An efficient method for markerless mutant generation by allelic exchange in *Clostridium acetobutylicum* and *Clostridium saccharobutylicum* using suicide vectors

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**Abstract**

**Background:** *Clostridium acetobutylicum* and *Clostridium saccharobutylicum* are Gram-positive, spore-forming, anaerobic bacterium capable of converting various sugars and polysaccharides into solvents (acetone, butanol, and ethanol). The sequencing of their genomes has prompted new approaches to genetic analysis, functional genomics, and metabolic engineering to develop industrial strains for the production of biofuels and bulk chemicals.

**Results:** The method used in this paper to knock-out, knock-in, or edit genes in *C. acetobutylicum* and *C. saccharobutylicum* combines an improved electroporation method with the use of (i) restrictionless Δ*upp* (which encodes uracil phosphoribosyl-transferase) strains and (ii) very small suicide vectors containing a markerless deletion/insertion cassette, an antibiotic resistance gene (for the selection of the first crossing-over) and *upp* (from *C. acetobutylicum*) for subsequent use as a counterselectable marker with the aid of 5-fluorouracil (5-FU) to promote the second crossing-over. This method was successfully used to both delete genes and edit genes in both *C. acetobutylicum* and *C. saccharobutylicum*. Among the edited genes, a mutation in the *spo0A* gene that abolished solvent formation in *C. acetobutylicum* was introduced in *C. saccharobutylicum* and shown to produce the same effect.

**Conclusions:** The method described in this study will be useful for functional genomic studies and for the development of industrial strains for the production of biofuels and bulk chemicals.

**Keywords:** *Clostridium acetobutylicum*, *Clostridium saccharobutylicum*, *upp* gene, 5-FU, Restrictionless, Markerless, Gene deletion, Gene replacement

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due to the low frequency of transformation of solventogenic Clostridia [13, 14]. Two families of methods have been developed to allow deletion and/or the introduction of genes at their normal chromosomal context without maintaining an antibiotic marker.

The first family [7, 10] uses a replicative vector containing (i) a replacement cassette consisting of an antibiotic resistance gene (Tetr) flanked by two FRT sequences, (ii) two sequences homologous to the selected regions around the target DNA sequence, and (iii) a counterselectable marker made either of the codon-optimized mazF toxin gene from Escherichia coli (under the control of a lactose-inducible promoter) or the upp gene [which encodes an uracil phosphoribosyl-transferase and leads to 5-fluorouracil (5-FU) toxicity] to allow the direct positive selection of double-crossover allelic exchange mutants. After this first step, a second plasmid system expressing the FLP recombinase must be introduced, enabling efficient deployment of the FLP–FRT system to generate markerless deletion or integration mutants. A scar consisting of an FRT site remains at the target site, which can potentially act as a transcriptional terminator [15] or create a large chromosomal DNA deletion or inversion when several FRT sites are present on the chromosome [16, 17].

The second family [9, 18] also uses a replicative vector containing (i) a replacement cassette consisting of two sequences homologous to the selected regions around the target DNA sequence and (ii) a counterselectable marker made either of the codA [18] gene or the pyrE [9] gene. However, as the replacement cassette does not include an antibiotic resistance gene, and as this method uses a replicative plasmid, its stable single integration in the chromosome will be a rare event that cannot be selected for. When the counterselection is then applied, most of the clones will lose the plasmid and have a wild-type phenotype.

Creating a method for the rapid deletion, insertion, or modification of genes would require the use of a small suicide vector (to improve the transformation efficiency by electroporation), a replacement cassette consisting of two sequences homologous to the selected regions around the target DNA sequence and a counter selection marker such as upp, codA, or pyrE. One way to increase the transformation efficiency of solventogenic Clostridia is to remove the restriction modification system naturally present in the bacterium [5, 10, 14, 19]. Restrictionless, markerless mutants of solventogenic Clostridia have already been constructed for two species, C. acetobutylicum [10] and C. saccharobutylicum [5]. Although a transformation efficiency of $10^4$/μg DNA has previously been reported when using electroporation for a restrictionless mutant of C. acetobutylicum [10], the transformation efficiency of a restrictionless mutant of C. saccharobutylicum has not been measured [5].

In the present study, we further improved the transformation efficiency of the two restrictionless mutants by weakening the cell wall using a lysozyme treatment before electroporation. We then constructed small suicide vectors containing the catP or the mls8 genes for the selection of chromosomal integration and the upp gene to select, in combination with the 5-fluorouracil (5-FU) system, for the second crossing-over. These plasmids, the restrictionless strains with a upp deletion and the improved transformation protocol were successfully used to develop a method for gene knock-in, knock-out, and editing in C. acetobutylicum and C. saccharobutylicum.

