $\mu\text{g}/\text{mL}$ spectinomycin (Duchefa), 25 $\mu\text{g}/\text{mL}$ chloramphenicol (Sigma-Aldrich) and/or 500 $\mu\text{g}/\text{mL}$ erythromycin (Duchefa).

2.2. Plasmid construction and transformation

Except where noted, plasmids and DNA templates used for *C. beijerinckii* NCIMB 8052 CRISPR system were generated as yeast/*E. coli*/*Clostridium* shuttle vector in the yeast *S. cerevisiae* by homologous recombination as described previously [34]. Yeasts were transformed by the lithium acetate method [35]. Competent cells were transformed with linear plasmids and insert(s) produced by PCR. Primers are listed in supplementary file S1. Each primer contained a 30 bp 5'-tail specific to another DNA fragment to allow homologous recombination. Final yeast/*E. coli*/*Clostridium* shuttle vectors obtained from yeast were digested by AvrII and self-ligated using the T4-DNA ligase (NEB) to evict the yeast replication origin (2microns) and selection marker (URA3 gene).

The *SpoIIE* complementation plasmid was constructed using the Circular Polymerase Extension Cloning [36]. The pS shuttle vector was digested by SphI and XhoI enzymes for linearization and the insert was

amplified by PCR on genomic DNA from *C. beijerinckii* NCIMB 8052 using the primers Cbei0097prom_F and Cbei0097_R.

The plasmid pS_XR_cela_S1 was constructed (with the promoter of the *thl* gene, cbei_3630, driving expression of *cela*) via homologous recombination in *S. cerevisiae* using the primers listed in supplementary file S1. Unfortunately, non-silent mutations were found in the *cela* gene. In the meantime, transcriptome analysis [37] showed that another copy of the *thl* gene, cbei_0411 was more expressed and thus its promoter would be a stronger promoter than cbei_3630 and a better choice. Therefore, to simultaneously address both matters, a BsaI/XcmI fragment encompassing the relevant regions was replaced with an accordingly designed synthetic BsaI/XcmI fragment (Genscript). The resulting vector, pS_XRThlPcela_S1, was sequence-verified and uses the promoter of the *thl* gene (cbei_0411) to drive the expression of the *cela* gene.

Plasmids were introduced in *C. beijerinckii* NCIMB 8052 and related recombinant strains prepared according to the protocol described previously [38].

To construct pCas9_{ind}- $\Delta catB$ _S1, a DNA cassette containing the $\Delta catB$ template DNA as well as the gRNA targeting the *C. beijerinckii* DSM 6423 *catB* locus (anhydrotetracycline inducible promoter) was synthesized by Eurofin Genomics and cloned in the pEX-A258 commercial vector. This cassette was amplified by PCR ($\Delta catB$ _fwd and $\Delta catB$ _rev primers) and cloned at the XhoI restriction site in pCas9_{ind} [30].

To construct pGRNA-pNF2, a gRNA expression cassette composed of the Pcm-2tetO1 promoter [39] and a 20-nt guiding sequence targeting pNF2 fused with the chimeric gRNA sequence [28], pNF2-gRNA, was synthesized (Eurofins Genomics, sequence available in supplementary file S1), and cloned into the SacI-digested pEC750C.

Prior to electroporation, plasmids were transformed in a *dam*⁻ *dcm*⁻ *E. coli* strain (INV110, Invitrogen). *Dam*⁻ *Dcm*⁻ DNA was then used to transform *C. beijerinckii* DSM 6423 as described previously [40], with a few modifications outlined thereafter. 2YTG 20 g/L glucose was used for liquid cultures. Mid-exponential phase ($\text{OD}_{600} = 0.6\text{--}0.8$) cells were used and 5–20 μg DNA was electroporated with a single pulse (100 Ω , 25 μF , 1.4 kV) in 2 mm-gap cuvettes.

2.3. Reprogramming the gRNA protospacer in pS_X^RK^R

The CRISPR-Cas9 target sequences were selected after a manual search in the target region for a 25 bp spacer sequence flanked in its 3' end by a 5'-NGG-3' motif named protospacer adjacent motif (PAM). The spacer was designed by the annealing of two primers; (i) the primer "Target_Forw" consisting in the spacer sequence to which the motif "5'-GAGG-3'" was added at its 5' end and (ii) the primer "Target_Rev" consisting in the reverse complement of the spacer sequence to which the motif "5'-AAAC-3'" was added at its 5' end. Both primers were mixed (1 μM each), heated at 95°C for 10 min and cooled down at room temperature. The primer dimer was then inserted in pS_X^RK^R by Golden Gate cloning using the BsaI enzyme (NEB). Ligation and digestion were performed concomitantly for 1 h at 37°C .

2.4. Selection of mutant strains

C. beijerinckii NCIMB 8052 recombinant strains harboring the two plasmids of the CRISPR-Cas9 system were grown at 37°C in 25 mL liquid mCGM medium containing 0 (without induction) or 40 g/L xylose (pre-induction) for 24 h–3 days to induce the CRISPR-Cas9 system. Once the optical density of the culture containing xylose reached 1.5 MAU at 600 nm, these cultures were concentrated in 100 μL of fresh mCGM media. 50 μL of the cell suspension were spread on mCGM selective plates supplemented 40 g/L xylose, 50 $\mu\text{g}/\text{mL}$ erythromycin and 650 $\mu\text{g}/\text{mL}$ spectinomycin and incubated for 24 h–3 days to obtain single colonies. Edited cells were screened by colony PCR to detect single and double allelic exchange profiles.

C. beijerinckii DSM 6423 recombinant colonies bearing a single- or dual-plasmid genome editing system were treated as described previously [30], with a 200 ng/mL anhydrotetracycline (Sigma-Aldrich) induction.

