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Improvement of the Genome Editing Tools Based on 5FC/5FU Counter Selection in *Clostridium acetobutylicum*

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Abstract: Several genetic tools have been developed for *Clostridium acetobutylicum* utilizing 5-fluorouracil (5FU) or 5-fluorocytosine (5FC) resistance as a selection method. A method based on the integration, by single crossing-over, of a suicide plasmid (pCat-*upp*) followed by selection for the second crossing-over using a counter-selectable marker (the *upp* gene and 5FU resistance) was recently developed for genome editing in *C. acetobutylicum*. This method allows the modification of the genome without any marker or scar left in a strain of *C. acetobutylicum* that is *Δupp*. Unfortunately, 5FU has strong mutagenic properties inducing mutations in the strain's genome. After numerous applications of the pCat-*upp*/5FU system for genome modification in *C. acetobutylicum*, the bacteria became completely resistant to 5FU in the presence of the *upp* gene, resulting in failure in the selection for the second crossing-over. It was found that the potential repressor of the pyrimidine operon, PyrR, was mutated at position A115, leading to the 5FU resistance of the strain. We developed two plasmids, one overexpressing the native *pyrR* gene and a suicide plasmid carrying a non-mutated and optimized *pyrR* gene (*pyrR**) and *upp*, which allowed us to restore the 5FU sensitivity of the strain. We also improved the pCat-*upp*/5FU system by reducing the concentration of 5FU from 1mM to 5 µM using a define synthetic medium.

Keywords: *Clostridium acetobutylicum*; genome edition; 5FU; PyrR

1. Introduction

In recent years, solventogenic Clostridia have garnered significant attention in the post-genomic era, primarily owing to the comprehensive sequencing and annotation of their genomes [1,2]. This wealth of genomic information has provided valuable insights into the metabolism of these industrially important strains, thereby catalyzing new approaches to genetic analysis, functional genomics, and metabolic engineering for the development of industrial strains geared towards biofuel and bulk chemical production.

To facilitate these endeavors, various reverse genetic tools have been devised for solventogenic Clostridia. These tools include markerless gene inactivation systems, employing methods such as homologous recombination with non-replicative [3–5] and replicative plasmids [6–9], as well as the insertion of group II introns [10,11]. For all homologous recombination-based methods involving two crossing-over, the use of a counterselection technique is imperative. This may involve employing CRISPR-Cas9 [12,13] or a counterselectable marker, which have been constructed using the codon-optimized *mazF* toxin gene from *Escherichia coli* (under the control of a lactose-inducible promoter) [7], the *pyrE* [8] gene (encoding an orotate phosphoribosyltransferase, leading to 5-fluoroorotate (5-

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FOA) toxicity), the *upp* gene (encoding an uracil phosphoribosyltransferase, leading to 5-fluorouracil (5-FU) toxicity) [5,9], or the *codA* gene [4] (encoding a cytosine deaminase that converts 5-Fluorocytosine to 5-FU, which is further transformed into a toxic compound by the product of the *upp* gene).

It is worth noting that while strategies relying on 5FC/5FU selection are highly effective, they should be employed cautiously. 5FU is a well-known anticancer drug recognized for its mutagenic properties in human cancers [14]. These mutagenic attributes have been demonstrated in various organisms, including *Caenorhabditis elegans*, *E. coli*, or *Mycobacterium tuberculosis* [15–17].

In our study, we will demonstrate that a mutation impairs the 5FU counterselection method. Upon identification of this issue, we endeavored to enhance the 5FU counterselection method for genome editing in *C. acetobutylicum*. This improvement involved the development of a corrective and preventive suicide plasmid, inspired by Foulquier et al. [5], featuring the introduction of a synthetic codon-optimized *pyrR* gene, referred to as *pyrR**. Additionally, we created a corrective replicative plasmid, building upon the work of Raynaud et al. [18], which incorporated the native *pyrR* gene. These two plasmids effectively circumvented the unanticipated 5FU resistance observed in *C. acetobutylicum*, substantially improving our team's previously described method by also reducing the concentration of 5FU required by a factor of 200 (from 1 mM to 5 µM) using a synthetic define medium.

2. Materials and Methods

2.1. Bacterial strains, plasmids and oligonucleotides

The bacterial strains and plasmids used in this study are referenced in **Table 1**. The oligonucleotides used for PCR amplification were synthesized and provided by Eurogentec are listed in **Table 2**.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
E. coli		
TOP10		Invitrogen
C. acetobutylicum		
CAB1060	*ΔCAC1502ΔuppΔptbΔbukΔctfABΔldhAΔrexA ΔthlA::atoB Δhbd::hbd1*	[19]
Δcac1502	*ΔCA_C1502*	[9]
Δcac1502ΔuppΔcac3535	*ΔCA_C1502 ΔCA_C2879 ΔCA_3535*	[9]
Δcac1502ΔuppΔcac3535 pyrR^{mut}	*ΔCA_C1502 ΔCA_C2879 ΔCA_3535 CA_C2113 g.344C>T*	This study
Δcac1502ΔuppΔcac3535 pyrR^{mut} Δldh::sadh hydG	*ΔCA_C1502 ΔCA_C2879 ΔCA_3535 CA_C2113 g.344C>T ΔCA_C0227:: CIBE_3470 HydG* (Accession: P25981.3)	This study
Plasmid		
pCat-*upp*	*Cm^R, upp, colE1 origin*	[5]
pCat-*upp- pyrR^{mut}*	*Cm^R, upp, pyrR edition cassette for C. acetobutylicum*	This study
pCat-*upp-Δldh*	*Cm^R, upp, ldh deletion cassette for C. acetobutylicum*	[20]
pCat-*upp-pyrR**	*Cm^R upp pyrR**	This study

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pCat-*upp-pyrR*- Δldh*	*Cm^R, upp pyrR*, ldh deletion cassette for C. acetobutylicum*	This study
pCat-*upp-pyrR*-Δldh::sadh-hydG*	*Cm^R, upp pyrR*, ldh substitution cassette for sadh hydG for C. acetobutylicum*	This study
pSOS95	*Ap^R,MLS^R , acetone operon, repL gene, colE1 origin*	[18]
pSOS95-*pyrR*	*Ap^R, MLS^R, pyrR, repL gene, colE1 origin*	This study

Table 2. Oligonucleotides used for PCR amplification.

