Construction of a restriction-less, marker-less mutant useful for functional genomic and metabolic engineering of the biofuel producer Clostridium acetobutylicum

Christian Croux, Ngoc Phuong Thao Nguyen, Jieun Lee, Céline Raynaud, Florence Saint-Prix, Maria Gonzalez-Pajuelo, Isabelle Meynial Salles, Philippe Soucaille

To cite this version:

Christian Croux, Ngoc Phuong Thao Nguyen, Jieun Lee, Céline Raynaud, Florence Saint-Prix, et al.. Construction of a restriction-less, marker-less mutant useful for functional genomic and metabolic engineering of the biofuel producer Clostridium acetobutylicum. Biotechnology for Biofuels, BioMed Central, 2016, 9 (1), 10.1186/s13068-016-0432-2 . hal-01602952

HAL Id: hal-01602952
https://hal.archives-ouvertes.fr/hal-01602952

Submitted on 28 May 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Construction of a restriction-less, marker-less mutant useful for functional genomic and metabolic engineering of the biofuel producer Clostridium acetobutylicum

Christian Croux1†, Ngoc-Phuong-Thao Nguyen1†, Jieun Lee2, Céline Raynaud3, Florence Saint-Prix1, Maria Gonzalez-Pajuelo1, Isabelle Meynial-Salles1 and Philippe Soucaille1,3*

Abstract

Background: Clostridium acetobutylicum is a gram-positive, spore-forming, anaerobic bacterium capable of converting various sugars and polysaccharides into solvents (acetone, butanol, and ethanol). The sequencing of its genome 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 or knock-in genes in C. acetobutylicum combines the use of an antibiotic-resistance gene for the deletion or replacement of the target gene, the subsequent elimination of the antibiotic-resistance gene with the flippase recombinase system from Saccharomyces cerevisiae, and a C. acetobutylicum strain that lacks upp, which encodes uracil phosphoribosyl-transferase, for subsequent use as a counter-selectable marker. A replicative vector containing (1) a pIMP13 origin of replication from Bacillus subtilis that is functional in Clostridia, (2) a replacement cassette consisting of an antibiotic resistance gene (MLS) flanked by two FRT sequences, and (3) two sequences homologous to selected regions around target DNA sequence was first constructed. This vector was successfully used to consecutively delete the Cac824I restriction endonuclease encoding gene (CA_C1502) and the upp gene (CA_C2879) in the C. acetobutylicum ATCC824 chromosome. The resulting C. acetobutylicum Δcac1502Δupp strain is marker-less, readily transformable without any previous plasmid methylation and can serve as the host for the “marker-less” genetic exchange system. The third gene, CA_C3535, shown in this study to encode for a type II restriction enzyme (Cac824II) that recognizes the CTGAAG sequence, was deleted using an upp/5-FU counter-selection strategy to improve the efficiency of the method. The restriction-less marker-less strain and the method was successfully used to delete two genes (ctfAB) on the pSOL1 megaplasmid and one gene (ldhA) on the chromosome to get strains no longer producing acetone or l-lactate.

Conclusions: The restriction-less, marker-less strain described in this study, as well as the marker-less genetic exchange coupled with positive selection, 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, upp, Cac824I, 5-FU, Gene deletion, Gene replacement, FRT, FLP, upp gene

© 2016 Croux et al. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

*Correspondence: philippe.soucaille@insa-toulouse.fr
†Christian Croux and Ngoc-Phuong-Thao Nguyen contributed equally to this work
1 LISBP, INSA, University of Toulouse, 135 Avenue de Rangueil, 31077 Toulouse Cedex, France
Full list of author information is available at the end of the article
Background
In recent years, Clostridium acetobutylicum ATCC 824 has been of interest in the postgenomic era due to the complete sequencing and annotation of its genome [1], supplying a wealth of information regarding its protein machinery. This global knowledge has prompted new approaches to genetic analysis, functional genomics, and metabolic engineering in order to develop industrial strains for the production of biofuels and bulk chemicals.

To this end, several reverse genetic tools have been developed for C. acetobutylicum ATCC 824, including a gene inactivation system based on non-replicative [2, 3] and replicative plasmids [4–7] and the group II intron gene inactivation system [8, 9]. Among these methods, only the method developed by Al-Hinai et al. [5] allows for in-frame deletions and/or the introduction of genes at their normal chromosomal context without an antibiotic marker remaining. This system is made of two parts. The first part is a replicative vector containing (1) a pIMP13 origin of replication from Bacillus subtilis functional in Clostridia, (2) a replacement cassette consisting of an antibiotic resistance gene (ThrR) flanked by two FRT sequences, (3) two sequences homologous to the selected regions around the target DNA sequence, and (4) a codon-optimized mazF toxin gene from Escherichia coli under the control of a lactose-inducible promoter from Clostridium perfringens to allow for the positive selection of double-crossover allelic exchange mutants. The second part is a plasmid system with inducible segregational instability, enabling efficient deployment of the FLP-FRT system to generate marker-less deletion or integration mutants.

In 2006, our group patented a marker-less, in-frame deletion method [10] similar to the two-part method published by Al-Hinai et al. [5] in 2012. The first part of our method is based on the same replicative plasmid and the same replacement cassette, but it uses the uracil PRTase upp/5-fluorouracil (5-FU) system as a counter-selection strategy. The second part is based on a plasmid carrying (1) the FLP-FRT system to generate marker-less deletion and (2) the uracil PRTase upp/5-FU system to select for the plasmid loss after marker excision. This method was successfully used by the Metabolic Explorer Company to develop and patent an industrial recombinant strain of C. acetobutylicum for n-butanol production. As this method has not been described in detail and to make it available to and usable by the scientific community, we report how this method was developed and its use to create a restriction-less, marker-less strain of C. acetobutylicum. We show that this strain lacking upp (CA_C2879, encoding the uracil–phosphoribosyltransferase), CA_C1502 encoding Cac824I and CA_C3535 encoding Cac824II (the second type II restriction enzyme) can be transformed by non-methylated DNA at very high efficiency and can be used for rapid gene knock-in and knock-out using the upp/5-FU counter-selectable system for both functional genomic and metabolic engineering of C. acetobutylicum. This strain and the method were further used to delete three genes ctfAB and ldhA to create strains no longer producing acetone and lactate, respectively.