**Results and discussion**

**Transformation efficiency of different industrially relevant solventogenic Clostridia**

In a previous study [10], we demonstrated that a restrictionless mutant of C. acetobutylicum could be transformed by electroporation with unmethylated pCons2.1 at very high efficiency ($6 \times 10^4$ transformants/μg of unmethylated DNA). However, when we evaluated the transformation efficiency of most of the non-sporulating, metabolically engineered strains, we noticed that the transformation efficiency of unmethylated pCons2.1 drastically decreased to values as low as 85 transformants/μg of unmethylated DNA. To improve the transformation efficiency of these industrially important, non-sporulating strains, we used as a prototype a C. acetobutylicum Δacac1502 Δacac3535ΔuppΔpSOL mutant that no longer sporulated or produced solvent. This mutant was obtained by spreading the C. acetobutylicum Δacac1502 Δacac3535Δupp strain on an RCA plate and selecting clones that no longer produced a halo of starch hydrolysis after iodine staining [20]. The loss of pSOL1 was demonstrated by PCR analysis. The initial transformation efficiency of this strain with unmethylated pCons2.1 was low at approximately $142 \pm 47$ transformants/μg of unmethylated DNA (Table 1). Changing the voltage or the time constant did not significantly improve the transformation efficiency (data not shown). It was then decided to evaluate the use of cell wall weakening agents to facilitate DNA entry during the electroporation step. Such treatments, such as the use of lysozyme, have been shown previously [21–23] to improve the transformation efficiency of other Gram-positive bacteria. Lysozyme treatment, at concentrations ranging from 15 to 1500 μg/ml, was initially applied in the electroporation buffer for 30 min at 4 °C before electroporation. Although the transformation efficiency with unmethylated pCons2.1 was improved to values as high as $1 \times 10^4$ transformants/μg of unmethylated DNA, the
results were not reproducible. It was then decided to add the lysozyme treatment directly to the culture medium, before centrifugation and washing, according to the protocol described in “Methods”. Very reproducible results were then obtained with an optimal lysozyme concentration of 150 μg/ml resulting in a transformation efficiency of $6.5 \times 10^3$ transformants/μg of unmethylated DNA, a value in the same range of the transformation efficiency of the sporulating C. acetobutylicum Δcac1502 Δcac3535 Δupp ΔpSOL with unmethylated pCons2.1 plasmid.

A generic method for gene knock-out, knock-in, and editing in C. acetobutylicum and C. saccharobutylicum

To create the generic method (presented in Fig. 2) for gene modification in both species, two very small shuttle suicide vectors (pCat-upp and pEry-upp) were constructed that carry either a colEI or a p15A origin of replication functional in E. coli or a msR gene for the selection of single cross-over integration of the plasmid from thiamphenicol or erythromycin-resistant clones, respectively. Both plasmids have a unique BamHI site for the insertion of the modification cassettes.

The recipient strain should be restrictionless, but should also carry a upp deletion for counterselection using 5-FU. Such a strain was already constructed for C. acetobutylicum [10]. However, the Ch2 mutant of C. saccharobutylicum still had a functional upp gene. The pCat-upp-Dupp plasmid was then constructed by inserting in pCat-upp the upp deletion cassette containing two 1-kbp regions flanking the upp gene on the chromosome of C. saccharobutylicum. When Ch2 was transformed with

| Lysozyme concentration (μg/ml) | Electroporation efficiencies |
|--------------------------------|-----------------------------|
| 0                              | 142 ± 47                    |
| 15                             | 648 ± 154                   |
| 150                            | $6.5 \times 10^3 ± 2.2 \times 10^3$ |
| 1500                           | 2.1 ± 0.2                   |

Values are expressed in number of transformants per μg of unmethylated pCons2.1

Mean values and standard deviations from two independent experiments are given

$5 \mu g$ pCons2.1 was used in each experiment.

Table 2 Transformation efficiencies of different C. saccharobutylicum mutants with unmethylated pMTL84151 plasmid

| C. saccharobutylicum strain | Electroporation efficiencies |
|----------------------------|----------------------------|
| WT                        | 0                          |
| ΔhsdR1                    | 0                          |
| Ch1                       | 58 ± 4                     |
| Ch2                       | 255 ± 117                  |