2.5. Fermentation assays

Fermentations were performed in CM2 medium [41] which contains per liter: yeast extract, 5 g; KH_2PO_4 , 1 g; K_2HPO_4 , 0.76 g; NH_4Ac , 3 g; p-aminobenzoic acid, 0.1 g; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 1 g; and $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.5 g, glucose, 60 g. 50 mL of media were inoculated with 250 μL of a heat shocked fresh spore suspension and incubated overnight at 35 °C. The overnight cultures were inoculated (5% (v/v)) in fresh culture medium and incubated at 35 °C for 72 h. The concentrations of metabolites were determined in clear culture supernatants after removal of microbial biomass by centrifugation. Glucose, acetate, butyrate, lactate, acetoin, acetone, butanol and ethanol were determined by HPLC as described previously [2,42] using valeric acid (30 mM) as an internal standard.

2.6. Microscopy

Phase contrast microscopy (Olympus BX51; Olympus, Tokyo, Japan) was used to determine the presence of endospores or pre-spore stages. Native bacteria were observed by phase contrast microscopy at $\times 1000$ magnifications.

3. Results

3.1. Genome editing strategy

The *cas9* gene was placed on a different plasmid from the gRNA expression cassette and the editing template so that (1) cloning was facilitated (i.e. with the use of smaller plasmids) and (2) space was available for large editing templates. Since previous CRISPR-Cas9-related studies in *Clostridia* hypothesized that DSBs catalyzed by Cas9 are likely to severely impair transformation efficiency [22,43], both the *cas9* gene located on pE_X_cas9 and the gRNA expression cassette located on pS_XKR were placed under the control of the inducible promoter PxyIB from *C. difficile* str. 630 [32] (Supplementary file S2). To complete our 2-plasmid inducible system, the *xyIR* regulatory gene was further cloned into the plasmid pS_XKR yielding pS_X^RKR.

Editing templates and gRNA expression cassettes were thus inserted into the pS_X^RKR plasmid (Fig. 1a). Each editing template contains two homology sequences (HS) of at least 500 bp, named HS1 and HS2. For genomic deletion, the two HS flank the region that has to be removed, which contains the CRISPR target site. Genomic insertions were designed by placing the sequence of interest between HS1 and HS2. This configuration allows 3 different allelic exchange events between the genome and the plasmid containing the DNA template (Supplementary file S3): two single-crossover allelic exchanges (SAE) and one double-crossover allelic exchange (DAE). The latter exchange is the only one which results in genomic DNA without the CRISPR-Cas9 target, which enables the survival of the transformants upon Cas9 induction.

The pS_X^RKR-based targeting plasmids were then introduced into a *C. beijerinckii* NCIMB 8052 recombinant strain already transformed with the pE_X_cas9 plasmid (Fig. 1b–c).

Following plasmid selection, xylose was used to induce the expression of both *cas9* and the gRNA. This critical step enables the selection of correctly edited cells, as, in the absence of non-homologous repair systems, Cas9 targets the bacterial chromosome, resulting in cell death. The final recombinant strains were next obtained by curing the pS_X^RKR-based plasmid and the pE_X_cas9 plasmid by serial subculturing in mCGM media lacking both antibiotics (Fig. 1d–e). Alternatively, selectively curing the targeting plasmid allows to directly reuse the modified cells for another round of genome editing (Fig. 1g).