Results
MGCΔcac1502 strain, a C. acetobutylicum strain that is transformable without previous in vivo plasmid methylation
Cac824I, the type II restriction endonuclease encoded by CA_C1502, is a major barrier to the electrotransformation of C. acetobutylicum with E. coli–C. acetobutylicum shuttle vectors [11]. The Cac824I restriction endonuclease recognition sequence 5′-GCNGC3′, where N can be any nucleotide, occurs infrequently in C. acetobutylicum DNA because of the high A + T DNA content (72 % A + T), but the sequence occurs frequently in E. coli plasmids. No methyltransferase that can be used in vitro to protect DNA from restriction by Cac824I is commercially available. Prior to the transformation of C. acetobutylicum, shuttle plasmids have to be methylated in vivo by transformation into E.coli ER2275 (pAN1) expressing the Bacillus subtilis phage φ3T1 methyltransferase, which protects the shuttle plasmids from digestion by the clostridial endonuclease Cac824I [11]. This step is time consuming and may be a drawback if the genes to be transferred to C. acetobutylicum are toxic when expressed in E. coli. Therefore, a C. acetobutylicum strain deficient for this particular restriction system would be valuable for efficient electrotransformation without previous treatment of the plasmid to be transformed.

To delete the Cac824I encoding gene, the first step is the construction of a shuttle vector carrying the replacement cassette. The CA_C1502 replacement cassette was cloned into the BamHI site of the pCons2-1 and pCIP2-1 to generate the pREPcac15 and pCIPcac15 plasmids, respectively. The difference between these two plasmids is the origin of replication. The pREPcac15 contains a pIMP13 origin of replication from B. subtilis (rolling circle mechanism) functional in Clostridia, whereas pCIPcac15 contains the origin of replication of the pSOL1 megaplasmid (θ replication mechanism). The pREPcac15 and pCIPcac15 plasmids were methylated in vivo in E.coli ER2275 (pAN1) and were used to transform C. acetobutylicum ATCC824 by electroporation. After selection on plates for clones resistant to erythromycin at 40 µg/ml, one colony of each transformant was cultured for 24 h in liquid SM with erythromycin and was then subcultured four times in liquid 2YTG medium without antibiotic
(Fig. 1a). To select integrants that lost the pREPCac15 or pCIPcac15 plasmids, 10^5 erythromycin resistant clones were replica plated on both RCA with erythromycin and RCA with thiamphenicol at 50 µg/ml. Whereas several colonies resistant to erythromycin and sensitive to thiamphenicol were obtained with pREPCac15 transformants, no such colonies were obtained with the pCIPcac15 transformants, which indicates that the θ replication mechanism of pCIPcac15 is less favorable for promoting double-crossover in C. acetobutylicum than a rolling circle mechanism. The genotype of clones with the desired phenotype was checked by PCR (polymerase chain reaction) analysis (Fig. 2a). The Δcac1502::mls^R strain, which had lost the pREPCac15, was isolated. This strain was transformed with the pCLF1 plasmid expressing the FLP1 gene of S. cerevisiae encoding for the FLP recombinase. The expression of FLP1 was under the control of the promoter and RBS (ribosome binding site) from the thiolase gene from C. acetobutylicum. After transformation and selection for resistance to thiamphenicol at 50 µg/ml, one colony was cultured in liquid SM with thiamphenicol. One hundred thiamphenicol resistant clones were replica plated on both RCA with erythromycin and RCA with thiamphenicol. The genotype of the clones with erythromycin sensitivity and thiamphenicol resistance was checked by PCR analysis with primers CAC 0 and CAC 5 (Fig. 2a). Two successive 24-h liquid cultures of the Δcac1502 strain were conducted in the absence of antibiotics to remove pCLF1. The Δcac1502 strain that lost pCLF1 was isolated according to its sensitivity to both erythromycin and thiamphenicol. This strain was called MGCΔcac1502.

The efficiency of transformation of this strain with methylated and unmethylated pCons2.1 plasmid was evaluated and compared to the wild type strain. Both strains can be transformed with methylated pCons2.1 with similar efficiency, but only MGCΔcac1502 can be transformed efficiently with unmethylated DNA (Table 1).

The following deletions described in this manuscript were conducted in this strain without previous in vivo plasmid methylation.

**Construction of the MGCΔcac1502::upp strain: the first marker-free C. acetobutylicum strain with two deleted genes**

To develop a positive screening of integrants, we used the “upp/5-FU as counter selection marker” system. The C. acetobutylicum upp gene (CA_C2879) encodes uracil phosphoribosyl-transferase (UPRTase), which catalyzes the conversion of uracil into UMP, thus allowing the cell to use exogenous uracil [12]. The pyrimidine analog 5-fluoro-UMP, which is metabolized into 5-fluoro-dUMP, an inhibitor of thymidylate synthetase, toxic for the cell. The use of the upp expression cassette as a counter-selection marker is linked to the construction of a C. acetobutylicum strain deleted for the upp gene, thus resistant to 5-FU.

To delete upp, the upp replacement cassette was cloned into the BamHI site of pCons2-1 to generate the plasmid pREPupp. The plasmid pREPupp was used to transform the MGCΔcac1502 strain by electroporation without previous in vivo methylation. After selection on plates for clones resistant to erythromycin at 40 µg/ml, one colony was cultured for 24 h in liquid 2YT medium with erythromycin and was then subcultured in liquid 2YT medium without antibiotic (Fig. 1a). To select integrants having lost the pREPupp plasmid, 10^5 erythromycin resistant clones were replica plated on both RCA with erythromycin and RCA with thiamphenicol. The genotype of the clones resistant to erythromycin and sensitive to thiamphenicol was determined by PCR analysis (Fig. 2b). The MGCΔcac1502Δupp::mls^R strain that lost pREPupp was isolated. When the resistance to 5-FU was analyzed, we showed that this strain was resistant to up to 1 mM 5-FU compared to 50 µM for the MGCΔcac1502 strain. This strain was then transformed with the pCLF1 plasmid, and selection of MGC Δcac1502Δupp strain with sensitivity to both erythromycin and thiamphenicol was performed, as previously described for the MGCΔcac1502 strain (Fig. 2b).