Values are expressed in number of transformants per μg of unmethylated pMTL84151

Mean values and standard deviations from two independent experiments are given

$20 \mu g$ pMTL84151 was used in each experiment.
200 μg of this plasmid using the optimized electroporation protocol presented above, no clones resistant to thiamphenicol could be obtained. As such clones would result from a RecA-dependent crossing-over between the homologous regions of the plasmid and the chromosome, and as it is well known that RecA is more efficient on single-stranded DNA, the pCat-upp-Dupp plasmid was first denatured at 95 °C for 5 min and rapidly cooled on ice before electroporation. Applying this DNA pretreatment, approximately 10 thiamphenicol colonies were obtained. PCR analysis of the different clones showed (Fig. 3b) that integration was obtained both in the upstream and downstream regions of upp. Two clones with an integration in each homologous arm were grown in 2×YTG, and appropriate dilutions were plated on MES-MM (0.01% yeast extract) with 5-FU at 1 mM. To select integrants having excised and lost pCat-upp-Dupp, 5-FU-resistant clones were replica plated on both MES-MM (0.01% yeast extract) + 5-FU at 1 mM and 2xYTG with thiamphenicol at 15 μg/ml. To identify mutants that lost pCat-upp-Dupp and possessed a markerless upp deletion, clones resistant to 5-FU and sensitive to thiamphenicol (at 25 μg/ml) were checked by PCR analysis (with primers Upp-check-F and Upp-check-R located outside of the upp deletion cassette). All the 5-FU-resistant, thiamphenicol-sensitive clones showed that upp was deleted when analyzed by
PCR (Fig. 3c). The fermentation profiles of one of the C. saccharobutylicum ΔhsdR1ΔhsdR2ΔhsdR3Δupp clones were evaluated in batch fermentation performed without pH regulation in MES-MM (0.001% yeast extract) medium. Solvent and acid formation by C. saccharobutylicum ΔhsdR1ΔhsdR2ΔhsdR3Δupp was similar to that of the wild-type strain (Table 3), indicating that no physiological modifications were introduced during the construction of the mutant.

**Gene deletion and editing in C. acetobutylicum using the generic method**

The alsD gene (CA_C2967) encodes an acetolactate decarboxylase involved in the last step of acetoin formation [24]. To delete alsD, the alsD deletion cassette was cloned into the BamHI site of the pCat-upp to generate the plasmid pCat-upp-alsD. The plasmid pCat-upp-alsD was used to transform the C. acetobutylicum Δcac1502 Δcac3535Δupp strain by electroporation...
without previous in vivo methylation, and pCat-upp-alsD integrants were selected on RCA plates with thiamphenicol at 20 µg/ml. Two colonies were cultured for 24 h in liquid SM-glucose medium and then subcultured in liquid 2xYTG medium without antibiotic. Appropriate dilutions were plated on RCA with 5-FU at 1 mM. To select integrants having excised and lost pCat-upp-alsD, 5-FU-resistant clones were replica plated on both RCA and RCA with thiamphenicol at 40 µg/ml. To identify mutants possessing a markerless alsD deletion, clones resistant to 5-FU and sensitive to thiamphenicol were checked by PCR analysis (with primers alsd-0 and alsd-5 located outside of the alsD deletion cassette and primers alsd-F and alsd-R located inside alsD). Approximately half of the clones had an alsD deletion, and half had a wild-type genotype for alsD. The C. acetobutylicum Δcac1502 Δcac3535Δupp ΔalsD strain was isolated. The fermentation profile of this strain was compared to that of the C. acetobutylicum Δcac1502 Δcac3535Δupp control strain during batch fermentation at pH 4.8 (Fig. 4). Surprisingly, the production of acetoin was only slightly decreased, indicating that either acetoacetate can be chemically decarboxylated in vivo [25] or that Adc, the acetoacetate decarboxylase involved in the last step of acetone formation (15), can also decarboxylate acetolactate.

In a project aiming to improve the isopropanol tolerance of C. acetobutylicum using an adaptive laboratory evolution (ALE) approach, three individual clones (IPT4, IPT7, and IPT10) able to grow at isopropanol concentrations higher than 40 g/l were isolated (Fig. 5). When the genomes of these three strains were sequenced, 26 mutations present in the three strains were identified. Among all the mutated genes, two retained our attention: CA_C 0437 and CA_C3368, which encode a phosphatase that catalyzes the dephosphorylation of Spo0A.

### Table 3 Solvent and acid formation by C. saccharobutylicum wild-type and mutant strains in batch culture without pH regulation

|                      | C. saccharobutylicum wild type | C. saccharobutylicum ΔhisdR1ΔhisdR2ΔhisdR3Δupp | C. saccharobutylicum ΔhisdR1ΔhisdR2ΔhisdR3Δupp, spo0A* |
|----------------------|--------------------------------|-----------------------------------------------|------------------------------------------------------|
| [Acetone]_{final} (mM) | 33                             | 30                                            | 0                                                    |
| [Butanol]_{final} (mM)  | 83                             | 76                                            | 0                                                    |
| [Ethanol]_{final} (mM)  | 11                             | 9                                             | 6                                                    |
| [Acetate]_{produced} (mM) | 11                            | 15                                            | 28                                                   |
| [Butyrate]_{final} (mM)  | 12                             | 16                                            | 47                                                   |
| Butanol yield (g g⁻¹)    | 0.17                           | 0.16                                          | 0                                                    |

Cultures were done at 37 °C in MES-MM medium supplemented with 0.001% yeast extract for 96 h.