To evaluate the robustness of this new genome-editing approach for

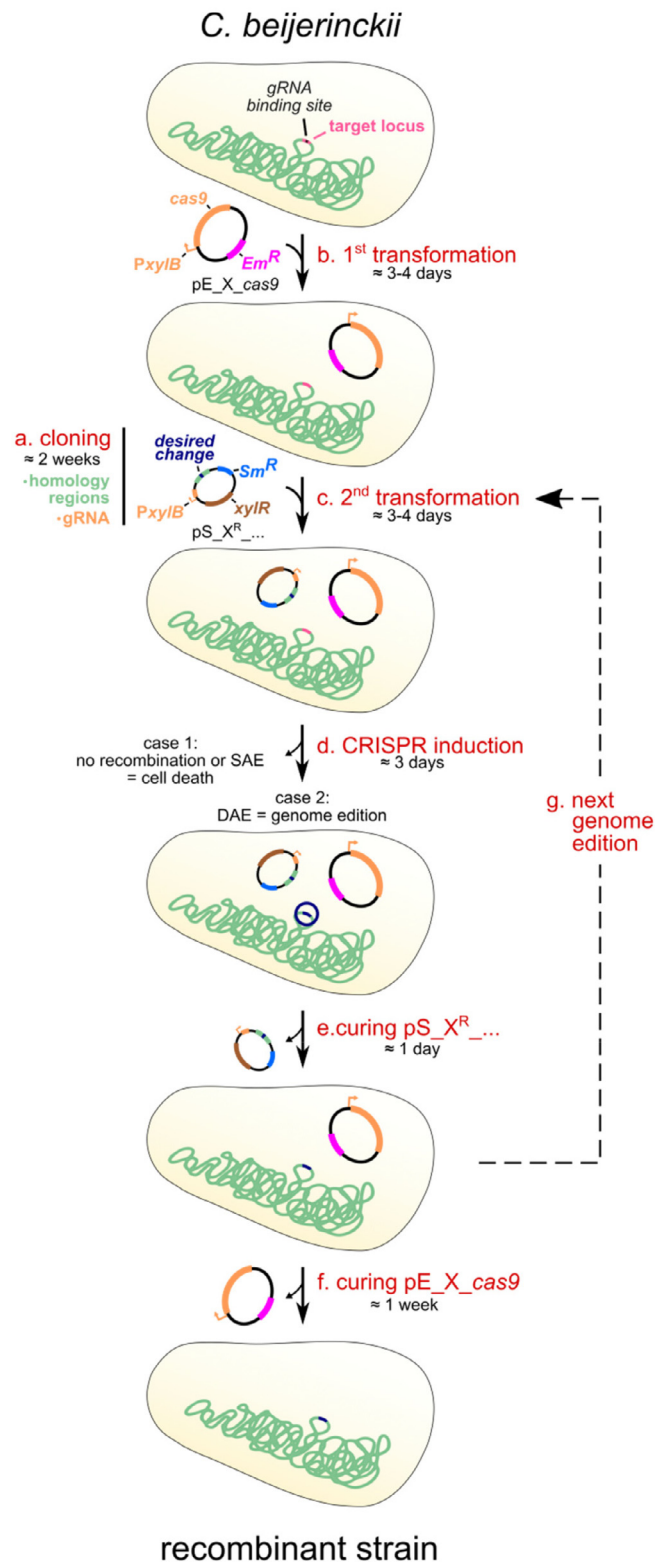


Fig. 1. Dual-plasmid inducible CRISPR-Cas9 strategy for *C. beijerinckii* NCIMB 8052 genome editing. a. Preliminary cloning of homology sequences and gRNA protospacer in pS_X^RKR plasmid to obtain a targeting plasmid. b,c. Transformation of (b) pE_X_cas9 in the WT strain and (c) of the targeting plasmid in the latter strain. d. Induction of *cas9* and gRNA expression to yield an edited strain. e,f,g. Curing of the targeting plasmid (e) and pE_X_cas9 plasmid (f) yields a markerless genome edited mutant. Alternatively (g), the targeting plasmid can be specifically removed to promote another round of genome editing. gRNA: guide RNA; Em^R: erythromycin resistance marker; Sm^R: spectinomycin resistance marker.

Table 2
Chromosomal modifications performed in this study.

Strain	DNA template	Size of the sequences (base pairs)				Screening results ^a			
		Deleted	Inserted	HS1	HS2	DAE	WT	DAE & WT	“–”
NCIMB 8052	<i>ΔspoIIE</i>	2424	0	741	1020	7	0	0	1
	<i>celA</i>	19	1667	1001	1017	4	0	0	1
DSM 6423	<i>ΔcatB</i>	653	8	400	400	3	0	0	1

HS1 and HS2 Homologous sequence; WT: wild type; DAE: Double allelic exchange; SAE: Single allelic exchange; “–”: no amplification.

^a Screening was performed on colonies growing with the highest xylose/aTc concentrations.

modification of *C. beijerinckii* NCIMB 8052, the system was used for: (i) a gene deletion and (ii) a gene insertion. The modifications performed in this study and their characteristics are summarized in Table 2.

3.2. Deletion of the *spoIIE* gene, characterization of the edited strains and complementation with a functional *spoIIE* gene

The *spoIIE* gene (cbei_0097) encoding a phosphatase involved in the sporulation regulation was targeted first. In model organisms *Bacillus subtilis* and *C. acetobutylicum*, SpoIIE is involved in the first steps (Stage II) of the regulation the sporulation mechanisms cycle [44,45]. It enables the activation of the sporulation-specific sigma factor σ^F and is necessary to complete asymmetric division. In *C. acetobutylicum*, SpoIIE inactivation does not impair solvent production [46,47]. The pS_X Δ *spoIIE*_S1 plasmid contains the “*ΔspoIIE*” DNA editing template (Table 1 and Fig. 2a) and the “*spoIIE*_S1” gRNA. HS1 and HS2 templates were designed to remove 2379 bp within the coding sequence of the *spoIIE* gene. Without induction of the CRISPR-Cas9 machinery, PCR screening showed that the full pS_X Δ *spoIIE*_S1 plasmid frequently integrated into the host genome by SAE mechanisms either with HS1 or with HS2. However, no DAE event was observed (data not shown). Overnight xylose induction in the *C. beijerinckii* (pE_X Δ *cas9*, pS_X Δ *spoIIE*_S1) strain negatively affected its growth, suggesting a toxic effect of Cas9 activity (Fig. 2c). This overnight culture was then plated on mCGM agar containing xylose (Fig. 2d), which permitted the selection of correctly edited Δ *spoIIE* cells (7/7 DAE events, Fig. 2e and Table 2). Microscopic analysis showed that the Δ *spoIIE* cells were more elongated than the wild-type cells and did not form viable endospores (Fig. 3a–b). Similarly to what was observed in *C. acetobutylicum*, mutant strains were still able to produce solvents (Supplementary file S4). To test whether the asporogeneous phenotype was effectively caused by the disruption of the *spoIIE* gene, complementation with a plasmidic copy of the *spoIIE* gene under the control of its native promoter was performed. As expected, sporulation was restored in the complemented mutant (Fig. 3a–b).

3.3. Insertion of the *celA* gene from *Neocallimastix patriciarum*

The *celA* gene from the fungus *Neocallimastix patriciarum* [48,49], encoding a cellobiohydrolase, was inserted downstream of the *hbd* gene (cbei_0325). The pS_XR Δ *celA*_S1 plasmid, harboring the “*celA*” DNA template (Table 1 and Fig. 2b) and the “*celA*_S1” gRNA, was introduced in strain NCIMB 8052 (pE_X Δ *cas9*). The *celA* template was designed to enable the insertion of the *celA* gene under the control of the thiolase promoter from *C. beijerinckii* (1667 bp) and the deletion of a 19 bp sequence located 36 base pairs downstream of the stop codon of the *hbd* gene (cbei_0325).