Primer name	5′-3′ Oligonucleotide Sequence
PSC 39	GCATGCTCTTGTAGGTGATCCTT
PSC 40	TGTTTACTGAATCCTCTTCATCTATTCC
PSC 46	AAAAAAGGCGCCCTACAACTCATAAATGTTTACTGAATCCTC
PSC 51	CAGAGTATTTAAGCAAAAACATCGTAGAAAT
PSC 52	TTATTTTGTACCGAATAATCTATCTCCAGC
PSC 58	AAAAAAGGATCCTTATACTGGAGGTGAGTGTATGAATTTAAAAG
PSC 61	CCATGGTTATACTGGAGGTGAGTGTATGAATCTTAAAGCTAAGATTCTTGATGA
PSC 62	TAAGGC
PSC 72	AAACACCGTATTTCTACGATGTTTTTGCTTAAATACTCTGCCATGGCTATAGCTC ATATATGTTAACACTATCCTCTTC
PSC 75	TCTTGGAGATGCTGGAGATAGATTATTCGGTACAAAATAACCATGGTTATACTG GAGGTGAGTG
PSC 76	TTAATAGGATCCGAACCCATCAAATAAGAGTGCATATGG
PSC 104	TATTAAGGATCCAGTCCTGCCCAACC
PSC 105	AAATATAAATGAGCACGTTAATCATTTAACATAGATAATTAAATAGTAAAAGG AGGAACATATTTTATGAAAGGTTTTGC
PSC 106	GGCAAAAGTTTTATAAACATGGGTACTGGTTATATTATATTATTTATGACTTTATT ATTTATCACCTCTGCAACCACAGC
PSC 107	TAGAGAAATTTTTAAAGATTTCTAAAGGCCTTTAACTTCATGTGAAAAGTTTGT TAAAATATAAATGAGCACGTTAATCATTTAA
PSB 384	TCCACCCTTGGAGTTTAGGTCTTTTACCAGGCCTGAATACCCATGTTTATAGGG CAAAAGTTTTATAAACATGGGTACT
PSB 385	GGGAAAGGTTTTAAGAGCGGCG CAACAATTGTCTCCGGTTTCAAGGG

2.2. Growth Conditions

E. coli strains were grown in Luria-Bertani (LB) medium. *C. acetobutylicum* strains were maintained as spores in synthetic medium (SM) at - 20°C as previously described or, for non-sporulating strains, directly in degassed and sterile serum bottles at - 80°C [21–23]. Spores were activated by heat shock at 80°C for 15 min. Strains were grown under anaerobic conditions at 37 °C in Clostridial Growth Medium (CGM) supplemented each time with 30 gL^{-1} of glucose [24] or in CGM supplemented with 20 gL^{-1} MES hydrate (Sigma Aldrich) , synthetic medium (SM) or in SM supplemented with 20 gL^{-1} MES hydrate or in Reinforced Clostridial Medium (RCM) (Millipore). The pH of CGM was adjusted at 6.0 or 5.2 with hydrochloric acid. The pH of RCM was adjusted at 5.8 with hydrochloric acid. The SM used for *C. acetobutylicum* growth contained per liter of deionized water: Glucose, 30 g; KH_2PO_4, 0.50 g; K_2HPO_4, 0.50 g; $MgSO_4.7H_2O$, 0.22 g; acetic acid, 2.3 mL; $FeSO_4.7H_2O$ 10 mg; para amino benzoic acid, 8 mg; biotin, 0.08 mg, nickel (II) chloride, 3 mg; zinc chloride, 60 mg; nitriloacetic acid, 0.2 g. The pH of the medium was adjusted to 6.0 with ammonia. For solid media preparation, 1.5 % agar was added to liquid media. The media were supplemented

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as needed with the appropriate antibiotic at the following concentrations: for *C. acetobutylicum*, erythromycin (Ery) at 40 µg/mL, clarithromycin (Clari) at 40 µg/mL, and thiamphenicol (Tm) at 10 µg/mL; for *E. coli*, carbenicillin (Cb) at 100 µg/mL and chloramphenicol (Cm) at 30 µg/mL. Stocks of 5-Fluorouracil (5FU) and uracil (Sigma Aldrich) were prepared at 0.1 M in dimethyl sulfoxide (DMSO) and stored at - 20°C.

2.3. DNA manipulation

Genomic DNA was extracted from *C. acetobutylicum* strains using GenEluteTM Bacterial Genomic DNA Kits (Sigma-Aldrich). Plasmids DNA were extracted from *E. coli* using NucleoSpin® Plasmid or NucleoBond® Xtra Midi kits (Macherey-Nagel). Phusion DNA Polymerase (New England Biolabs (NEB)) was used to generate PCR products according to the supplier's standard protocols. OneTaq® 2X Master Mix with Standard Buffer (NEB) was used to screen colonies by PCR according to the supplier's standard protocols. Restriction enzymes, antartic phosphatase, T4 DNA ligase (NEB) were used according to the manufacturer's instructions. DNA fragments were purified from agarose gel using Zymoclean™ Large Fragment DNA Recovery Kit (Zymo Research). DNA PCR fragments were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). Plasmid DNA and DNA PCR fragments were sequenced using sanger method (Eurofins Genomics).

*2.4. Design of pyrR**

The nucleotide sequence of *pyrR* gene (*CA_C2113*) was codon optimized to create a synthetic *pyrR* gene, named *pyrR**, with low identity to the wildtype *pyrR*, in which some codons have been replaced by other codons with closed frequency in *C. acetobutylicum* using https://gcua.schoedl.de/. The synthetic gene was then synthesized (Geneart, Thermofisher Scientific).

Table 3. Nucleotide sequence of wild-type *pyrR* gene and *pyrR*gene*.

Gene name	Nucleotide sequence
wild-type *pyrR*	ATGAATTTAAAAGCAAAGATTTTAGATGATAAGGCTATGCAAAGGACTTTGACCAGAAT AGCACATGAAATTATAGAAAAGAATAAAGGTATAGATGATATAGTACTAGTAGGAATA AAGAGAAGAGGAGTTCCAATAGCCGATAGAATAGCGGATATAATTGAAGAAATAGAA GGAAGTAAGGTTAAGCTAGGAAAAGTAGATATAACCTTATATAGAGACGATTTGTCTAC GGTAAGTTCTCAACCAATAGTAAAAGATGAGGAAGTATATGAAGATGTAAAGGATAAG GTAGTAATACTTGTTGATGACGTTTTATATACAGGAAGAACATGCAGAGCAGCCATAGA AGCTATTATGCATAGAGGAAGACCAAAGATGATACAGCTTGCAGTTTTGATAGATAGGG GACATAGAGAACTTCCTATAAGGGCAGATTATGTTGGAAAAAATGTACCTACATCAAA AAGTGAATTGATATCGGTAAATGTTAAAGGAATAGATGAAGAGGATTCAGTAAACATTT ATGAGTTGTAG
synthetic *pyrR* (*pyrR**)	ATGAATCTTAAAGCTAAGATTCTTGATGATAAGGCAATGCAAAGGACACTAACCAGAA TAGCTCATGAAATAATAGAAAAGAATAAAGGAATAGATGATATAGTTTTGGTTGGAAT AAAGAGAAGAGGAGTACCTATAGCGGATAGAATAGCCGATATAATAGAAGAAATAGA AGGATCAAAGGTAAAGTTGGGAAAAGTTGATATAACCCTTTATAGAGACGATCTATCA ACCGTTTCAAGTCAACCTATAGTTAAAGATGAGGAAGTTTATGAAGATGTTAAGGATAA GGTTGTTATATTAGTTGATGACGTACTTTATACTGGAAGAACTTGCAGAGCTGCGATAGA AGCAATAATGCATAGAGGAAGACCTAAGATGATACAGTTAGCTGTACTAATAGATAGG GGACATAGAGAACTACCAATAAGGGCTGATTATGTAGGAAAAAATGTTCCAACTAGTA

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AATCAGAATTGATATCCGTAAATGTAAAAGGAATAGATGAAGAGGATAGTGTTAACAT ATATGAGCTATAG

2.5. Construction of pCat-upp-pyrR^{mut}

This plasmid was constructed, based on the pCat-*upp* described by Foulquier *et al*. [5] by introducing the *pyrR^{mut}* gene containing the mutation *g.344C>T* encoding the PyrR A115V protein found in our mutant strain. The mutant *pyrR* gene was PCR amplified from total DNA from *CAB1060* as template using the PSC 75 and PSC 76 primers containing *Bam*HI restriction sites. The PCR fragment and the pCat-*upp* were digested by *Bam*HI. The plasmid was dephosphorylated with antarctic phosphatase and the PCR fragment and the plasmid were purified. The PCR fragment was cloned by ligation into the plasmid to obtain pCat-*upp-pyrR^{mut}*.