Fig. 4 Solvent, acetoin and acid production by C. acetobutylicum Δcac1502 Δcac3535Δupp and C. acetobutylicum Δcac1502 Δcac3535Δupp ΔalsD in batch culture at pH 4.8 in SM medium. Cultures were ran for 72 h.
and a putative permease, respectively. The mutation in each gene is translated at the protein level to C1151A and G506A mutations. To evaluate the effect of these mutations on isopropanol tolerance, the genome-editing method presented above was used to introduce each of the two mutations in the genome of \textit{C. acetobutylicum} \textDelta cac1502 \textDelta cac3535 \textDelta upp. For this purpose, two editing cassettes were created by directly amplifying a two kbp region centered around the point mutations in CA_C0437 and CA_C3368 from the genome of the evolved strains and directly cloning them in pCat-upp to yield pCat-upp-CAC0437* and pCat-upp-CAC3368*.

Each plasmid was transformed by electroporation in the \textit{C. acetobutylicum} \textDelta cac1502 \textDelta cac3535 \textDelta upp strain and integrants were selected by their resistance to thiamphenicol. The generic method described in Fig. 2 was then used to select for the second crossing-over. Clones with the proper mutations were identified by a mismatch amplification mutation assay PCR (MAMA PCR) \cite{27}, and validation was finally performed by sequencing the region corresponding to the editing cassette plus 1 kbp on each side. The \textit{C. acetobutylicum} \textDelta cac1502 \textDelta cac3535\textDelta upp::cac0437* and \textit{C. acetobutylicum} \textDelta cac1502 \textDelta cac3535\textDelta upp::cac3368* were obtained and then characterized for their tolerance to isopropanol.

The tolerance of both edited strains was not significantly different from the control strain (Fig. 5), indicating that those two mutations are either not involved in isopropanol tolerance or alone are not able to significantly participate in the isopropanol tolerance of \textit{C. acetobutylicum}. Using the generic method described in this manuscript, a reverse strategy is currently under way, i.e., the editing back to wild type of each of the 26 mutations identified in one of the isopropanol tolerant strains and analysis of the isopropanol tolerance of the strains obtained.

\textbf{Use of the gene-editing method to assess the effect of the Spo0A G179S mutation on the control of sporulation and solvent formation in \textit{C. acetobutylicum} and \textit{C. saccharobutylicum}}

During the selection process of the \textit{C. acetobutylicum} \textDelta cac1502 \textDelta cac3535\textDelta upp\textDelta pSOL strain, a mutant not producing solvent but still having the pSOL1 plasmid was identified and isolated. When the genome of this mutant was sequenced, a point mutation in the spo0A gene was identified, translating to the G179S mutation at the protein level. The mutated glycine residue is in a very conserved region of the Spo0A protein in all Firmicutes \cite{28}, IIHEIGVPAHIKGY, in which the lysine residue was
shown to be involved in DNA binding to the Spo0A box [29].

This mutant was still able to sporulate, although at a lower frequency (Fig. 6), but after classical heat shock (70 °C for 10 min), no colony forming units were obtained for the G179S Spo0A mutant, while $4 \times 10^5$ CFU/ml were obtained for the control strain (Table 4). Analysis of the product profile of the mutant showed that it no longer produced solvents, and only acetic and butyric acid accumulated in the fermentation broth (Table 5).

Using the gene-editing method, the same mutation in spo0A (translating to the G172S mutation at the protein level, as this protein is 7 amino acid residues shorter in N-terminal than the corresponding C. acetobutylicum protein) was introduced in the C. saccharobutylicum ΔhsdR1 ΔhsdR2 ΔhsdR3 Δupp strain. This mutant was still able to sporulate (Fig. 6), but similar to the C. acetobutylicum G179S Spo0A mutant, it no longer produced solvent (Table 3), and the spores were thermally sensitive (Table 4). A tdcR knock-out mutant of C. difficile was previously shown to also produce heat-sensitive spores, which was associated with a lower expression of the SigE- and SigF-dependent sporulation genes [30].

**Conclusions**

The restrictionless, markerless generic method for genome modification in C. acetobutylicum and C. saccharobutylicum is a simple and useful tool for research groups involved in functional genomic studies and for further metabolic engineering of these two industrially important strains. As a demonstration of the efficiency of the method, we deleted the alsD gene in C. acetobutylicum to better understand how acetoin is produced in this microorganism. Furthermore, using this method we successfully edited genes to better characterize how C. acetobutylicum can develop isopropanol tolerance through adaptive laboratory evolution. Finally, we identified a mutation (G179S) in the Spo0A protein that abolishes solvent formation in both microorganisms while still allowing sporulation, although the spores produced were heat sensitive. Compared to the CRISPR/Cas9 method, that due to the large size of the cas9 gene imposes the use of replicative, this method allows the use of suicide vectors avoiding the step of plasmid curing that can be troublesome.