**Deletion of the CA_C3535 gene in the MGCΔcac1502Δupp strain using the upp/5-FU system as a counter-selectable marker for the loss of plasmid**

The CA_C3535 gene encodes Cac824II, a potentially bi-functional enzyme carrying both a type II restriction endonuclease and methylase activities. To delete CA_C3535, the CA_C3535 replacement cassette was cloned into the BamHI site of the pCons::upp to generate the plasmid pREPcac3535::upp. The plasmid pREPcac3535::upp was used to transform the C. acetobutylicum MGCΔcac1502Δupp strain by electroporation without previous in vivo methylation.

After plate selection for clones resistant to erythromycin at 40 µg/ml, 100 transformants were replica plated on RCA with erythromycin, RCA with thiamphenicol and RCA with 5-FU at 400 µM (Fig. 1b). All transformants were resistant to erythromycin and thiamphenicol and were sensitive to 5-FU compared to the parental strain, which was resistant to 5-FU. This result demonstrates that the expression of the upp gene carried by pREPcac::upp confers sensitivity to 5-FU.

To select for Δcac3535::Em^R integrants that lost the pREPCac3535::upp plasmid, erythromycin-
5-FU-resistant clones were selected on RCA plates containing erythromycin and 5-FU from 100 µl of a liquid culture of the MGCΔcac1502Δupp (pREPcac3535::upp) strain. Approximately 500 colonies were obtained, and 100 of them were replica plated on both RCA with erythromycin and RCA with thiamphenicol. Most of the clones (95%) were resistant to erythromycin and sensitive to thiamphenicol. Four clones were checked by PCR analysis (Fig. 2c) All four clones had the correct phenotype, and one of the clones was selected as the MGC Δcac1502ΔuppΔcac3535 strain. This strain was then transformed with pCLF::upp, a derivative of the pCLF1 plasmid that also carries the upp gene, in order for the positive selection of plasmid loss after the excision of the mlsR marker. After the first selection of clones resistant to thiamphenicol and sensitive to erythromycin, a second selection of clones resistant to 5-FU and sensitive to thiamphenicol was performed to obtain the MGC Δcac1502ΔuppΔcac3535 strain that was control by PCR (Fig. 2c) for the presence of all the marker-less deletions. Finally, when compared to C. acetobutylicum ATCC824 wild type, the growth of the restriction-less marker-less strain in MS medium at pH 4.5 (Fig. 3) was shown to be unaffected by the different deletions.

The unmethylated pCons2.1 plasmid was used to evaluate the transformation efficiency of the MGCΔcac1502 and the MGCΔcac1502Δupp Δcac3535 strains. The transformation efficiency of MGCΔcac1502ΔuppΔcac3535 for unmethylated pCons2.1 was ~eightfold higher than that of MGCΔcac1502 (Table 2).

Table 1 Transformation efficiencies of C. acetobutylicum ATCC824 and MGCΔcac1502 for unmethylated and methylated pCons2.1

|                      | C. acetobutylicum  | MGCΔcac1502 |
|----------------------|--------------------|-------------|
|                      | ATCC824            |             |
| Unmethylated pCons2.1| 0                  | 0.79 (±0.24) × 10⁴ |
| Methylated pCons2.1  | 0.46 (±0.11) × 10⁴ | 0.58 (±0.18) × 10⁴ |

Values are expressed in number of transformants per μg DNA
Mean values and standard deviations from three independent experiments are given
25 ng pCons2.1 was used in each experiment
I recognize that the plasmid expressing Cac824II has three AcuI sites, but when we tried to digest it with AcuI, it was not sufficient to protect DNA against its restriction activity. We applied the same strategy for the expression of Cac824II, we cloned CA_C3535 in the pPAL vector using the E. coli BL21-AI cells containing the pSC-CAC3534 plasmid as host. The Cac824II endonuclease was purified, and its activity towards unmethylated pCons2.1 in the presence of SAM was determined. AcuI recognizes the 5'-CTGAAG-3' sequence and cuts the pCons2.1 plasmid two times, resulting in two fragments of 2411- and 882-bp. Figure 4 shows that Cac824II gives the same restriction pattern as AcuI. To confirm that the AcuI and Cac824II recognition sequences were identical, pCons2.1 was digested by 50 µg of Cac824II in the presence of 1 U of AcuI. Figure 4 shows that the restriction pattern was unchanged, which definitively confirms that AcuI and Cac824II are isoschizomers.

Deletion of the ctfAB genes in the MGCΔcac1502ΔuppΔcac3535 to create a strain no longer producing acetone

The ctfAB genes (CA_P0163-CA_P0164) located on the pSOL1 megaplasmid encodes an acetoyl-CoA:acyl CoA-transferase involved in the first step of acetone formation [15]. To delete ctfAB, the ctfAB replacement cassette was cloned into the BamHI site of the pCons::upp to generate the plasmid pREPctfAB::upp. The plasmid pREPctfAB::upp was used to transform the C. acetobutylicum MGCΔcac1502ΔuppΔcac3535 strain by electroporation without previous in vivo methylation and cell containing the plasmid were selected on RCA plate with erythromycin at 40 µg/ml. To select for ΔctfAB::EmR.

| Table 2 Transformation efficiencies of MGCΔcac1502 and MGCΔcac1502ΔuppΔcac3535 for unmethylated pCons2.1 |
|---------------------------------------------------------------|
| Unmethylated pCons2.1 | 0.79 (±0.24) × 10⁴ | 6.1 (±3.2) × 10⁴ |

Values are expressed in number of transformants per μg DNA

Mean values and standard deviations from three independent experiments are given.

25 ng pCons2.1 was used in each experiment.