*2.6. Construction of pCat-upp-pyrR**

This plasmid was constructed from the pCat-*upp* described by Foulquier *et al.* [5] by introducing the synthetic *pyrR** gene under the control of the thiolase promoter. The entire pCat-*upp* plasmid was amplified with PSC 51 and PSC 52 for linearization. The *pyrR** gene was PCR amplified primers using synthetic *pyrR* gene (from Geneart) as template. A first PCR was performed with PSC 61 and PSC 62 primers to amplify the *pyrR* gene with its native RBS and downstream homology arms. The first PCR fragment was then purified. A second PCR was performed on the first PCR product with PSC 72 and PSC 62 to introduce upstream homology arm. The final PCR fragment of *pyrR** was cloned into linearized pCat-*upp* plasmid by recombination using the GeneArt™ Seamless Cloning and Assembly Kit (Thermofisher Scientific).

2.7. Construction of pCat-upp-pyrR- Δldh*

This plasmid was constructed based on the *pCat-upp-pyrR** (this study) and the pCat-*upp-Δldh* described by Soucaille *et al.* in the patent WO 2016/042160 A1 [20]. The *pCat-upp-pyrR** plasmid was linearized by digestion with *Bam*HI restriction enzyme and dephosphorylated with antartic phosphatase. Then the pCat-*upp-Δldh* plasmid was digested with *Bam*HI and the fragment containing the *ldh* homology arms was purified from agarose gel. The two fragments were then ligated using T4 DNA Ligase.

2.8. Construction of pCat-upp-pyrR-~~Δ~~ldh::sadh-hydG*

This plasmid was constructed based on the pCat-*upp-pyrR*-Δldh* plasmid (this study) by introducing an operon composed of *sadh* and *hydG* genes (GenBank: AF157307.2), with their own RBS, under the control of the *ldh* promoter. The pCat-*upp-Δldh* was digested by *StuI*, dephosphorylated and purified. *sadh* and *hydG* were amplified from a synthesized *sadh_hydG* genes (Genart, Thermofisher) as template. A first PCR was performed with PSC 104 and PSC 105 to amplify *sadh_hydG* genes and introduce the *ldh* promoter region upstream of *sadh* and the *ldh* terminator downstream of *hydG*. After purification, the first PCR product was then amplified with PSC 106 and PSC 107 to introduce upstream and downstream homology arms to recombine with the pCat-*upp-pyrR*-Δldh* plasmid. The final PCR fragment was cloned into the pCat-*upp-pyrR*-Δldh* by recombination using the GeneArt™ Seamless Cloning and Assembly Kit (Thermofisher Scientific).

2.9. Construction of pSOS95-pyrR

This plasmid was constructed based on the pSOS95 plasmid described by Raynaud *et al*. [18] by introducing the native *pyrR* gene under the control of the thiolase promoter. The pSOS95 plasmid was digested with *Bam*HI and *Sfo*I and purified from agarose gel. The *pyrR* gene was amplified using total DNA from the strain *Δcac1502* as template. The PSC 58 and PSC 46 used for this amplification introduced the *Bam*HI restriction site upstream of the *pyrR* gene RBS and the *Sfo*I restriction site

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downstream of the *pyrR* gene. The PCR fragment was digested with *Bam*HI and *Sfo*I, purified. And cloned in the pSOS95 plasmid by ligation using T4 DNA Ligase.

2.10. Transformation protocol

Transformation of *C. acetobutylicum* was performed by electroporation according to the following protocol. From a culture of *C. acetobutylicum* in CGM at A_{620} between 1 and 2, a new serum bottle with 50 ml of CGM was inoculated at A_{620} of 0.1. When the culture reached A_{620} between 0.6 and 0.8, the culture has been placed on ice for 30 minutes and transferred under an anaerobic chamber (Jacomex) where all the following manipulations were performed. The cells were then harvested by centrifugation at 7000g for 15 minutes and washed in 10 mL of ice-cold electroporation buffer (EB) composed of 270 mM sucrose, 10 mM MES hydrate at pH 6.0. Then the pellet was resuspended in 500 µL of EB and cells were transferred into a sterile electrotransformation vessel (0.40 cm electrode gap x 1.00 cm) with 5-100 µg plasmid DNA. A 1.8 kV discharge was applied to the suspension from a 25 µF capacitor and a 400 Ω resistance in parallel using the Gene Pulser (Bio-Rad Laboratories, Richmond, CA). Cells were transferred directly to 10 mL of warm CGM and incubated for 6 hours at 37°C before plating on RCM supplemented with the required antibiotics.

2.11. Microbiological enumeration on solid media

C. acetobutylicum was cultivated in CGM until reaching an A_{620} of 0.55. Subsequently, the culture was transferred to an anaerobic chamber, and 100 µL of various dilutions (10^{-1} to 10^{-6}) of the culture were plated onto CGM MES or SM MES agar supplemented with the necessary antibiotics, ranging from 0 µM to 200 µM for 5FU and from 0 µM to 50 µM for uracil. Following incubation at 37 °C for a period of 1 to 4 days, the resulting colonies were counted.

2.12. 5FU selection protocol

C. acetobutylicum was cultivated in CGM until reaching an A_{620} of 0.55. The spreading protocol was the same as described in subsection *2.11*. After isolation, 50 colonies were picked and plated on a fresh plate with the same concentration of 5FU. The plates were incubated at 37 °C from 1 to 2 days. The colonies were then picked and patched onto plates with and without thiamphenicol to determine the percentage of double crossing-over. Colonies showing a double crossover phenotype were screened by PCR to verify that genome editing occurred.

2.13. Locus verification in C.acetobutylicum after metabolic engineering

After the genome edition of *C. acetobuylicum*, the different loci were checked by PCR amplification. In order to check for the insertion of point mutations, the genome was amplified by PCR and the PCR fragment obtained was sent for sequencing.