In the future, with the combined use of the pCat-upp and pEry-upp vectors developed in this study, it should be possible to simultaneously inactivate two genes in case each of the single knock-out mutants is not viable, while the double knock-out mutant is viable.

**Methods**

**Bacterial strain, plasmids, and oligonucleotides**

The bacterial strain and plasmids used in this study are listed in Table 6. The specific oligonucleotides used

**Table 4 Heat resistance of spores from different C. acetobutylicum and C. saccharobutylicum strains**

|                          | C. acetobutylicum Δcac1502 Δcac3535 Δupp | C. saccharobutylicum ΔhsdR1 ΔhsdR2 ΔhsdR3 Δupp |
|--------------------------|------------------------------------------|-----------------------------------------------|
| Control strain           | SpooA G179S                               | Control strain SpooA G172S                     |
| 96 h cultures (in MES-MM medium supplemented with 0.001% yeast extract for C. saccharobutylicum and SM medium for C. acetobutylicum) were heat treated at 70 °C for 10 min. Values are expressed in number of CFU per ml of culture |

|                          | C. acetobutylicum Δcac1502 Δcac3535 Δupp | C. acetobutylicum Δcac1502 Δcac3535 Δupp, spo0A* |
|--------------------------|------------------------------------------|--------------------------------------------------|
| [Acetone]$_{final}$ (mM) | 57                                       | 0                                                |
| [Butanol]$_{final}$ (mM) | 139                                      | 0                                                |
| [Ethanol]$_{final}$ (mM) | 41                                       | 10                                               |
| [Acetate]$_{produced}$ (mM)| −21                                      | 27                                               |
| [Butyrate]$_{final}$ (mM) | 9                                        | 68                                               |
| Butanol yield (g g$^{-1}$) | 0.21                                     | 0                                                |

Cultures were done at 37 °C in SM medium for 96 h
for PCR amplification were synthesized by Eurogentec (Table 7).

**Culture and growth conditions**

_Clostridium acetobutylicum_ and _C. saccharobutylicum_ were maintained as spores in (SM) and MES-MM (0.001% yeast extract) synthetic media, respectively, as previously described [31–33]. Spores were activated by heat treatment at 70 °C for 10 min. All _C. acetobutylicum_ and _C. saccharobutylicum_ strains were grown in anaerobic conditions at 37 °C in SM or MES-MM (0.001% yeast extract), in _Clostridium_ growth medium (CGM) [34] in 2xYTG [35], or in reinforced clostridial medium (RCM) (Fluka). Solid media were obtained by adding 1.5% agar to the liquid media. Media were supplemented, when required, with the appropriate antibiotic in the following concentrations: for _C. acetobutylicum_ and _C. saccharobutylicum_, erythromycin at 40 µg/ml and thiamphenicol between 15 and 25 µg/ml; for _E. coli_, erythromycin at 200 µg/ml, and chloramphenicol at 30 µg/ml. 5-Fluorouracil (5-FU) was purchased from Sigma, and stock solutions were prepared in DMSO.

**Selection of isopropanol tolerant _C. acetobutylicum_ mutant strains**

An isopropanol tolerant population was selected using an Adaptive Laboratory Evolution (ALE) strategy.

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**Table 6 Bacterial strains and plasmids used in this study**

| Strain or plasmid | Relevant characteristics | Source/references |
|-------------------|--------------------------|-------------------|
| **Bacterial strains** |
| E. coli TOP10 | | Invitrogen |
| **C. acetobutylicum** |
| Δcac1502Δcac3535Δupp | ΔCA_C1502ΔCA_C3535ΔCA_C2879 | [10] |
| Δcac1502Δcac3535Δupp ΔpSOL | ΔCA_C1502ΔCA_C3535ΔCA_C2879ΔpSOL1 | This study |
| Δcac1502Δcac3535Δupp ΔalsD | ΔCA_C1502ΔCA_C2879ΔCA_C3535ΔCA_C2967 | This study |
| Δacr1502Δcac3535Δupp: cac0437* | ΔCA_C1502ΔCA_C2879ΔCA_C3535: CA_C0437* | This study |
| Δacr1502Δcac3535Δupp: cac3368* | ΔCA_C1502ΔCA_C2879ΔCA_C3535: CA_C3368* | This study |
| Δacr1502Δcac3535Δupp: spo0A* | ΔCA_C1502ΔCA_C2879ΔCA_C3535: CA_C2071* | This study |
| **C. saccharobutylicum** |
| ΔhsdR1 | ΔCLSA_RS20150 | [5] |
| Ch1 (ΔhsdR1ΔhsdR2) | ΔCLSA_RS20150ΔCLSA_RS14125 | [5] |
| Ch2 (ΔhsdR1ΔhsdR2ΔhsdR3) | ΔCLSA_RS20150ΔCLSA_RS14125ΔCLSA_RS04425 | [5] |
| ΔhsdR1ΔhsdR2ΔhsdR3Δupp | ΔCLSA_RS20150ΔCLSA_RS14125ΔCLSA_RS04425ΔCLSA_RS02460 | This study |
| ΔhsdR1ΔhsdR2ΔhsdR3Δupp, spo0A* | ΔCLSA_RS20150ΔCLSA_RS14125ΔCLSA_RS04425ΔCLSA_RS026780 | This study |
| **Plasmids** |
| pAN1 | Cm′, φ3Ti, p15A origin | [14] |
| pUC18 | Ap′, colE1 origin | Fermentas |
| pCR-BluntII-TOPO | Ze′, Km′ | Invitrogen |
| pCons2-1 | Cm′, replL | [10] |
| pMTL8415 | pCD6, Cm′ | [5] |
| pCons:upp | MLS′, upp, replL | [10] |
| pCR4-TOPO-Blunt | Ap′, Km′, Cm′ | Invitrogen |
| pCat-upp | Cm′, upp, colE1 origin | This study |
| pEry-upp | MLS′, upp, p15A origin | This study |
| pCat-upp-Dupp | Cm′, upp, allelic deletion cassette for _C. saccharobutylicum_ | This study |
| pCat-upp-alsd | Cm′, upp, alsD deletion cassette for _C. acetobutylicum_ | This study |
| pCat-upp-spo0A*Csa | Cm′, upp, spo0A editing cassette for _C. saccharobutylicum_ | This study |
| pCat-upp-cac0437* | Cm′, upp, cac0437* editing cassette for _C. acetobutylicum_ | This study |
| pCat-upp-cac3368* | Cm′, upp, cac3368* editing cassette for _C. acetobutylicum_ | This study |