Similarly to what was observed for *spoIIE* inactivation, only SAE events were detected by PCR in the transformants containing pE_X Δ *cas9* and pS_XR Δ *celA*_S1, prior to xylose induction. Following xylose induction, 3 out of the 4 xylose-resistant colonies tested were correctly edited (Fig. 2e and Table 2).

The resulting *celA* mutants displayed cellulolytic activity on mCGM plates containing 1 g/L CMC (carboxymethylcellulose), similarly to a

control *C. beijerinckii* strain carrying the *celA* transcription unit on a plasmid (pWUR3, [48]) (Fig. 3b).

3.4. Adaptation of the dual-plasmid genome editing strategy to *C. beijerinckii* DSM 6423

The advantages provided by a two-plasmid CRISPR-Cas9 genome editing system (i.e. scarless modifications, high efficiency, relatively high DNA cargo capacity) prompted us to develop a similar system in the poorly studied *C. beijerinckii* DSM 6423 strain. A first obstacle was the lack of knowledge on the transformability of this natural IBE producer. This hurdle was overcome by applying a DNA demethylation strategy prior to electroporation, as described by Kolek et al. [33] in the closely related *C. beijerinckii* NRRL B-598 strain (Supplementary file S7). This treatment enabled the generation of transformants containing pFW01-derived vectors [30,50] (Supplementary file 7). However, vectors bearing the spectinomycin resistance marker could not be introduced successfully in this strain (data not shown). This was a major bottleneck for the use of a two-plasmid editing strategy since this microorganism was also resistant to thiamphenicol, an antibiotic often used for selection in clostridial recombinant systems [51].

To alleviate this second obstacle, a single-plasmid CRISPR-based approach was developed to disrupt the *catB* gene (CIBE_3859), predicted to encode an amphenicol acetyltransferase probably involved in thiamphenicol resistance. pE_X Δ *cas9* plasmid was not chosen as a backbone since this already large vector (\approx 13 kb, Supplementary file S2) does not carry the *xylR* gene necessary for *cas9* inducibility. Instead, the 8874 bp pCas9_{ind} plasmid previously used for genome editing in *C. acetobutylicum* ATCC 824 [30] was engineered to include an anhydrotetracycline-inducible gRNA expression cassette targeting *catB* as well as homology sequences designed to delete 99% of the *catB* gene (Fig. 4a, Table 2). The resulting pCas9_{ind}- Δ *catB*_S1 vector was introduced in *C. beijerinckii* DSM 6423, and mutants with the expected *catB* deletion were obtained after anhydrotetracycline (aTc) induction (Fig. 4b).

As anticipated, these Δ *catB* mutants were sensitive to thiamphenicol (Fig. 4c) and allowed the maintenance of the pEC750C vector in this modified strain, which carries the *catP* marker from *C. perfringens* [52]. Besides, this vector could be introduced in a strain already bearing a pFW01-based plasmid. In order to test whether a dual-plasmid CRISPR-Cas9 editing approach could be applied to the Δ *catB* mutant, we constructed a pEC750C-based plasmid carrying an aTc-inducible gRNA cassette targeting the endogenous pNF2 plasmid (pGRNA-pNF2_S1). The pNF2 plasmid [53] was discovered during the reconstruction of *C. beijerinckii* DSM 6423 genome along with other extra-chromosomal genetic elements (i.e. another plasmid and a linear phage). The pNF2 plasmid is the smallest of these elements (4282 bp) and bears 4 putative genes, 3 of which have no predicted function, and the last one is predicted to encode a potential plasmid replication protein. The pGRNA-pNF2_S1 vector was subsequently introduced in *C. beijerinckii* DSM 6423 Δ *catB* (pCas9_{ind}) which allowed the highly efficient selection of Δ pNF2 mutants (Fig. 4d–e). Fermentation assays further revealed that the deletion of Δ pNF2 did not affect alcohol production (Supplementary file S8). However, pNF2 removal resulted in increased transformation