Determination of the recognition sequence of Cac824II encoded by CA_C3535

CA_C3535 encoded a 993 amino acid protein with a calculated molecular mass of 116,842 Da. The amino acid sequence analysis revealed high similarities with two restriction endonucleases: AcuI from Acinetobacter calcoaceticus SRW4 [13] and Eco57I from E. coli RFL57 [14] with 44 and 46 % identity, respectively. Both enzymes belong to the IIg family of restriction enzymes [14] with 44 and 46 % identity, respectively. Both enzymes belong to the IIg family of restriction enzymes. To heterologously express the AcuI-encoding gene in E. coli [13], it was necessary to first express the AcuIM methylase-encoding gene because the methylase activity of AcuI was not sufficient to protect DNA against its restriction activity. We applied the same strategy for the expression of CA_C3535-encoding Cac824II: we cloned into the pSOS2K2 gene and expressed in E. coli the CA_C3534 gene that encodes a putative methylase and that is located immediately downstream of CA_C3535 gene in the C. acetobutylicum chromosome. The pSC-CAC3534 plasmid expressing CA_C3534 has three AcuI recognition sites, but when we tried to digest it with AcuI, it was completely protected from the activity of this enzyme. To express, purify and determine the recognition sequences of Cac824II, we cloned CA_C3535 in the pPAL vector using the E. coli BL21-AI cells containing the pSC-CAC3534 plasmid as host. The Cac824II endonuclease was purified, and its activity towards unmethylated pCons2.1 in the presence of SAM was determined. AcuI recognizes the 5'-CTGAAG-3' sequence and cuts the pCons2.1 plasmid two times, resulting in two fragments of 2411- and 882-bp. Figure 4 shows that Cac824II gives the same restriction pattern as AcuI. To confirm that the AcuI and Cac824II recognition sequences were identical, pCons2.1 was digested by 50 µg of Cac824II in the presence of 1 U of AcuI. Figure 4 shows that the restriction pattern was unchanged, which definitively confirms that AcuI and Cac824II are isoschizomers.

Deletion of the ctfAB genes in the MGCΔcac1502ΔuppΔcac3535 to create a strain no longer producing acetone

The ctfAB genes (CA_P0163-CA_P0164) located on the pSOL1 megaplasmid encodes an acetoyl-CoA:acyl CoA-transferase involved in the first step of acetone formation [15]. To delete ctfAB, the ctfAB replacement cassette was cloned into the BamHI site of the pCons::upp to generate the plasmid pREPctfAB::upp. The plasmid pREPctfAB::upp was used to transform the C. acetobutylicum MGCΔcac1502ΔuppΔcac3535 strain by electroporation without previous in vivo methylation and cell containing the plasmid were selected on RCA plate with erythromycin at 40 µg/ml. To select for ΔctfAB::EmR. 

Fig. 3 Growth curves of C. acetobutylicum ATCC824 and MGCΔcac1502 Δupp Δcac3535 at pH 4.5 in SM medium.

Fig. 4 Digestion properties of recombinant Cac824II as compared to commercial Acel (New England Biolabs). 250 ng of unmethylated pCONS2.1 plasmid were incubated for 1 h at 37 °C in a reaction volume of 20 µL containing 50 mM potassium acetate, 20 mM Tris-acetate (pH 7.9), 10 mM Magnesium acetate, 100 µg/mL BSA and 0.04 mM S-adenosyl-methionine with (A) No enzyme, (B) purified Cac824II (50 µg), (C) Acel (SU), and (D) purified Cac824II (50 µg) + Acel (SU). Lanes M, 1 kb DNA ladder (0.5–10 kbp, NEB). Reactions products were electrophoresed on a 0.8 % agarose gel. An arrow indicates the incomplete digestion product remaining after incubation with Cac824II.

Table 2 Transformation efficiencies of MGCΔcac1502 and MGCΔcac1502ΔuppΔcac3535 for unmethylated pCons2.1

| Transformation efficiencies | MGCΔcac1502 | MGCΔcac1502ΔuppΔcac3535 |
|----------------------------|-------------|------------------------|
| Unmethylated pCons2.1      | 0.79 (±0.24) × 10⁴ | 6.1 (±3.2) × 10⁴ |

Values are expressed in number of transformants per μg DNA

Mean values and standard deviations from three independent experiments are given.

25 ng pCons2.1 was used in each experiment.
integrants that lost the pREPctfAB:upp plasmid, erythromycin- and 5-FU-resistant clones were selected on RCA plates containing erythromycin and 5-FU from 100 μl of a liquid culture of the MGC Δ cac1502 Δ upp Δ cac3535 (pREPctfAB:upp) strain. Approximately 500 colonies were obtained, and 50 of them were replica plated on both RCA with erythromycin and RCA with thiophenicol. Most of the clones (80 %) were resistant to erythromycin and sensitive to thiophenicol. Four clones were checked by PCR analysis (with primers CTF-0 and CTF-5 located outside of the ctfAB replacement cassette and primers CTF-D and CTF-R located inside of ctfAB). All four clones had the correct phenotype, and one of the clones was selected as the MGC Δ cac1502 Δ upp Δ cac3535 Δ ldhA::mlsR strain. The fermentation profile of this strain was compared to the MGC Δ cac1502 Δ upp Δ cac3535 control strain during batch fermentation at pH 4.5 (Fig. 5). The production of 1-lactate was totally abolished proving that ldhA encodes the main 1-lactate dehydrogenase of C. acetobutylicum.

Discussion
We developed a simple and efficient method to create mutations in the Clostridium acetobutylicum chromosome. This method is based on the use of (1) a replicative plasmid, (2) a deletion cassette containing both DNA sequences with homology to the flanking region of the target gene (to delete it) and an antibiotic resistance gene surrounded by FRT sequences (as an excisable marker), and (3) the upp gene, which encodes the uracil–phosphoribosyl-transferase, as a counter-selectable marker.

A plasmid that replicates via a rolling circle mechanism was more efficient in terms of double cross over frequency than a plasmid that replicates through a theta mechanism. This result is in agreement with previous findings in Bacillus subtilis showing that plasmid replication through a rolling circle mechanism favors recombination between homologous sequences [16, 17].

The deletion cassette can be rapidly constructed through a three-step procedure using pre-constructed building blocks. After a fusion PCR and TOPO cloning of the product, a predesigned antibiotic resistance gene surrounded by two FRT sites in direct repeats is inserted. The upp gene is located on the plasmid outside of the deletion cassette. This allows the positive selection of clones that have lost the plasmid and integrated the deletion cassette by a double recombination event. We demonstrate here that this event occurs at a frequency of 10⁻⁵, which means that without the selection procedure, it would be much more difficult to isolate the correct deletion mutant by replica plating alone.
Once the deletion cassette is integrated into the chromosome, the expression of the flp recombinase allows (1) the excision of the antibiotic marker for a clean in-frame deletion of the targeted gene (without polar effect) and (2) consecutive gene deletions. Such a strategy was previously applied to marker-less gene deletions in E. coli [18] and Mycobacterium smegmatis [19]. The plasmid expressing the FLP recombinase-encoding gene was further improved by coexpressing the upp gene to use it as a positive selection for the plasmid loss after excision of the MLSR marker. A similar tool was developed by Al-Hinai et al. [5] using a plasmid (1) that expresses the FLP recombinase-encoding gene and (2) that has an inducible segregational instability to promote the plasmid loss.