Primers name	Function
PSC 39 – PSC 40	*pyrR* gene sequencing
PSB 384 – PSB 385	*ldh locus* verification

2.14. Analytical procedures

Culture growth was monitored by measuring optical density over time using a spectrophotometer at A_{620}. Glucose, acetate, butyrate, acetone, isopropanol, ethanol and butanol concentrations were measured using High Performance Liquid Chromatography (HPLC) analysis (Agilent 1200 series, Massy, France). The separations were performed on a Biorad Aminex HPX-87H column (300 mm x 7.8 mm), and detection was achieved using either a refractive index measurement or a UV absorbance measurement (210 nm). The operating conditions were as follows: temperature, 14 °C; mobile phase, H_2SO_4 (0.5 mM); and flow rate, 0.5 ml/min.

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3. Results

3.1. Identification of the 5FU resistance of the strain

The strain *CAB1060*, as detailed by Nguyen et al. [19], was developed through the utilization of the *upp*/5FU counterselection method. This genome editing method, initially described by Croux et al. [8] and originally employing a replicative plasmid, was subsequently adapted into a suicide plasmid format, as outlined by Foulquier et al. [5].

After several genome modifications and the use of 5FU as a counter selection marker, the strain became no longer sensitive to 5FU even at a concentration of 1 mM and its entire genome was sequenced. Many random mutations were found including one that particularly caught our attention, the mutation *g.344C>T* located in the *pyrR* gene that introduced a A115V mutation in the PyrR protein. PyrR is a potential repressor of the pyrimidine operon and it has been shown in other organisms that mutations in the *pyrR* gene or a complete deletion of the *pyrR* gene can lead to 5FU resistance [16,17,25]. The hypothesis put forward was that the mutated protein no longer performed its regulatory function and the pyrimidine operon was overexpressed. This could result in overproduction of UMP, which protect bacteria from the toxic effects of 5FUMP. Based on these data, we hypothesized that the observed mutation could be responsible of 5FU resistance in the strain.

3.2. Evaluation of the 5FU sensitivity of C. acetobutylicum strain (non mutated in the pyrR gene)

The viability of the *C. acetobutylicum Δcac1502* strain was evaluated both on rich medium (CGM MES) and on synthetic medium (SM MES) in the presence of various concentrations of 5FU (**Table 4**). In the absence of 5FU, no significant differences could be observed between the two media. On the otherhand, the 5FU sensitivity of the strain was much higher when spread on SM MES (**Table 4**) compared to rich media.

Table 4. Bactericidal effect of 5FU on *cac1502* on CGM MES and SM MES

5FU concentration (µM)	CGM MES (UFC/mL)	SM MES (UFC/mL)
0	2.06 ± 0.41E7	1.75 ± 0.25E7
5	1.65 ± 0.34E7	0
25	3.02 ± 0.52 E6	0
50	1.45 ± 0.28E6	0
100	0	0
200	0	0

According to Singh et al., exogenous uracil protects the bacteria from the toxicity of 5FU. This was validated in *Mycobacterium tuberculosis* in which the supplementation of uracil at 15.6 µM protected bacteria up to 25 µM 5FU [17]. On the basis of the literature, we have assumed that the yeast extract added to the rich medium contains between 25 µM and 50 µM of uracil [26]. Therefore, we tested the protective effect of uracil against 5FU in *C. acetobutylicum* by addition of uracil to the synthetic medium containing 5 µM of 5FU (**Table 5**).

As expected, we observed a protective effect of uracil but at very low concentration (5 µM) which is 5 to 10-fold lower than the expected concentration due to the addition of yeast extract. Based on these results, and in order to minimize the concentrations of 5FU used, all the following experiments were carried out in synthetic medium.

Table 5. Protective effect of uracil against 5FU in *C. acetobutylicum Δcac1502* strain. 0.1 ml of a 10^{-1} dilution of a CGM culture were spread on the different SM MES plates.

Uracil concentration (µM)	Number of colonies (5 µM 5FU)
0	0
5	Layer
12.5	Layer

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25	Layer
50	Layer

3.3. Construction of a C. acetobutylicum strain with pyrR^{mut} and 5FU resistance validation

First, we tried to reproduce the occurrence of the mutation *g.344C>T* in *pyrR* gene obtained in the 5FU resistant *CAB1060* strain. To test for the occurrence of mutations, we plated 2 x 10^8 cells of *C. acetobutylicum* strain *Δcac1502* on SM MES agar plates with high concentrations of 5FU (25-50 µM). The occurrence of mutations in the *pyrR* and in *upp* genes were analyzed. No mutation in the *upp* was observed, but many mutations appeared at different positions in the *pyrR* gene (**Table 6**). However, the original mutation found in the *pyrR* gene in the 5FU resistant *CAB1060* strain has not been obtained. To ensure that the mutation *g.344C>T* was responsible for the 5FU resistance of the *CAB1060* strain, it was decided to introduce it into the *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535* using a pCat-*upp-pyrR*^{mut}. After the transformation with pCat-*upp-pyrR*^{mut} and the 5FU selection, the insertion of *g.344C>T* mutation in *pyrR* gene was checked by sequencing. In the *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535pyrR*^{mut} *obtained*, the pCat *upp-Δldh* was then integrated at the *ldh* locus. In a strain with a WT *pyrR* gene, the *upp* gene contained in the suicide plasmid should be sufficient to get a sensitivity to 5FU of the strain. But, as shown in **Table 7**, in the strain mutated in the *pyrR* gene, most of the cells were resistant to high concentrations of 5FU. This result showed that the single *g.344C>T* mutation in *pyrR* gene was sufficient to obtain a strain resistant to 5FU and confirmed our hypothesis concerning the 5FU resistance of the *CAB1060* strain.

Table 6. Spontaneous mutations founded in PyrR after 5FU exposition

5FU concentration (µM)	Amino acid change	Nucleotide change
25	R124G	*g.370A>G*
25	A47D	*g.140C>A*
50	R136X	T addition in aa 132
50	P45L	*g.134C>T*
50	V85X	G deletion in aa 85
50	E23K	*g.67G>T*

Table 7. Mutated *pyrR* strain viability on 5FU while maintaining a pCat-*upp*

	pCat-*upp*-Δ*ldh*		
5FU concentration (µM)	0	25	50
SM MES + Tm (UFC/mL)	9.52E7	9.14E7	8.46E7

3.4. Restoration of the 5FU sensitivity in a strain mutated in pyrR gene

3.4.1 Restoration of 5FU sensitivity to 5FU resistant strains by overexpressing *pyrR gene on a replicative plasmid*

This first method consists of using a replicative plasmid overexpressing a native non-mutated version of *pyrR*, to overcome the problems of 5FU selection when a strain mutated in the *pyrR* gene has already integrated a pCat-*upp*. Viability of *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535 pyrR*^{mut} with the pCat-*upp-Δldh* integrated at the *ldh* locus and the pSOS95-*pyrR* replicative plasmid was tested in the presence of erythromycin for the maintenance of the replicative plasmid overexpressing *pyrR* and/or thiamphenicol for the maintenance of the pCAT-*upp-Δldh* suicide vector. Whereas,

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previously, in the presence of Tm, most of the cells were resistant to 5FU (**Table 7**), by just overexpressing a WT *pyrR* gene in the same strain, we restore its sensitivity to 5FU even at low concentrations (**Table 8**).