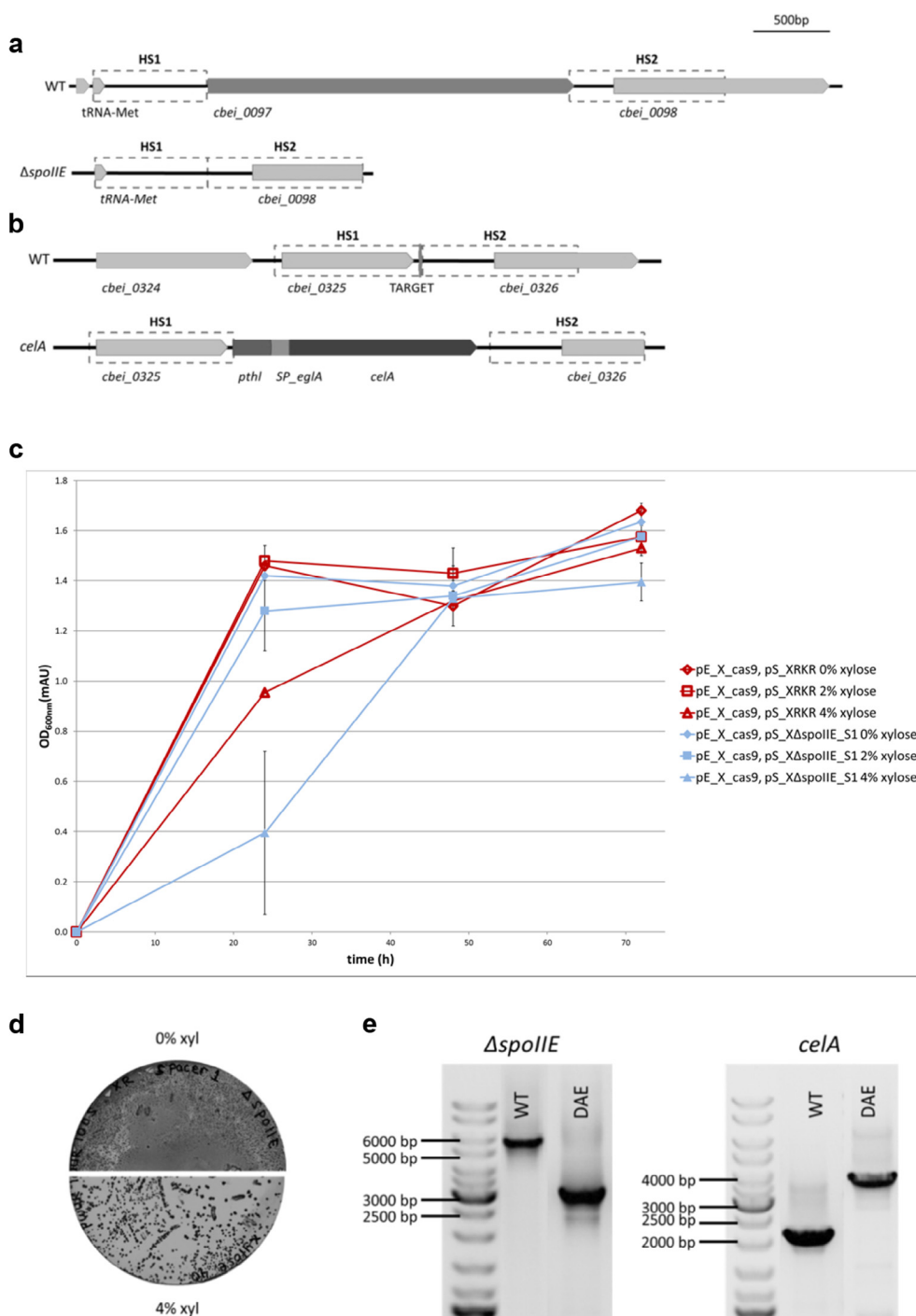


Fig. 2. Genome editing of the *C. beijerinckii* NCIMB 8052 strain: design and selection. a, b. Targeted loci and their relative DNA templates. (a) Disruption of *spoIIE*. (b) Insertion of the *celA* gene from *N. patriarum*. c, d, e. Selection and screening of edited strain. (c) Growth of *C. beijerinckii* NCIMB 8052 pE_X_cas9 transformed with the empty gRNA plasmid pS_XRKR or the *spoIIE* disruption plasmid pS_X $\Delta spoIIE$ _S1. The transformants were grown in duplicates in mCGM supplemented with erythromycin and spectinomycin and with 0–4% of xylose. The error bars represent the error of the replicates (d) Selection of *spoIIE* on mCGM agar plate containing 0 or 4% xylose (w/v) inoculated with overnight pre-induced cultures. (e) Growing xylose-resistant colonies were subsequently screened by colony PCR. The screening for *spoIIE* gene disruption was performed using primers cbeI_0096_F/cbeI_0098_R. Expected sizes: 5462 bp (WT) and 3083 bp (DAE). The screening of the insertion of the *celA* gene was performed using primers cbeI_0325_F/cbeI_0326_R. Expected sizes: 2083 bp (WT) and 3607 bp (DAE). HS1 and HS2: homologous sequence 1 and 2; *SP_eglA*: signal peptide *eglA*; *pthI*: promoter of *C. beijerinckii* thiolase gene (cbeI_3630); WT: wild type; DAE: double allelic exchange (edited cells).

efficiencies (from 20 to 2000 fold depending on the plasmid, Fig. 4f).

In summary, we successfully adapted the two-plasmid CRISPR-Cas9 editing tool to *C. beijerinckii* DSM 6423 and created successively two platform strains. The first modification made the strain sensitive to thiamphenicol, which allowed us to use two different resistance markers. This mutant strain was further engineered by removing pNF2 which allowed us to obtain a second strain, with higher transformation efficiencies.

4. Discussion

In this study we describe the successful adaptation of a CRISPR/Cas9 genetic tool in the solvent-producing *C. beijerinckii*. The two-

plasmid system, in which the *cas9* gene was cloned on a first plasmid - the nuclease plasmid - while the gRNA and the DNA template were cloned on a second plasmid - the editing plasmid, was successfully used to edit the genome of two reference strains of *C. beijerinckii*. Such a method benefits its user on several points. Firstly, the cloning procedure is facilitated because the toxic genes of the CRISPR-Cas9 machinery are localized on separate genetic elements. Therefore, it is easier to construct plasmids for a given genetic modification, which will also be significantly smaller, likely resulting in higher transformation efficiencies in *Clostridia*.

Secondly, our methodology helps reducing the time spent on the traditionally laborious genetic engineering steps. The strain bearing the nuclease plasmid can indeed be stored at -80°C and used multiple