In this study Cac824II (encoded by CA_C3535), the second type II restriction enzyme of C. acetobutylicum predicted by REBASE [20], was biochemically characterized and it was demonstrated that it is an isoschizomer of AcaI [13] recognizing the 5′-CTGAAG-3′ sequence. It was also shown that Cac824II methylase (encoded by CA_C3534) protects DNA against restriction by Cac824II and AcaI by probably methylating one of the adenine in the 5′-CTGAAG-3′ sequence. Two Cac824II restriction sites are present (in the ampR gene and in the coE1 origin of replication) in most the shuttle vector use to transform C. acetobutylicum and it was then justified to construct a marker-less strain deleted from CA_C3535. The transformation efficiency of MGCΔacI502ΔuppΔcac3535 for unmethylated pCons2.1 was much higher (~eightfold higher) than that of MGCΔcac1502 of MGCΔcac3535. The transformation efficiency of shuttle vector use to transform gene and in the coE1 origin of replication) in most the shuttle vector use to transform C. acetobutylicum and it was then justified to construct a marker-less strain deleted from CA_C3535. The transformation efficiency of MGCΔacI502ΔuppΔcac3535 for unmethylated pCons2.1 was much higher (~eightfold higher) than that of MGCΔcac1502 and it will be an interesting strain to Δcac1502 of MGCpCons2.1 was much higher (~eightfold higher) than that of MGCΔcac1502 of MGCΔcac1502. The restriction-less, marker-less strain and the genome modification method presented here become simple and convenient tools that are useful for research groups involved in functional genomic studies of C. acetobutylicum and for further metabolic engineering of this strain to produce bulk chemicals and biofuel. As a demonstration of the efficiency of the method, we constructed two strains unable to produce l-lactate or acetone. Furthermore, this method was successfully used by the Metabolic Explorer Company to develop and patent an industrial recombinant strain of C. acetobutylicum for n-butanol production [23] at high yield.

Methods

Bacterial strain, plasmids and oligonucleotides
The bacterial strain and plasmids used in this study are listed in Table 3. The specific oligonucleotides used for PCR amplification were synthesized by Eurogentec (Table 4).

Culture and growth conditions
C. acetobutylicum was maintained as spores in synthetic medium (SM) as previously described [24, 25]. Spores were activated by heat treatment at 80 °C for 15 min. All C. acetobutylicum strains were grown in anaerobic conditions at 37 °C in SM, in Clostridium growth medium (CGM) [26] in 2YTG [27] 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, erythromycin at 40 µg/ml and thiophenicol at 50 µg/ml; for E. coli, erythromycin at 200 µg/ml and chloramphenicol at 30 µg/ml. Transformations of C. acetobutylicum were conducted by electroporation, as previously described [11]. 5-FU was purchased from Sigma, and stock solutions were prepared in DMSO (dimethyl sulfoxide).

DNA manipulation techniques
Total genomic DNA from C. acetobutylicum was isolated as previously described [27]. 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 [28]. 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 QIAquick gel purification kit (Qiagen, France).

Conclusions
The restriction-less, marker-less strain and the genome modification method presented here become simple
Construction of pUC18-FRT-MLS2
Inverse PCR was performed using the pKD4 plasmid [18] as a template and oligonucleotides PKD4.1 and PKD4.2 as primers to amplify the plasmid region with the FRT sites but without the kanamycin resistance marker. This blunt end fragment was later ligated to the MLSr gene obtained after a HindIII digestion of the pETSPO plasmid [4] and Klenow treatment. The corresponding plasmid (pKD4-Ery1) was then used as a template to amplify by PCR the macrolide lincosamide streptogramin B resistance (MLSr) gene, functional in Clostridia and flanked by two FRT sites and two StuI sites, using the oligonucleotides FRT-MLSR-F and FRT-MLSR-R as primers. This fragment was directly cloned into the SmaI digested pUC18 to generate the pUC18-FRT-MLS2 plasmid.

Construction of pCons2.1
Inverse PCR was performed using the pETSPO plasmid [4] as a template and oligonucleotides PCONSAccI (mutating a BamHI site) and PCONSEcoRI as primers. The PCR product, containing a pIMP13 B. subtilis origin of replication functional in Clostridia (rolling circle mechanism of replication) and a catP gene conferring resistance to thiamphenicol was phosphorylated and ligated to yield the pCons0 plasmid. This plasmid was then digested with BamHI to remove the spoA cassette, Table 3 Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| **Bacterial strains** | | |
| E. coli | | |
| TOP10 | RecA− McrBC− | Invitrogen |
| ER2275 | | |
| C. acetobutylicum | | |
| ATCC824 | Wild type | ATCC |
| MGCΔcac1502 | ΔCA_C1502 | This study |
| MGCΔcac1502Δupp | ΔCA_C1502ΔCA_C2879 | This study |
| MGCΔcac1502ΔuppΔcac3535 | ΔCA_C1502ΔCA_C2879ΔCA_C3535 | This study |
| MGCΔcac1502ΔuppΔcac3535ΔctfAB | ΔCA_C1502ΔCA_C2879ΔCA_C3535ΔCA_P0162-3 | This study |
| MGCΔcac1502ΔuppΔcac3535ΔldhA | ΔCA_C1502ΔCA_C2879ΔCA_C3535ΔCA_C0267 | This study |
| **Plasmids** | | |
| pAN1 | Cm', φ3TI, p15A origin | [27] |
| pKD4 | Ap', Km' | [18] |
| pETSP0 | Cm', MLS' | [4] |
| pUC18 | Ap' | Fermentas |
| pUC18-FRT-MLS2 | Ap' MLS' | This study |
| pCons2-1 | Cm' | This study |
| pCR-BluntIII-TOPO | Zeo' Km' | Invitrogen |
| pCIP2-1 | Cm' | This study |
| pREP cac15 | Cm', MLS', ΔCA_C1502 | This study |
| pCIP cac15 | Cm', MLS', ΔCA_C1502 | This study |
| pREP upp | Cm', MLS', Δupp | This study |
| pCP20 | Ap', Cm', FLP | [29] |
| pSOS95 | Ap', MLS', acetone operon, repl. gene, ColE1 origin | [32] |
| pCLF1 | Cm', FLP | This study |
| pCR4-TOPO-Blunt | Ap', Km' | Invitrogen |
| pCons-upp | Cm', MLS', upp | This study |
| pREP cac3535-upp | Cm', MLS', upp ΔCA_C3535 | This study |
| pREP ctfAB-upp | Cm', MLS', upp ΔctfAB | This study |
| pREP ldhA-upp | Cm', MLS', upp ΔldhA | This study |

**a** RecA− homologous recombination abolished, McrB− lacking methylcytosine-specific restriction system, Cm' chloramphenicol resistance, Ap' ampicillin resistance, MLS' macrolide lincosamide and streptogramin B resistance, Zeo' zeomycin resistance, φ3TI φ3TI methyltransferase, repl. Gram-positive origin of replication from pIM13

**b** NEB New England BioLabs, ATCC American Type Culture Collection (Rockville, MD)
and the DNA fragment was purified and ligated to generate the pCons2-1 plasmid.