Cm′: chloramphenical resistance; Ap′: ampicillin resistance; MLS′: macrolide lincosamide and streptogramin B resistance; Zeo′: zeomycin resistance; replL: Gram-positive origin of replication from pLM13
using serial subcultures in SM–glucose medium with increasing concentration of isopropanol up to 5% W/V. Individual colonies were then on SM–glucose plates containing 4% W/V isopropanol. 10 clones were then cultured and tested in the following conditions: isopropanol tolerance in liquid culture and the three best ones were sent for genome resequencing.

**Analytical methods**

Cell growth was monitored by measuring optical density at 600 nm (OD600). Solvent and acid production as well as glucose consumption in cell-free supernatant samples were determined based on high-performance liquid chromatography (HPLC) [36] using H2SO4 at 0.5 mM, as mobile phase.

**DNA manipulation techniques**

Total genomic DNA from *C. acetobutylicum* and *C. saccharobutylicum* were isolated as previously described [35]. Plasmid DNA was extracted from *E. coli* with the QIAprep kit (Qiagen, France). Pfu DNA Polymerase (Roche) was used to generate PCR products for cloning, and Taq Polymerase (New England BioLabs) was used for screening colonies by PCR with standard PCR protocols employed for all reactions. DNA restriction and cloning were performed according to standard procedures [37]. Restriction enzymes and Quick T4 DNA ligase were obtained from New England BioLabs (Beverly, MA) and were used according to the manufacturer’s instructions. DNA fragments were purified from agarose gels with the QIA quick gel purification kit (Qiagen, France).

**Transformation protocol**

Transformations of *C. acetobutylicum* and *C. saccharobutylicum* were conducted by electroporation according to the following protocol. A 10% inoculum of *C. acetobutylicum* or *C. saccharobutylicum* were conducted by electroporation according to standard procedures [37]. Restriction enzymes and Quick T4 DNA ligase were obtained from New England BioLabs (Beverly, MA) and were used according to the manufacturer’s instructions. DNA fragments were purified from agarose gels with the QIA quick gel purification kit (Qiagen, France).

Table 7: Oligonucleotides used for PCR amplifications

| Primer name       | Oligonucleotide sequence                                                                 |
|-------------------|------------------------------------------------------------------------------------------|
| pcat-Upp-F        | AAAAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| pcat-Upp-R        | AAAAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| p1SA-F            | AAAAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| p1SA-R            | AAAAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| eryUpp-F          | AAAAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| eryUpp-R          | AAAAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| upp-Teradhe2-F    | GATTATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| upp-Teradhe2-R    | GATTATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-1         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-2         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-3         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-4         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-2         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-3         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-4         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-2         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-3         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-4         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-2         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-3         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-4         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-2         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-3         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-4         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-2         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-3         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-4         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-2         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-3         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-4         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-2         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-3         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-4         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-2         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-3         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |
| Upp-Csa-4         | ATATAGGATCCGAGCGAAGCCGACGAAAGAGGCACGAGAAGGCCGCCAGCAACGGGC |

Restriction sites used for the cassettes construction are underlined.
the Gene Pulser (Bio-Rad Laboratories, Richmond, CA). The cells were immediately transferred to 10 ml of pre-warmed 2×YTG and incubated overnight at 30 °C prior to plating on 2×YTG with 20 μg/ml and 15 μg/ml thiamphenicol for C. acetobutylicum and C. saccharobutylicum, respectively.