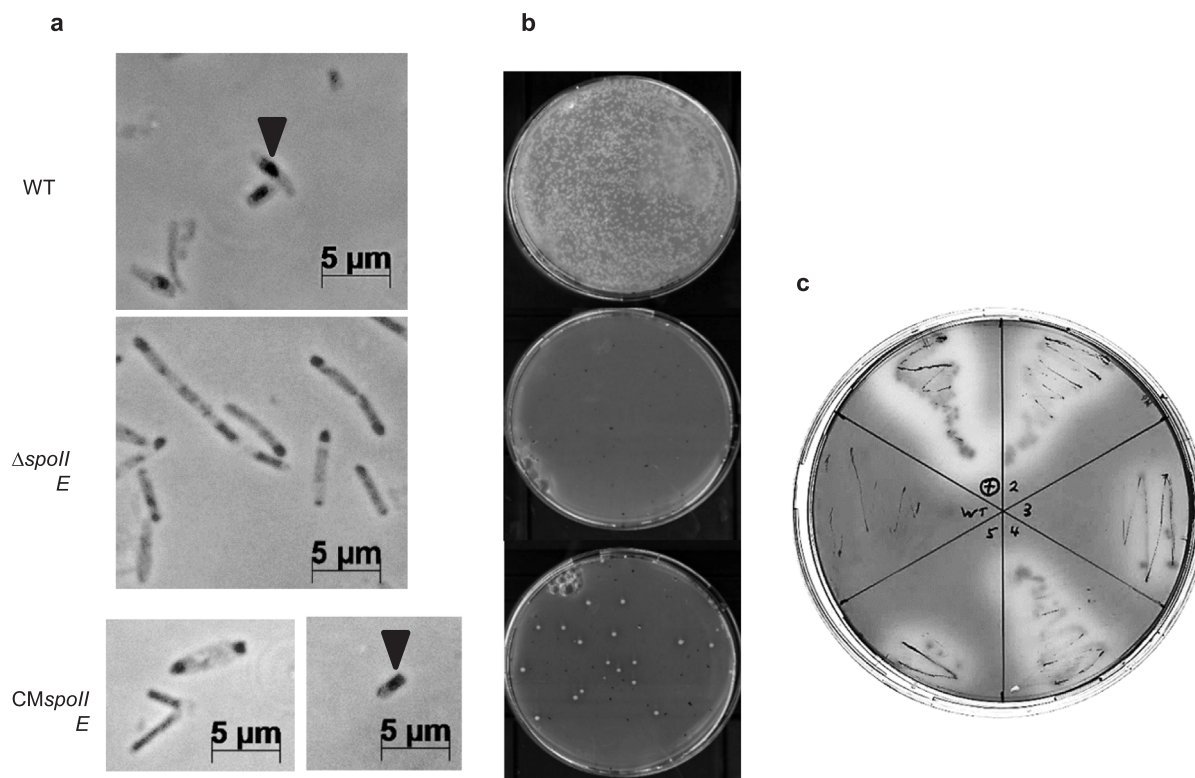


Fig. 3. Novel phenotypes in genetically modified *C. beijerinckii* NCIMB 8052 mutants. a. Light microscopy pictures at 73 h of fermentation of *C. beijerinckii* NCIMB 8052 wt, $\Delta spoIIIE$, $CMspoIIIE$ (complemented mutant) strains. Spores are marked by a triangle. b. Growth of *C. beijerinckii* NCIMB 8052 wt, $\Delta spoIIIE$, $CMspoIIIE$ on CM2 agar plates after heat shocking 200 μ L of a 48-hour-old culture. c. Detection of the cellulase activity of *C. beijerinckii* *celA* mutants. WT: *C. beijerinckii* wild type, 2–5: clone number (*CelA* mutants after plasmid curing), +: positive control (non-integrated self-replicating *CelA* plasmid) the halo surrounding the cells are caused by the degradation of carboxymethylcellulose.

times, for several genetic constructions. Similarly, the editing plasmid can be specifically cured after a genome editing experiment, permitting multiple successive rounds of genome editing. This editing plasmid was besides designed to fasten the cloning process by incorporating genetic elements allowing a quick and easy reprogramming of the gRNA. In our hands, CRISPR-Cas9-based counter-selection also proved to be extremely efficient with most clones – if not all – edited after the induction step, which also contributes to shorten the mutant screening process.

Lastly, the system displays a good genetic cargo capacity (up to 3.5 kb insertions with *C. acetobutylicum* [30]), which makes it suited for large genome modifications required for metabolic engineering. The additional space provided by the second plasmid also gives more freedom for the design of DNA templates and notably enables the use of longer homologous sequence to favor allelic exchanges [54].

In the CRISPR system described in this study, an inducible Cas9 nuclease [24,31] was chosen over the Cas9 nickase previously used by other groups [22,23]. In those studies, edited cells were obtained at lower frequencies than in studies when the Cas9 nuclease was used. This can be explained by a lower toxicity of a single nick in the genome, rather than a DSB. Since the Cas9 nickase is less toxic, and therefore less selective, its use increases the probability of having a mixed population, especially when the desired mutation negatively impacts the fitness of the bacterium, or when the editing events are rare (e.g. insertion of large DNA fragments). As an example, the disruption of *adc* in *C. acetobutylicum* or *C. beijerinckii* using the nickase led to the isolation of 1 and 3 correctly edited colonies out of 16 colonies tested, respectively, whereas other mutations (*xyIR*, *cbei_3923*) were obtained in almost all of the tested colonies [23].

In order to use Cas9, the inducible promoter *PxyIB* from *C. difficile* and the *xyIR* gene, whose product codes for the associated regulatory

protein [32], were used to control the expression of both *cas9* and the gRNA. The lethal effect of the DSB resulting from their expression following xylose induction was then used as a strong counter selection marker. In this study, this approach allowed the isolation of correctly edited cells at an efficiency close to 100%. Moreover, unlike what is reported in studies using the Cas9 nickase, serial transfers of recombinant strains were not required to isolate clones that undergone large fragment insertions in their genome. All mutants generated in this study were therefore isolated within one week starting from the strain *C. beijerinckii* NCIMB 8052 (*pE_X_cas9*). Although the system is mediated by two plasmids, their removal required only a few serial transfers making the system as efficient as those previously described. The easy clearance of the plasmids carrying the gRNA and DNA template and the absence of selection marker in the final strain thus enable multiple rounds of genome editing.