**Construction of pCIP2-1**

The pIMP13 origin of replication from pCons2-1 was replaced by the origin of replication of the pSOL1 megaplasmid. The origin of replication of pSOL1 was amplified by PCR using *C. acetobutylicum* total DNA as a template and oligonucleotides ORI3-D and ORI3-R as primers. This PCR product was cloned into the pCR-BluntII-TOPO vector, and the resulting plasmid was digested by *EcoRI* to obtain the 2.2 kb *EcoRI* fragment containing the origin of replication of pSOL1. The pCons2-1 plasmid was digested by *EcoRI*, and the 2.4 kb fragment was ligated to the 2.2 kb *EcoRI* fragment to generate the plasmid pCIP2-1.

**Construction of pREPcac15**

Two DNA fragments surrounding *cac1502* were amplified by PCR using *C. acetobutylicum* total DNA as the template and two pairs of oligonucleotides as primers. Using the primers pairs CAC 1 and CAC 2 or CAC 3 and CAC 4, 1493 and 999-bp DNA fragments were obtained, respectively. Both primers CAC 1 and CAC 4 introduce a *BamHI* site, whereas primers CAC 2 and CAC 3 have complementary 5′extended sequences that introduce a *StuI* site. DNA fragments CAC 1–CAC 2 and CAC 3–CAC 4 were joined in a PCR fusion with primers CAC 1 and CAC 4, and the resulting fragment was cloned into the pCR4-TOPO-Blunt vector to generate pTOPO::cac15. At the unique *StuI* site of pTOPO::cac15, the 1372-bp *StuI* fragment of pUC18-FRT-MLS2 carrying the antibiotic resistance *MLSr* gene with FRT sequences on both sides was introduced. The *cac1502* replacement cassette obtained after *BamHI* digestion of the resulting plasmid was cloned into the *BamHI* site of the pCons2-1 to generate the plasmid pREPcac15.
Construction of pCIPcac15

The cac1502 replacement cassette above was cloned into the BamHI site of the pCIP2-1 to generate the plasmid pCIPcac15.

Construction of pREPupp

Two DNA fragments upstream and downstream of cac2879 were amplified by PCR using total DNA from *C. acetobutylicum* as the template and two pairs of oligonucleotides as primers. With the primer pairs UPP 1–UPP 2 and UPP 3–UPP 4, 1103- and 1105-bp DNA fragments were obtained, respectively. Both primers UPP 1 and UPP 4 introduce a BamHI site, whereas primers UPP 2 and UPP 3 have 5' extended sequences that introduce a Stul site. DNA fragments UPP 1–UPP 2 and UPP 3–UPP 4 were joined in a PCR fusion with primers UPP 1 and UPP 4, and the resulting fragment was cloned into pCR4-TOPO-Blunt vector to generate pTOPO::upp. At the unique Stul site of pTOPO::upp, the 1372-bp Stul fragment of pUC18-FRT-MLS2 carrying the antibiotic resistance MLS' gene with FRT sequences on both sides was introduced. The *upp* replacement cassette obtained after BamHI digestion of the resulting plasmid was cloned into the BamHI site of the pCons::upp to generate the plasmid pREPcac3535::upp.

Construction of pCLF1

The *FLP1* gene was amplified by PCR using the pCP20 plasmid [29] as a template and oligonucleotides FLP1-D and FLP1-R as primers. These primers introduced BamHI and SfoI restriction sites on the ends of the PCR product. After a BamHI–SfoI double digestion, the PCR product was cloned into the BamHI–SfoI sites of the pSOS95 expression vector to generate the pEX-FLP1 plasmid. The 1585-bp *SalI* fragment of pEX-FLP1 containing the *FLP1* expression cassette was cloned into the *SalI* site of pCons2-1 to generate the plasmid pCLF1 plasmid.

Construction of pCons::upp

The *upp* gene with its own ribosome binding site (RBS) was amplified by PCR from *C. acetobutylicum* total DNA with the oligonucleotides REP-UPP-F and REP-UPP-R as primers. The 664-bp PCR product was digested by *PvuII* and was cloned into pCons2.1, digested by BcglI and treated with T4 DNA polymerase to generate the pCons::upp plasmid. In this way, the *upp* gene was located just downstream of the *catP* gene to construct an artificial operon with *upp* expressed under the control of the *catP* promoter.

Construction of pREPcac35::upp

Two DNA fragments upstream and downstream of CA_C3535 were amplified by PCR using the total DNA from *C. acetobutylicum* as a template and two pairs of oligonucleotides as primers. With the primer pairs RM3535 1 and RM3535 2 or RM3535 3 and RM3535 4, 1044- and 938-bp DNA fragments were obtained, respectively. Both primers RM3535 1 and RM3535 4 introduce a BamHI site, whereas primers RM3535 2 and RM3535 3 have 5' extended sequences that introduce a Stul site. DNA fragments RM3535 1-RM3535 2 and RM3535 3-RM3535 4 were joined in a PCR fusion with primers RM3535 1 and RM3535 4, and the resulting fragment was cloned into the pCR4-TOPO-Blunt vector to generate pTOPO::cac3535. At the unique Stul site of pTOPO::cac3535, the 1372-bp Stul fragment of pUC18-FRT-MLS2 carrying the antibiotic resistance MLS' gene with FRT sequences on both sides was introduced. The CA_C3535 replacement cassette obtained after BamHI digestion of the resulting plasmid was cloned into the BamHI site of the pCons::upp to generate the plasmid pREPcac3535::upp.