Table 8. Viability of *Δcac1502ΔuppΔcac3535pyrR*^{mut} strain with a pCat-*upp* plasmid and a replicative pSOS95-*pyrR* plasmid

	pCat-*upp*-*Δldh* + pSOS95-*pyrR*			
5FU concentration (µM)	0	5	10	25
SM MES + Ery (UFC/mL)	1.20E7	3.80E4	2.84E4	2.10E4
SM MES + Ery + Tm (UFC/mL)	6.28E6	0	0	0

Indeed, by overexpressing the *pyrR* gene, a selection with 5FU at a concentration of 5µM is sufficient to allow a high frequency (> 85 %) of double crossing-over in a *pyrR* mutant strain with an integrated pCat-*upp* in the genome. This frequency was even higher than 95 % at 10 µM of 5FU (**Table 9**). These results showed that it was possible to reverse 5FU resistance of a strain mutated in the *pyrR* gene with a pCat-*upp* integrated into its genome. The use of a replicative plasmid overexpressing a native *pyrR* gene allowed the excision of the suicide vector at very low 5FU concentrations.

Table 9. Viability and frequency of double crossing-over of *Δcac1502ΔuppΔcac3535pyrR*^{mut} strain with a pCat-*upp* plasmid and a replicative pSOS95-*pyrR* plasmid after 5FU selection

	pCat-*upp*-Δ*ldh* + pSOS95-*pyrR*		
5FU concentration (µM)	5	10	25
Picked colonies viability (%)	86	90	84
Picked colonies Tm sensitivity (%)	86	96	98

3.4.2 Restoration of 5FU sensitivity to 5FU resistant strains by overexpressing a synthetic *pyrR** gene on a suicide vector

The second method to overcome the problems of 5FU selection when a strain is mutated in the *pyrR* gene consists of using a codon optimised (for low identity to the wild type gene) version of the *pyrR* gene (*pyrR**) directly on the suicide plasmid.

Viability of the *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535 pyrR*^{mut} with a *pCat-upp-pyrR*-Δldh* integrated at the *ldh* locus was tested in the presence or absence of Tm for the maintenance of the suicide vector and no difference in viability was observed (**Table 10**).

Whereas previously, in the strain with the pCat-*upp-Δldh*, most of the cells were resistant to 5FU in the presence of Tm (**Table 7**), with the same suicide plasmid but containing the *pyrR**, no colony were obtained (**Table 10**). These results validated the functionality of the synthetic *pyrR** gene and showed that a single copy of *pyrR** was sufficient to restore the sensitivity to 5FU of a resistant strain.

Table 10. Viability of *Δcac1502ΔuppΔcac3535pyrR*^{mut} strain with pCat-*upp-pyrR*-Δldh* plasmids

	pCat-*upp-pyrR*-Δldh*		
5FU concentration (µM)	0	25	50
SM MES (UFC/mL)	8.80E7	4.24E4	3.88E4

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SM MES + Tm (UFC/mL)	7.60E7	0	0

Indeed, by overexpressing the *pyrR** gene directly on the suicide vector, a selection with 5FU at a concentration of 5 µM is sufficient to allow a high frequency (> 90 %) of double crossing-over in a *pyrR* mutant strain. This frequency was even higher than 98 % with 10 µM of 5FU (**Table 11**). Thus, overexpressing a single copy of *pyrR** was sufficient to restore the sensitivity to 5FU of a resistant *pyrR*^{mut} strain.

Table 11. Viability and frequency of double crossing-over of *Δcac1502ΔuppΔcac3535pyrR*^{mut} strain with pCat-*upp* and a pCat-*upp-pyrR** plasmids after 5 FU selection

	pCat-*upp-Δldh*				pCat-*upp-pyrR*-Δldh*			
5FU concentration (µM)	5	10	25	50	5	10	25	50
Picked colonies viability (%)	100	98	98	76	88	96	60	52
Picked colonies Thiamphenicol sensitivity (%)	0	6	75	89	90	98	100	100

*3.5. Preventive use of pyrR**

After demonstrating the efficiency of overexpressing *pyrR* curatively in a 5FU resistant strain, we wondered if we could use the same method preventively in a strain "non-mutated" for the *pyrR* gene to avoid the development of 5FU resistance. However, we first wanted to check that the overexpression of *pyrR* did not result in a too high 5FU sensitivity. Viability of the *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535* with a *pCat-upp-pyrR*-Δldh* integrated at the *ldh* locus plasmid was assessed at low 5FU concentrations (5 and 10 µM) and the results showed that it was not affected (**.**

Table 12). In parallel, the frequency of double crossing-over, evaluated though the sensitivity to Tm, was shown to reach 100 % (**Table 12**). These results confirmed that overexpressing *pyrR* can be a method used both curatively and preventively.

Table 12. Viability and frequency of double crossing-over of *Δcac1502ΔuppΔcac3535* strain with the pCat-*upp-pyrR**/ 5 FU counter selection system

	pCat-*upp*-pyrR*-Δ*ldh*	
5FU concentration (µM)	5	10
Picked colonies viability (%)	98	100
Picked colonies Thiamphenicol sensitivity (%)	100	100

3.6. Insertion of sadh and hydG from C. beijerinckii at ldh locus

After validating the new *C. acetobutylicum* genome editing method using pCat-*upp-pyrR**, we wanted to test this tool to both delete and replace genes in a single step. The goal was to use the pCat-*upp-pyrR*-ldh::sadh-hydG* suicide plasmid to delete the *ldh* gene and replace it with an operon, to produce isopropanol in the *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535pyrR*^{mut}. This operon is composed of *sadh* and *hydG* genes from *C. beijerinckii* (**Figure 1)** which encoded for a primary-secondary alcohol dehydrogenase (SADH) [27] and a putative electron transfer protein (HydG) [28] from *Clostridium beijerinckii NRRL B593*, respectively. In the **Figure 2** the different stages involved in

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the integration of the "isopropanol operon" at the *ldh* locus are described. After integration of the suicide vector at the *ldh locus* and 5FU selection at 5 µM, Tm sensitive colonies were selected and screened by PCR using external primers. Primers were designed outside the homology arms, to discriminate between WT revertants and mutants with the desired genotype (*Δcac1502ΔuppΔcac3535 pyrR*^{mut} *ldh::sadh hydG*) (**Figure 3**). After picking and patching colonies, a second time on 5FU, we obtained 92 % of viable colonies using 5FU at 5 µM. After selecting on Tm, all viable clones were shown to be sensitive to 5FU, i.e. 100 % excision of pCat-*upp-pyrR** was achieved (**Table 13**). Both WT revertants and mutants with the desired genotype were obtained. In the figure 3, an example of two mutant clones (11 & 12) and one WT revertant clone (clone 15) is shown. The pCat-*upp-pyrR** tool can therefore be used to delete genes and replace them with others in a single step.