In this study, we deleted a 2.379 kb DNA fragment within the *spoIIIE* gene (*cbei_0097*), and inserted the 1.68 kb *N. patriciarum celA* gene. The disruption of the *spoIIIE* gene, described here for the first time in *C. beijerinckii*, resulted in asporogenous mutant strains. These results are in accordance with studies in earlier *Bacillus subtilis* [55] and other solventogenic *Clostridia* [46,56], which showed that a reduced *spoIIIE* expression coincided with hampered sporulation and that *spoIIIE* disruption prevents sporulation.

The *spoIIIE* mutants generated in this study kept the ability to produce solvents at similar levels to the wild-type strain (Supplementary file S4). These observations indicate that this gene is necessary for sporulation but does not play a role in solvent production, contrary to *spoOA* for example. This characteristic has also been observed in *spoIIIE* negative mutants generated in *C. acetobutylicum* [46,47]. In contrast to the *spoOA* homologues in *Clostridia*, the *spoIIIE* gene is not well conserved between clostridial species. Indeed, while *C. acetobutylicum*

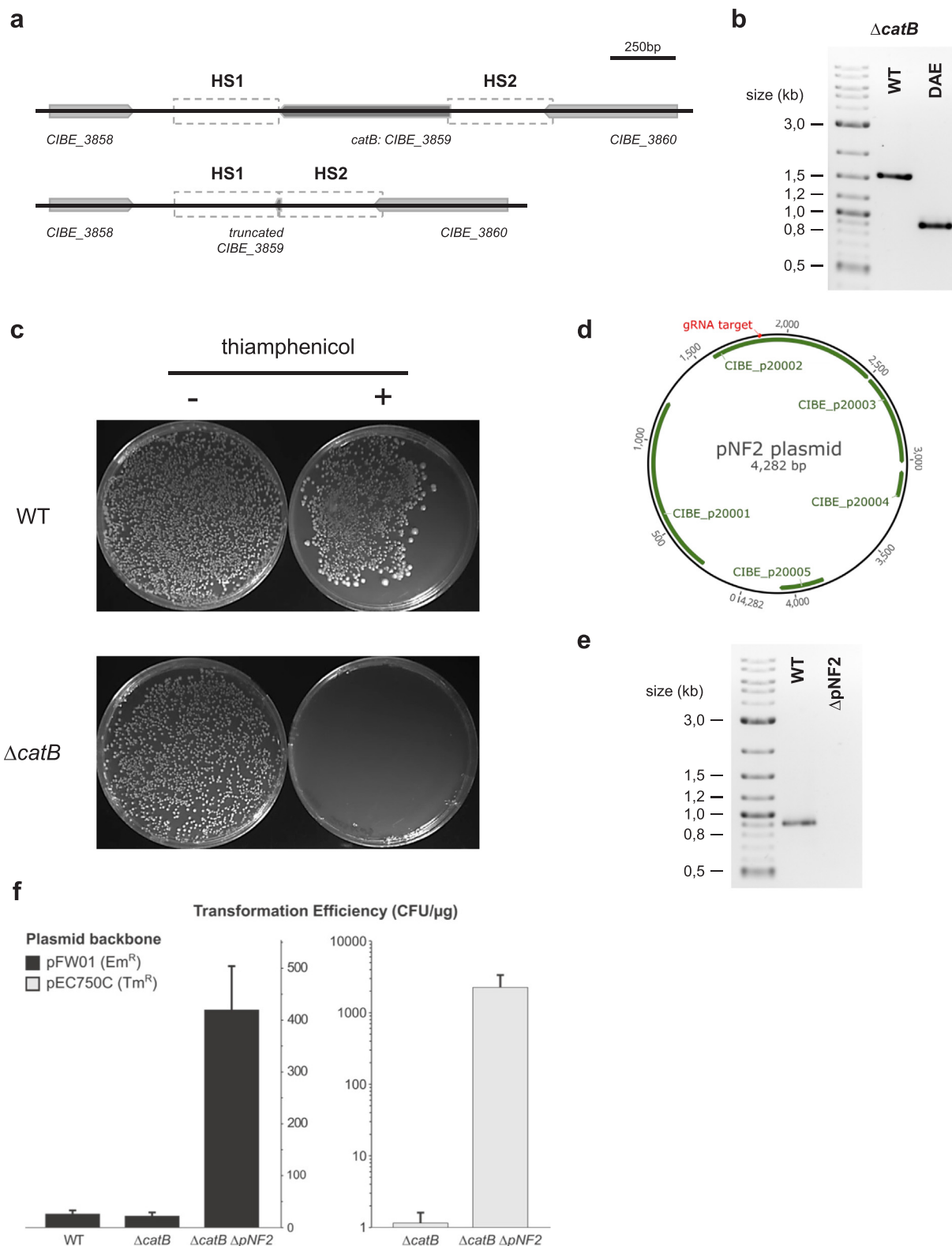


Fig. 4. Adaptation of the two-plasmid CRISPR-Cas9 system to *C. beijerinckii* DSM 6423. **a.** *catB* (CIBE_3859) genome deletion design by a single-plasmid strategy. **b.** Verification of *catB* deletion by PCR with primers RH76 and RH77, which amplify a genome region encompassing the homology sequences (expected sizes: 1553 (WT) and 900 (*ΔcatB*) bp). **c.** Thiamphenicol resistance assay on the resulting *ΔcatB* strain. **d.** Map of the natural pNF2 plasmid from *C. beijerinckii* DSM 6423. The locus targeted by the pGRNA-pNF2_S1 vector (containing the thiamphenicol resistance gene *catP*) is shown in red. **e.** Verification of the deletion of the pNF2 plasmid with a dual-plasmid CRISPR-Cas9 strategy. Primers pNF2_fwd and pNF2_rev were used for PCR (expected size: 907 bp (WT); no amplification is expected from a *ΔcatBΔpNF2* strain). **f.** Transformation efficiencies in the wild-type, the *ΔcatB* and the *ΔcatB ΔpNF2* strain. Errors bars represent the s.e.m. of duplicate experiments. Em^R: erythromycin resistance gene Tm^R: thiamphenicol resistance gene. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