For the poorly transformable strains, i.e., non-sporulating C. acetobutylicum and C. saccharobutylicum, a lysozyme (from egg chicken white, 7000 U/mg, Sigma-Aldrich) treatment (final concentration ranging from 15 to 1500 μg/ml) for 5 to 30 min was introduced immediately after cooling on ice the culture. This lysozyme pretreatment was optimized for both C. acetobutylicum Δupp Δcac1502 Δcac3535ΔuppΔpSOL (a restrictionless non-sporulating strain) and C. saccharobutylicum Ch2 (a restrictionless sporulating strain).

**Construction of pCat- upp**

This plasmid contains a colE1 origin of replication functional in E. coli, a catP gene conferring resistance to thiamphenicol and chloramphenicol, the upp gene (encoding the uracil phosphoribosyl-transferase of C. acetobutylicum) and a unique BamHI site for the cloning of the replacement cassette. This plasmid was constructed by PCR (Phusion) amplification of a 2845 bp fragment on the pCons::UPP plasmid DNA using oligonucleotides pCat-Upp-F and pCat-Upp-R. This fragment was digested by BamHI and ligated. The pCat- upp plasmid (2829 bp) was obtained.

**Construction of pEry- upp**

This plasmid contains a p15A origin of replication functional in E. coli, an mlsR gene conferring resistance to erythromycin, a upp gene and a unique BamHI site for the cloning of the replacement cassette. This plasmid was constructed in five steps.

1. PCR (Phusion) amplification of the p15A replication origin (P15A fragment) on the plasmid pAN1, with the primers p15A-F and p15A-R.
2. PCR (Phusion) amplification of the MLSR (EryR) cassette (EryUpp fragment) on the pSOS95-Upp plasmid with the primers eryUpp-F and eryUpp-R.
3. PCR (Phusion) amplification of the adhE2 terminator (Teradhe2 fragment) on Clostridium acetobutylicum genomic DNA with the primers upp-Teradhe2-F and teradhe2-R.
4. PCR fusion (Phusion) of the “EryUpp” and “Term-B” fragments using the primers eryUpp-F and teradhe2-R to get the “EryUpp- Teradhe2” fragment.
5. Digestion by BamHI and SalI of the “P15A” with “EryUpp- Teradhe2” fragments and ligation to get the pEry-Upp plasmid (2582 bp).

**Construction of pCat- upp-Dupp**

Two DNA fragments surrounding the upp-encoding gene (CLSA_RS02460) were PCR amplified with the Phusion DNA polymerase with total DNA from C. saccharobutylicum as template and two specific couples of oligonucleotides as primers. With the couples of primers Upp-Csa-1–Upp-Csa-2 and Upp-Csa-3–Upp-Csa-4, 1045 bp and 1047 bp DNA fragments were, respectively, obtained. Both primers Upp-Csa-1 and Upp-Csa-4 introduce a BamHI site, while primers Upp-Csa-2 and Upp-Csa-3 have complementary 5’ extended sequences. DNA fragments Upp-Csa-1–Upp-Csa-2 and Upp-Csa-3–cac-4 were joined in a PCR fusion experiment with primers Upp-Csa-1 and Upp-Csa-4 and the resulting fragment was cloned in the pCR4-TOPO-Blunt vector to yield pTOPO:upp. The upp replacement cassette obtained after BamHI digestion of the resulting plasmid was cloned, at the BamHI site, into pCat- upp to yield the pCat- upp-Dupp plasmid.

**Construction of pCat- upp-alsd**

Two DNA fragments surrounding the alsD encoding gene (CAC2967) were PCR amplified with the Phusion DNA polymerase with total DNA from C. acetobutylicum as template and two specific couples of oligonucleotides as primers. With the couples of primers Alsd-Cac-1– Alsd- Cac-2 and Alsd-Cac-3–Alsd-Cac-4, 1010 bp and 1011 bp DNA fragments were, respectively, obtained. Both primers Alsd-Cac-1 and Alsd-Cac-4 introduce a BglI site, while primers Alsd-Cac-2 and Alsd-Cac-3 have complementary 5’ extended sequences that introduced an in frame deletion of alsD. DNA fragments Alsd-Cac-1–Alsd- Cac-2 and Alsd-Cac-3–cac-4 were PCR amplified with the Phusion DNA polymerase with total DNA from Cac′-2967 as template and two specific couples of oligonucleotides as primers. With the couples of primers Alsd-Cac-1– Alsd- Cac-2 and Alsd-Cac-3–cac-4, 1010 bp and 1011 bp DNA fragments were, respectively, obtained. Both primers Alsd-Cac-1 and Alsd-Cac-4 introduce a BglI site, while primers Alsd-Cac-2 and Alsd-Cac-3 have complementary 5’ extended sequences that introduced an in frame deletion of alsD. DNA fragments Alsd-Cac-1–Alsd- Cac-2 and Alsd-Cac-3–cac-4 were joined in a PCR fusion experiment with primers cac-1 and cac-4 and the resulting fragment was cloned in the pCR4-TOPO-Blunt vector to yield pTOPO:alsD. The alsD replacement cassette obtained after BglI digestion of the resulting plasmid was cloned, at the BamHI site, into pCat- upp to yield the pCat- upp-alsd plasmid.