Construction of pREPctfAB::upp

Two DNA fragments upstream and downstream of *ctfAB* (CA_P0162-CA_P0163) were amplified by PCR using the total DNA from *C. acetobutylicum* as a template and two pairs of oligonucleotides as primers. With the primer pairs CTF 1 and CTF 2 or CTF 3 and CTF 4, 1144- and 1138-bp DNA fragments were obtained, respectively. Both primers CTF 1 and CTF 4 introduce a BamHI site, whereas primers CTF 2 and CTF 3 have 5' extended sequences that introduce a Stul site. DNA fragments CTF 1-CTF 2 and CTF 3-CTF 4 were joined in a PCR fusion with primers CTF 1 and CTF 4, and the resulting fragment was cloned into the pCR4-TOPO-Blunt vector to generate pTOPO::ctfAB. At the unique Stul site of pTOPO::ctfAB, the 1372-bp Stul fragment of pUC18-FRT-MLS2 carrying the antibiotic resistance MLS' gene with FRT sequences on both sides was introduced. The *ldhA* replacement cassette obtained after BamHI digestion of the resulting plasmid was cloned into the BamHI site of the pCons::upp to generate the plasmid pREPctfAB::upp.

Construction of pREPldhA::upp

Two DNA fragments upstream and downstream of *ldhA* (CA_C0267) were amplified by PCR using the total DNA from *C. acetobutylicum* as a template and two pairs of oligonucleotides as primers. With the primer pairs LDH 1 and LDH 2 or LDH 3 and LDH 4, 1135- and 1161-bp DNA fragments were obtained, respectively. Both primers LDH 1 and LDH 2 or LDH 3 and LDH 4 introduce a BamHI site, whereas primers LDH 2 and LDH 3 have 5' extended sequences that introduce a Stul site. DNA fragments LDH 1-LDH 2 and LDH 3-LDH 4 were joined in a PCR fusion with
primers LDH 1 and LDH 4, and the resulting fragment was cloned into the pCR4-TOPO-Blunt vector to generate pTOPO::ldhA. At the unique StuI site of pTOPO::ldhA, the 1372-bp StuI fragment of pUC18-FRT-MLS2 carrying the antibiotic resistance MLS5 gene with FRT sequences on both sides was introduced. The ldhA replacement cassette obtained after BamHI digestion of the resulting plasmid was cloned into the BamHI site of the pCons::upp to generate the plasmid pREPldhA::upp.

Construction of pCLF::upp
The 1585-bp SalI fragment of pEX-FLP1 containing the FLP1 expression cassette was cloned into the SalI site of pCons::upp to generate the pCLF::upp plasmid.

Cac3535 expression and purification
For the general cloning methods of restriction endonuclease genes in E. coli, the first step to clone and express the recombinant CA_C3535 gene into E. coli was to protect the host genomic DNA against the restriction activity of the Cac3535 bi-functional enzyme. The CA_C3534 methylase-encoding gene was thus amplified by PCR with Phusion DNA polymerase using C. acetobutylicum ATCC824 total genomic DNA as the template and Cac3534-d-Agel and Cac3534-R-Pvul as primers. After digestion with AgeI and Pvul, the resulting 1748-bp fragment was then cloned into pAH105 [30] a pSC101 derivative, that has been previously digested with AgeI and PacI, resulting in the pSC-CAC3534 plasmid. In this construct, the CA_C3534 gene expression was under the control of the pG1 1.6 promoter [31].

The E. coli BL21-AI strain (Invitrogen) was then transformed by the pSC-CAC3534 plasmid to give the BL21-AI-3534 strain. This strain, with host genomic DNA protected against the restriction activity of the Cac3535 bi-functional enzyme, was finally used as the host strain for the CA_C3535 gene over-expression using the T7-based expression system (see below). The Cac3535 protein was expressed in E. coli BL21 AI-3534 and was purified using the Profinity eXact Protein Purification System, following the recommendations of the manufacturer (Biorad). The CA_C3535 gene was amplified by PCR with Phusion DNA polymerase using C. acetobutylicum ATCC824 total gDNA as the template and Cac3535-d-Spel and Cac3535-R-BamHI as primers. The resulting 3002 bp fragment was cloned into the Zero Blunt TOPO vector (Invitrogen) to generate the TOPO-CAC3535 plasmid. After verification by DNA sequencing, the 2988-bp Spel-BamHI fragment from the latter plasmid was then introduced into the pPAL7 vector previously digested with the same enzymes to give the final pPAL-3535-1.24 plasmid.

After transformation, E. coli BL21-AI-3534 cells harboring the pPAL-3535-1.24 plasmid were grown in TB medium in the presence of 50 µg/ml carbenicillin and 100 µg/ml Spectinomycin at 37 °C to an OD550 ~ 0.45 and were then induced with 500 µM IPTG for 4 h at 37 °C. After centrifugation, the cell lysate was obtained by sonicating the resuspended pellet in bind/wash buffer (0.1 M sodium phosphate buffer, pH 7.2).

The tag-free Cac3535 protein was prepared using the Profinity eXact protein purification system, according to the standard protocol. After the Profinity Exact mini-spin column was bound by the protein and washed, the proteolytic activity of the affinity matrix was activated by applying two column volumes of room temperature 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1 M sodium fluoride. The column was incubated for 30 min to allow for the cleavage of the tag from the protein; then, the tag-free protein was released from the mini-spin column by centrifugation. The tag-free Cac3535 purified protein retains a Thr-Ser linker at its N-Terminus, ensuring optimal binding and cleavage during the purification steps (“Imprecise Fusion protein”).
References

1. Nolling J, Breton G, Omelchenko MV, Makarova KS, Zeng G, Gibson R, Lee HM, Dubois I, Quo D, Hitti J, et al. Genome sequence and comparative analysis of the solvent-producing bacterium Clostridium acetobutylicum. J Bacteriol. 2001;183(16):4623–38.

2. Green EM, Boynton ZL, Harris LM, Rudolph FB, Papoutsakis ET, Bennett GN. Genetic manipulation of acid formation pathways by gene inactivation in Clostridium acetobutylicum ATCC 824. Microbiology. 1996;142(Pt 8):2079–86.