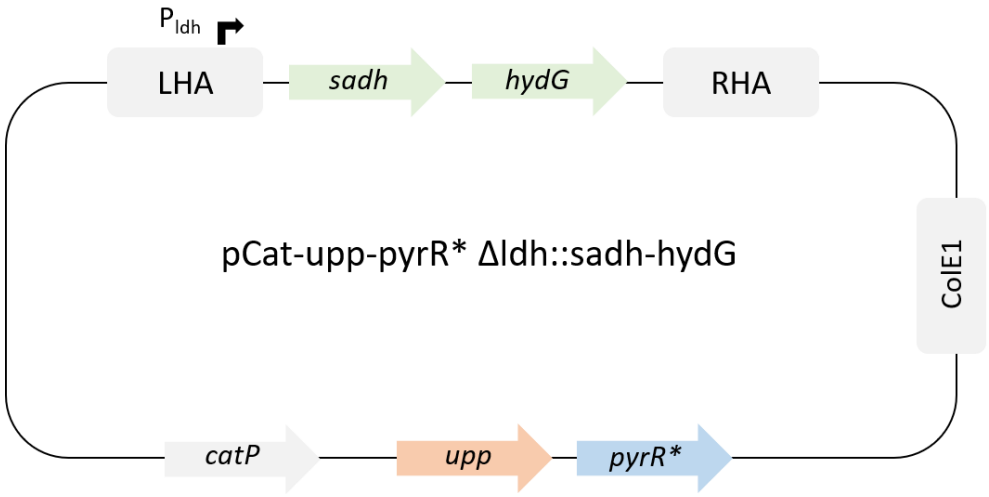

Figure 1. Suicide plasmid for *ldh* replacement by *sadh* and *hydG* from *C.beijerinckii*

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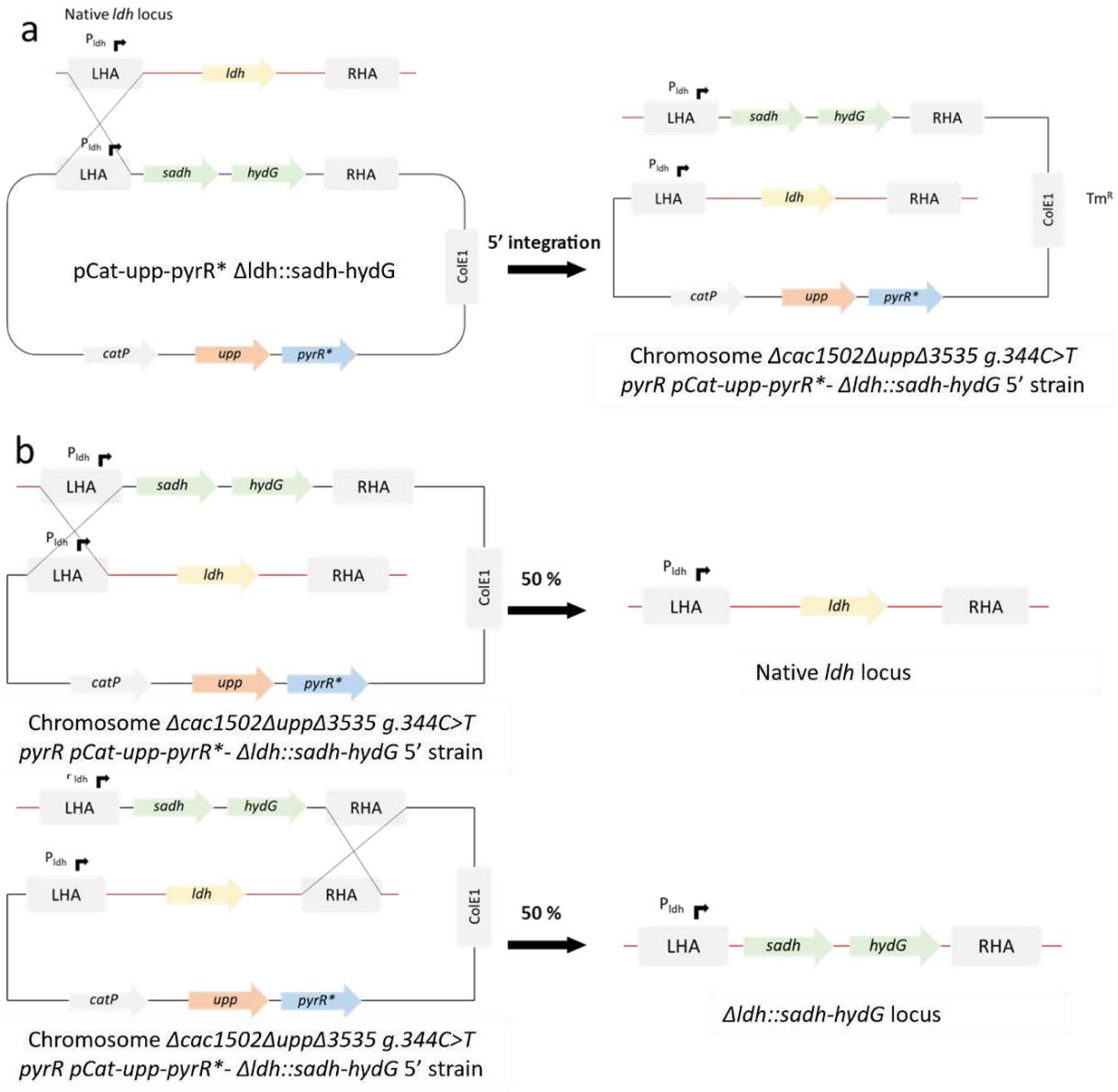

Figure 2. Diagram representing the replacement of *ldh* by *sadh and hydG* from *C. beijerinckii* by allelic ex-change in *Δcac1502ΔuppΔcac3535 pyrR*^{mut}. LHA: left homology arm; RHA: right homology arm. **a** 5′ integration of the suicide plasmid. The integrants are selected on thiamphenicol. **b** Double crossing-over induced by 5FU that cause the excision of the suicide plasmid.

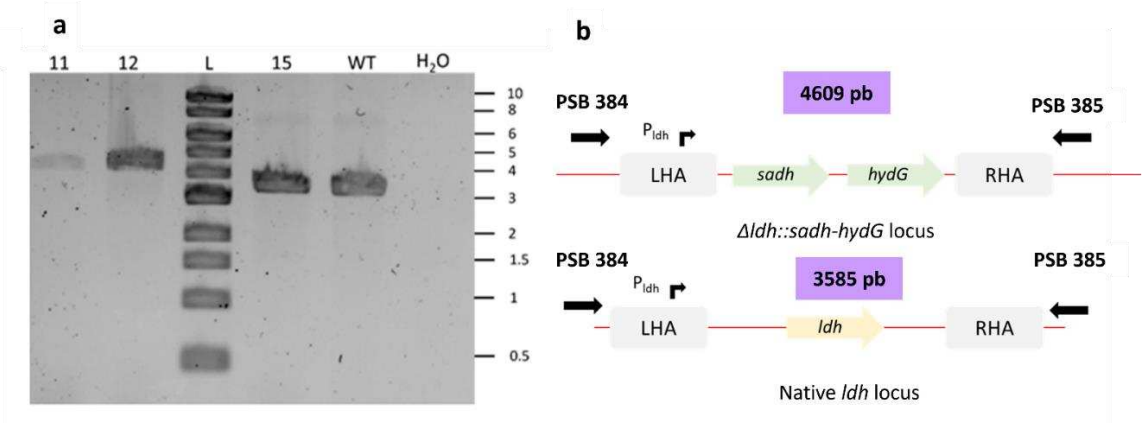

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Figure 3. a Screening of *Δldh::sadh hydG* mutant. The colonies were screened using PSB 384 and PSB 385 primers. Ladder: 1kb DNA ladder provided by New England Biolabs. **b** Schematic representation of *Δldh::sadh hydG* locus and native *ldh locus*.