**Construction of pCat- upp-spo0A* Csa**

Two DNA fragments surrounding the point mutation introduced in the spo0A-encoding gene (CLSARS02460) were PCR amplified with the Phusion DNA polymerase with total DNA from C. saccharobutylicum as template and two specific couples of oligonucleotides as primers. With the couples of primers spo0A*-Csa-1–spo0A*- Csa-2 and spo0A*-Csa-3–spo0A*-Csa-4, 797 bp, and 1204 bp DNA fragments were, respectively, obtained. Both primers spo0A*-Csa-1 and spo0A*-Csa-4 introduce a BamHI site, while primers spo0A*-Csa-2 and spo0A*- Csa-3 have complementary 5’ extended sequences which
introduce the point mutation. DNA fragments spo0A*-Csa-1–spo0A*-Csa-2 and spo0A*-spo0A*-3–spo0A*-4 were joined in a PCR fusion experiment with primers spo0A*-1 and spo0A*-4 and the resulting fragment was cloned in the pCR4-TOPO-Blunt vector to yield pTOPO: spo0A*-Csa. The spo0A replacement cassette obtained after BamHI digestion of the resulting plasmid was cloned, at the BamHI site into pCat-upp to yield the pCat-upp-spo0A*-Csa plasmid.

Construction of pCat-upp-cac0437* and pCat-upp-cac3368*
Cassettes containing the desired mutations surrounded by 1 kb upstream and downstream were PCR amplified with the Phusion DNA polymerase using total DNA from an isolated evolved isopropanol tolerant C. acetobutylicum strain (IPT4) as template and a specific couple of oligonucleotides as primers. For the CAC0437 PCR, the primers CAC0437_Bam_F and CAC0437_Bam_R were used to introduce a BamHI site, whereas for the CAC3368 PCR, the primers CAC3368_BglII_F and CAC3368_BglII_R were used to introduce a BglII site. The resulting fragments were cloned into the pCR4-TOPO-Blunt vector to generate pTOPO::CAC0437_C1151A and pTOPO::CAC3368_G506A, respectively. The CAC0437 C1151A fragment obtained after BamHI digestion and the CAC3368 G506A fragment obtained after BglII digestion were cloned at the BamHI site into pCat-upp to generate the pCat-upp-CAC0437* and the pCat-upp-CAC3368* plasmids, respectively.

Mismatch amplification mutation assay (MAMA PCR)
Primers for MAMA PCR were designed as described in publication [27] from Cha et al. Briefly, in each PCR, a forward MAMA primer and a reverse primer were used in a PCR reaction to detect the desired mutation. The PCR fragment was only generated from the wild-type gene and not from the gene with the mutation at the location covered by the mismatch position on the MAMA primer. For the CAC0437 C1151A mutation detection, the CAC0437_MAMA WT_F and CAC0437_ext_R primers were used. For the CAC3368 G506A mutation detection, the CAC3368_MAMA WT_F and the CAC3368_MAMA_R were used.

Mutants’ characterization
For each mutant strain, two clones of were systematically selected and their deletion cassettes sequenced after integration into the chromosome by double crossing-over.

Abbreviations
- S-FU: 5-fluourouracil; CCM: Clostridium growth medium; DMSO: dimethyl sulfoxide; FLP: flippase; FRT: flippase recognition target; MLS: the macrolide lincosamide streptogramin B resistance gene; PCR: polymerase chain reaction; EPB: electroporation buffer; RBS: ribosome binding site; RCM: reinforced clostridial medium; SM: synthetic medium; MES-MM: synthetic medium supplemented with MES; Th:thiamphenicol resistance gene; UPRTase: uracil phosphoribosyl-transferase.

Authors' contributions
AE, WL, IMS, and PS conceived the study. AT performed the initial construction of the pCAT-UPPER vector, and AT and NPTN optimized the method for efficiently transforming C. acetobutylicum. CNH optimized the method for efficiently transforming C. saccharobutylicum. MSY constructed the C. acetobutylicum J1050 strain and perform the cultures in fermentors. CF performed all the other deletions and gene editing of C. acetobutylicum. TWi performed the shake flask experiments. CNH performed all the deletions and gene editing of C. saccharobutylicum. PS drafted the manuscript and supervised the work. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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