3. Green EM, Bennett GN. Inactivation of an aldehyde/alcohol dehydrogenase gene from Clostridium acetobutylicum ATCC 824. Appl Biochem Biotechnol. 1996;57–58:213–21.

4. Harris LM, Welker NE, Papoutsakis ET. Northern, morphological, and fermentation analysis of spo0A inactivation and overexpression in Clostridium acetobutylicum ATCC 824. J Bacteriol. 2002;184(13):3586–97.

5. Al-Hinai MA, Fast AG, Papoutsakis ET. Novel system for efficient isolation of Clostridium double-crossover allelic exchange mutants enabling markerless chromosomal gene deletions and DNA integration. Appl Environ Microbiol. 2012;78(20):8112–21.

6. Liu CC, Qi L, Yanofsky C, Arkin AP. Regulation of transcription by unnatural amino acids. Nat Biotechnol. 2011;29:164–8.

7. Heap JT, Ehsaan M, Cooksley CM, Ng YK, Cartman ST, Winzer K, Minton NP. Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. Nucleic Acids Res. 2012;40(8):e59.

8. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. The Clostridium: a universal gene knock-out system for the genus Clostridium. J Microbiol Methods. 2007;70(3):452–64.

9. Shao L, Hu S, Yang Y, Gu Y, Chen J, Yang Y, Jiang W, Yang S. Targeted gene disruption by use of a group II intron (targetron) vector in Clostridium acetobutylicum. Cell Res. 2007;17(1):963–5.

10. Soucaille P, Figuer R, Croux C, Explorer M. Process for chromosomal integration and DNA sequence replacement in Clostridia. International Patent Application PCT/EP2006/066997. 2006.

11. Mermelstein LD, Papoutsakis ET. In vivo methylation in Escherichia coli by the Bacillus subtilis phage phi 3T I methyltransferase to protect plasmids from restriction upon transformation of Clostridium acetobutylicum ATCC 824. Appl Environ Microbiol. 1993;59(4):1077–81.

12. Fabret C, Ehrlich SD, Noirot P. A new mutation delivery system for genome-scale approaches in Bacillus subtilis. Mol Microbiol. 2002;46(1):25–36.

13. Samuelson J, Xu S, O’Loane D, New England Biolabs. I. Method for cloning and expression of AcuI restriction endonuclease and AcuI methylase in E. coli. US patent No.7,011,966. 2006.

14. Janulaitis A, Vaisvila R, Timinskas A, Klimasauskas S, Butkus V. Cloning and sequence analysis of the genes coding for Eco57I type IV restriction-modification enzymes. Nucleic Acids Res. 1992;20(22):6051–6.

15. Yoo M, Bestel-Corre G, Croux C, Riviere A, Meynial-Salles I, Soucaille P. A quantitative system-scale characterization of the metabolism of Clostridium acetobutylicum. MBio 2015;6(6):e01808–15.

16. Noirot P, Petit MA, Ehrlich SD. Plasmid replication stimulation by the yeast FLP recombinase. Gene. 2004;343(1):181–90.

17. Petit MA, Mesas JM, Noirot P, Morel-Delville F, Ehrlich SD. Induction of DNA amplification in the Bacillus subtilis chromosome. EMBO J. 1992;11(4):1317–26.

18. Datseriko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci USA. 2000;97(12):6640–5.

19. Stephan J, Stegger V, Niederweis M. Consecutive gene deletions in Mycobacterium smegmatis using the yeast FLP recombinase. Gene. 2000;243(1):181–90.

20. Sillers R, Chow A, Tracy B, Papoutsakis ET. Metabolic engineering of the non-sporulating, non-solventogenic Clostridium acetobutylicum strain MS to produce butanol without acetone demonstrate the robustness of the acid-formation pathways and the importance of the electron balance. Metab Eng. 2008;10(6):321.

21. Cooksley CM, Zhang Y, Wang H, Redl S, Winzer K, Minton NP. Targeted mutagenesis of the Clostridium acetobutylicum acetone-butanol-ethanol fermentation pathway. Metab Eng. 2012;14(6):630–41.

22. Millar T, Voigt C, Janssen H, Cooksley CM, Winzer K, Minton NP, Bahl H, Fischer RL, Wolkenhauer O. Coenzyme A-transferase-independent butyrate re-activation in Clostridium acetobutylicum-evidence from a mathematical model. Appl Microbiol Biotechnol. 2014;98(2):1905–72.

23. Soucaille P. Metabolic engineering of Clostridium acetobutylicum for enhanced production of n-butanol. International patent application WO2008052973.

24. Vascconcelos I, Girbal L, Soucaille P. Regulation of carbon and electron flow in Clostridium acetobutylicum grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. J Bacteriol. 1994;176(5):1443–50.

25. Peggio S, Goma G, Delorme P, Soucaille P. Metabolic flexibility of Clostridium acetobutylicum in response to met: Appl Microbiol Biotechnol. 1994;42(4):611–6.

26. Wiesenborn DP, Rudolph FB, Papoutsakis ET. Thiols from Clostridium acetobutylicum ATCC 824 and its role in the synthesis of acids and solvents. Appl Environ Microbiol. 1988;54(11):2717–22.

27. Mermelstein LD, Welker NE, Bennett GN, Papoutsakis ET. Expression of cloned homologous fermentative genes in Clostridium acetobutylicum ATCC 824. Biotechnology (NY). 1992;10(2):190–5.

28. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual 2nd ed. NY: Cold Spring Harbor Laboratory Press; 1989.

29. Cheresson P, Wackenagel W. Gene disruption in Escherichia coli: Tcr and KmR cassettes with the option of FLP-catalyzed excision of the antibiotic-resistance determinant. Gene. 1995;158(1):9–14.

30. Payne MS, Piccaraggio SK, Hsu AK, Nair RV, Valle F, Soucaille P, Trimbur DE, Inc GI. Company EDPDNA. Promoter and plasmid system for genetic engineering. 2012.

31. Meynial-Salles J, Cervin MA, Soucaille P. A new tool for metabolic pathway engineering in Escherichia coli: one-step method to modulate expression of chromosomal genes. Appl Environ Microbiol. 2005;71(4):2140–4.

32. Raynaud C, Sarcabal P, Meynial-Salles J, Croux C, Soucaille P. Molecular characterization of the 1,3-propanediol (1,3-PDO) operon of Clostridium butyricum. Proc Natl Acad Sci USA. 2003;100(9):5010–5.