Table 13. Viability and frequency of double crossing-over of *Δcac1502ΔuppΔcac3535pyrR*^{mut} strain with pCat--*pyrR*-Δldh::sadh-hydG* after 5FU selection.

	pCat-*pyrR*-Δldh::sadh-hydG*
5FU concentration (µM)	5
Picked colonies viability (%)	92
Picked colonies Thiamphenicol sensitivity (%)	100

3.7. Culture of C. acetobutylicum on synthethic medium for isopopanol production

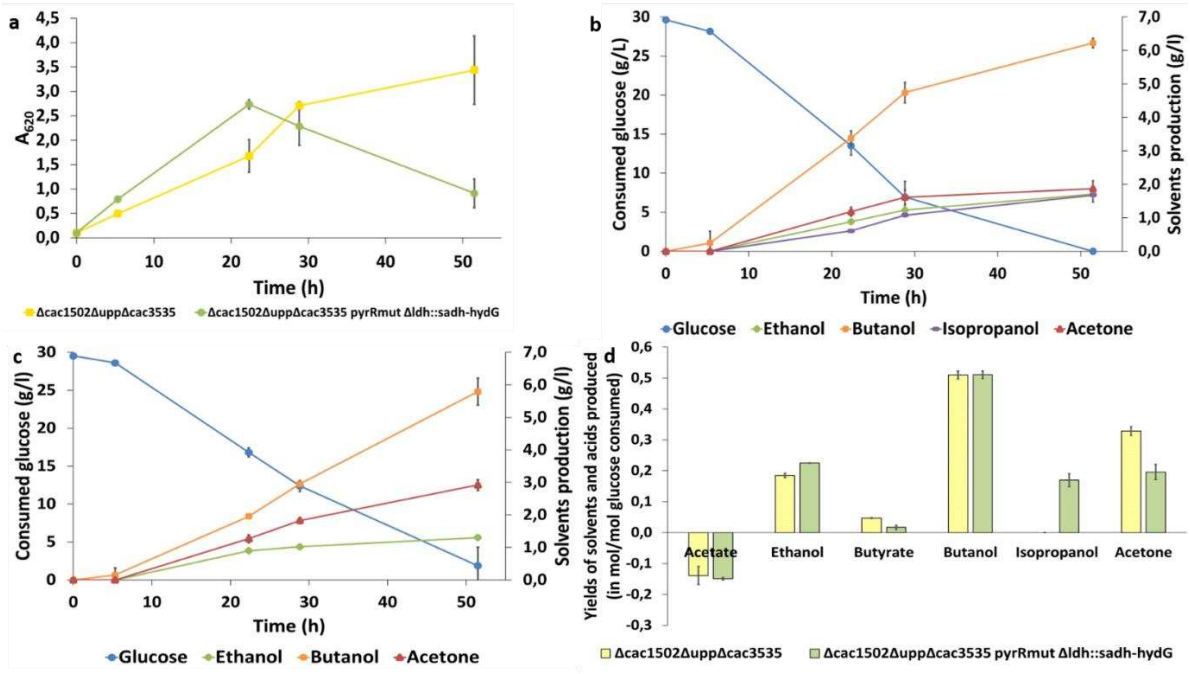

Figure 4. a Growth profile of the *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535 pyrR*^{mut} *Δldh::sadh-hydG* and *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535* on SM **b** Monitoring glucose consumption and solvents production over time of *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535 pyrR*^{mut} *Δldh::sadh-hydG* **c** Monitoring glucose consumption and solvents production over time of *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535* **d** Molar yields of solvents production. All the measurements shown are mean average (*n*=2). Errors bars represent the standard deviation.

To validate the functionality of the "isopropanol operon", the *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535 pyrR*^{mut} *Δldh::sadh-hydG* and the control strain, without isopropanol production pathway, were then cultured in synthetic media in serum bottles at an initial pH of 6.0. After 52 hours of culture, solvents and acids productions and final product yields were evaluated. The strain *Δcac1502ΔuppΔcac3535 pyrR*^{mut} *Δldh::sadh-hydG* had a growth rate of 0.15 h^{-1} during the first 25 h of cultures and reached an A_{620} maximum of 2.5 and then entered the lysis phase after 25 h of culture (**Figure 4 a**). The strain *Δcac1502ΔuppΔcac3535*, on the other hand, had a growth rate of 0.13 h^{-1} during the first 25 h of cultures, reached an A_{620} maximum of 2.9 and had not entered the lysis phase after 50 h of culture (**Figure 4 a**). Concerning isopropanol, its production was only detected in the *C. acetobutylicum* strain *Δcac1502ΔuppΔcac3535 pyrR*^{mut} *Δldh::sadh-hydG* . As expected, final molar yields showed that the isopropanol production observed is associated to a decreased acetone production in comparison to the control strain (acetone production is 1.7-fold-time higher in the control strain) (**Figure 4 d**). The isopropanol/acetone molar ratio was 0.87 in *Δcac1502ΔuppΔcac3535 pyrR*^{mut} *Δldh::sadh-hydG* strain. This strain also had higher acetate consumption, lower butyrate

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production and slightly higher ethanol production (**Figure 4 d**). The insertion of the "isopropanol operon" with the new pCat-*upp-pyrR**/5FU system has been confirmed by the production of isopropanol in the strain *Δcac1502ΔuppΔcac3535 pyrR*^{mut} *Δldh::sadh-hydG*.

4. Discussion

In the present study, we have shown that overexposure of *C. acetobutyicum* to 5FU can make it resistant to this drug. Spontaneous mutations are induced in the bacterial chromosome, notably in the *pyrR* gene. As described in other publications, the *pyrR* gene encodes for PyrR, the repressor of the pyrimidine operon [16,17,25]. The presence of a mutation in this protein can lead to the cessation of its function, resulting in an overproduction of UMP. This overproduction of UMP can protect against the harmful effects of 5FUMP, a molecule that is toxic to bacteria. We observed the appearance of a mutation in the PyrR protein of *C. acetobutylicum* that completely avoids selection of the double crossing-over step when using the pCat*upp*/5FU system. The principle of the *upp*/5FU system was based on the use of a strain in which the *upp* gene has been deleted and the use of 5FU as a counter selection agent. The *upp* gene encoding for an uracil phosphoribosyl transferase (UPRTase) that could convert uracil to UMP and 5FU to 5FUMP. 5FUMP is a molecule that prevents cells from producing intermediates needed for DNA synthesis, thereby causing cell death (**Figure 5**) [17].