spoIIE gene has only 36% identity with *C. beijerinckii spoIIE*, *spoOA* homologues have as much as 74% sequence identity. We observed differences at the phenotypic level between *C. acetobutylicum* and *C. beijerinckii spoIIE* mutants. Indeed *spoIIE* mutants in *C. beijerinckii* have two dark spots at their poles (Fig. 3), corresponding to the forespore condensed DNA, that are absent in *C. acetobutylicum spoIIE* mutants but were observed in *C. acetobutylicum sigE* mutants [57]. Moreover *C. beijerinckii spoIIE* mutant cells display an asymmetric septum and sometimes two septa at each pole of the cell, which is similar to the morphology of *B. subtilis spoIIE* mutants [44,58]. We can thus hypothesize that even though *SpoIIE* is necessary for the correct completion of the stage II of the sporulation cycle in *C. beijerinckii* and *C. acetobutylicum*, it is not needed for asymmetric septum formation in *C. beijerinckii* in contrary to what is observed in *C. acetobutylicum*.

The insertion of the “*thl_p celA*” transcriptional unit yielded mutant strains which showed cellulase activity on plate assays, not observed with the WT strain. The CelA enzyme from the anaerobic fungus *N. patriciarum* is a cellulase that contains an active domain belonging to the Family 6 of glycosyl hydrolases. This enzyme has been expressed before in clostridial strains using a plasmid vector, either alone or as part of a mini-cellulosome [48,59]. This study shows for the first time the insertion of a cellulase gene into the chromosome of a solventogenic strain and its functional expression. This opens novel possibilities for the direct fermentation of sugar polymers like cellulose into ABE or IBE mixtures.

The success of our genome editing strategy prompted us to apply it also to another strain. The atypical *C. beijerinckii* DSM 6423 is genetically very close to the NCIMB 8052 model strain [60], but its modification by targeted genome editing techniques has however never been reported, in spite of the microorganism value as being one of the few natural IBE-producing strains. Taking into account this strain characteristics, we successfully adapted our dual-plasmid inducible CRISPR-Cas9 approach. Indeed, some adjustments were found necessary to (1) circumvent the restriction-modification system of the strain (using Dam⁻ Dcm⁻ DNA, similarly to the NRRL B-598 strain [33]) and (2) find an alternative to the spectinomycin resistance gene, which proved to be malfunctioning in our hands. This bottleneck was specifically tackled by using a single-plasmid inducible CRISPR-Cas9 strategy to disrupt a putative amphenicol resistance gene (CIBE_3859, encoding the *catB* gene). The resulting strain was demonstrated to be sensitive to thiamphenicol, and the *catP* resistance marker (from *C. perfringens* [52], used extensively in *C. acetobutylicum* [51]) could subsequently be used in this mutant. This mutant was further exploited to delete the pNF2 natural plasmid [53] with a dual-plasmid inducible CRISPR-Cas9 system, underlining the compatibility of the $\Delta catB$ strain with such a system. The curation of the pNF2 plasmid was preferred to the modification of chromosomal genes because it required the introduction of a small editing plasmid without editing template. Indeed the poor transformation efficiency of the DSM 6423 strain severely impedes our efforts. For the disruption of the *catB* gene for example, several transformation experiments were required to introduce the single-plasmid inducible CRISPR-Cas9 and only one was successful, yielding 1 transformant when using 20 μ g DNA. This problem underlines the need for better transformation efficiencies in *C. beijerinckii* DSM 6423 for future genome editing experiments.

CRISPR-Cas9-based removal of non-essential genetic elements has already been pioneered in solventogenic *Clostridia*, with notable effects on the primary/secondary metabolism and/or on exogenous plasmid transformation efficiencies [30,61]. If the product pattern and quantities of the resulting $\Delta catB$ $\Delta pNF2$ strain were not strongly affected, the mutant interestingly showed much higher transformation efficiencies, most likely because the endogenous pNF2 plasmid interferes with the replication of non-natural plasmids introduced into the cell. As mentioned above, this improvement is critical as transformation efficiencies are normally extremely low in the wild-type genetic background (c.a. 4 CFU/ μ g with an empty plasmid – Supplementary file S7), which will

likely considerably help constructing future strains. The rise of the CRISPR/Cas9 technology in the field of *Clostridium* genetics has been a real breakthrough. The relatively simple generation of scarless mutants in a short period of time will hopefully facilitate the research into the physiology of solvent production and lead to a better understanding of the regulatory mechanisms involved in gene expression. The dual-plasmid system described in this study should overcome issues reported previously (higher transformation efficiency, flexibility for the design of DNA templates, convenience of the cloning procedure, rapid mutant selection, cargo capacity). We showed here that this advantageous system, initially developed for *C. acetobutylicum*, could be simply adapted to work as efficiently in a model microorganism from another species. We also demonstrated that this approach could be expanded to atypical microorganisms, recalcitrant to genetic manipulations, by following simple steps (i.e. finding the correct DNA methylation pattern, removing antibiotic resistance genes, removing mobile genetic elements), which suggests that similar strategies are likely to function in other prokaryotes.

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Declaration of Competing Interest

The authors declare no financial or commercial conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jymeth.2019.07.022>.

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