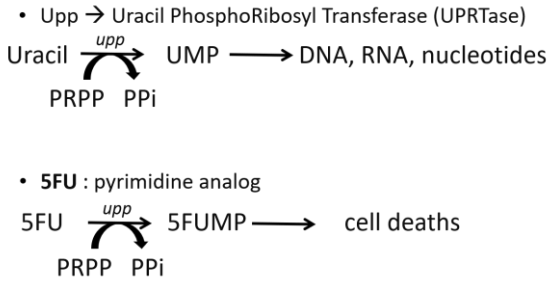

Figure 5. Metabolism of 5FU. PRPP, phosphoribosyl pyrophosphatase; PPi, pyrophosphatse; UMP, uridine monophosphate; 5FU, 5-fluorouracile; 5FUMP, 5-fluorouridine monophosphate.

When the A115V PyrR mutation was discovered in *C. acetobutylicum*, we compared it with other mutations in homologous proteins already described in the literature. A conserved protein sequence required in PRPP binding can be found in many species. Ghode and Singh described the G125V and R126C mutations *in M. tuberculosis* as being in this conserved zone [16,17]. According to Ghode, a mutation in this region could block the production of 5FUMP. As A115V PyrR mutation is situated close to this site, it could be one of the reasons why our strain is resistant to 5FU (**Figure 6**). In addition, *pyrR* encodes the regulatory protein of the pyrimidine operon. According to Ghode and Fields [16,29], mutations in PyrR of *M. tuberculosis* or *M. smegmatis*, or when PyrR is completely deleted in *B. subtilis* the protein no longer performs its regulatory function and the pyrimidine operon is overexpressed [25]. This results in overproduction of UMP, which protects the bacteria from the toxic effects of 5FUMP.

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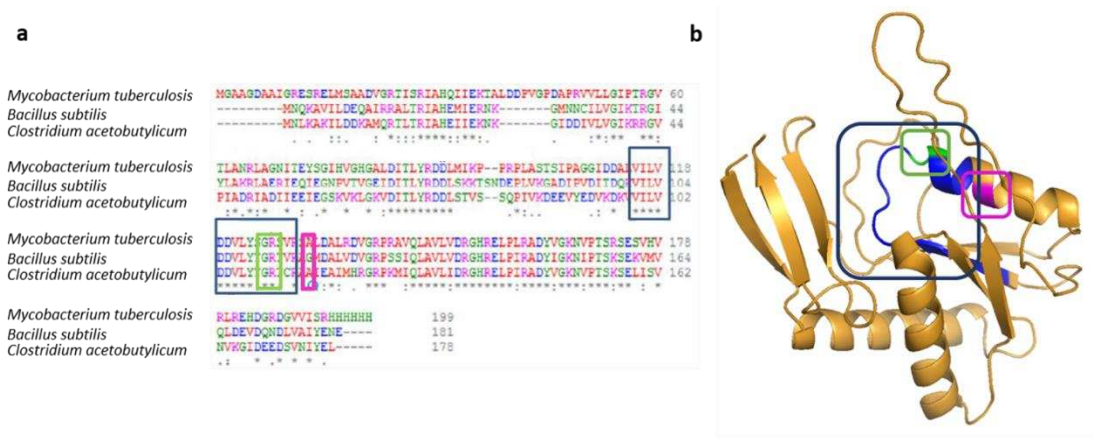

Figure 6. **a** Multiple sequences alignment of PyrR. **b** Cartoon diagram of PyrR protein of *C.acetobutylicum* predicted by Alpha-fold [30,31]. The blue boxes show the amino acids implied in PRPP binding. The green boxes highlight G125 and R126 sites described by Ghod and Singh [16,17]. The pink boxes represent the A115 position, where *C.acetobutylicum* was mutated.

To overcome this problem, we had to revise the protocol previously described by our team. First, we realized that the composition of the medium plays an important role in the resistance of the strain to 5FU. It is preferable to use a synthetic medium that is not supplemented with uracil. In fact, the yeast extract present in CGM brings uracil into the medium and thus protects against the toxic effect of 5FUMP. This hypothesis was tested by adding low concentrations of uracil to the synthetic medium. Bacterial growth was no longer affected by the presence of 5FU in the medium. After optimizing the medium for 5FU selection, we constructed two plasmids to restore the sensitivity of the strain to 5FU. The first plasmid is a replicative plasmid overexpressing a native version of *pyrR*. It is used to overcome the problems of 5FU selection when a strain mutated in the *pyrR* gene has already integrated a pCat-*upp*. The second plasmid is a pCat-*upp* containing a codon optimised version of the *pyrR* gene, called *pyrR**. The 5FU selection problem for genome editing is directly bypassed by this method. With both of these strategies, the concentration of 5FU could be reduced from 1 mM to 5 µM, thus minimizing the risk of spontaneous mutation. Both the use of *pyrR** and the use of the SM medium will be beneficial for all the counterselection methods involving *upp* and 5FU, as well as those utilizing *codA* and 5FC [4] .

Once the new protocol was established, we demonstrated that it was possible to both delete and insert genes of interest in *C. acetobutylicum Δcac1502ΔuppΔcac3535 pyrR^{mut}* strain in a single step using the pCat-*upp-pyrR**/5FU system. An isopropanol production pathway from *C. beijerinckii* was inserted at the *ldh* of *C. acetobutylicum Δcac1502ΔuppΔcac3535 pyrR^{mut}Δldh::sadh-hydG* strain using this technique. We decided to insert the *sadh* and *hydG* genes from *C. beijerinckii NRRL 593* following a publication by Dusséaux et al. [27]. SADH is an NADPH-dependent primary-secondary alcohol dehydrogenase that catalyzes acetone reduction and HydG is a putative electron transfer protein [28,32]. *hydG* was introduced into the *C. acetobutylicum Δcac1502ΔuppΔcac3535 pyrR^{mut} strain* genome at the same time as *sadh*, since these two genes are located in the same operon in *C. beijerinkcii NRRL 593*. It was assumed that the HydG activity would have a positive effect on the SADH activity, allowing the strain to obtain a better isopropanol production [28]. The final production of our *C. acetobutylium Δcac1502ΔuppΔcac3535 pyrR^{mut} Δldh::sadh-hydG* strain is lower than the one obtained by Dusséaux (up to 4.7 g.L^{-1} of isopropanol produced in a culture of 30 h) with a lower molar ratio of isopropanol/acetone [27]. This can be explained by the fact that in this strain both gene were overexpressed in a multi copy replicative plasmid and under the control of the *ptb* promoter, which is a stronger promoter than the *ldh* promoter [33]. The strain had also been grown in pH-regulated batch cultures, whereas we grew our strain in serum bottles without pH regulation. Better results would probably be obtained if the "isopropanol operon" was introduced at the *ptb buk* locus and if the pH of the culture was controlled.

Author Contributions: Conceptualization, C.F. and P.S.; methodology, E.B.; validation, C.F. and P.S.; resources, P.S.; data curation, C.F.; writing—original draft preparation, E.B and C.F.; writing—review and editing, C.F. and P.S.; supervision, P.S and C.F.; project administration, P.S.; funding acquisition, P.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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