A CRISPR/Anti-CRISPR Genome Editing Approach Underlines the Synergy of Butanol Dehydrogenases in *Clostridium acetobutylicum* DSM 792

François Wasels,[a]# Gwladys Chartier,[a] Rémi Hocq,[a] Nicolas Lopes Ferreira[a]

[a]Biotechnology Department, IFP Energies nouvelles, Rueil-Malmaison, France

Running title: Bdh contribution to solvent production

Keywords: *Clostridium acetobutylicum*, alcohol dehydrogenases, metabolic engineering, CRISPR-Cas9, Anti-CRISPR

Address correspondence to François Wasels, francois.wasels@ifpen.fr

ABSTRACT

Although *Clostridium acetobutylicum* is the model organism for the study of Acetone Butanol Ethanol (ABE) fermentation, its characterization has long been impeded by the lack of efficient genome editing tools. In particular, the contribution of alcohol dehydrogenases to solventogenesis in this bacteria has mostly been studied with the generation of single gene deletion strains. In this study, the three butanol dehydrogenases encoding genes located on the chromosome of the DSM 792 reference strain were deleted iteratively by using a recently developed CRISPR-Cas9 tool improved by using an anti-CRISPR protein encoding gene *acrIIA4.* Although the literature has previously shown that inactivation of either *bdhA*, *bdhB* or *bdhC* had only moderate effects on the strain, this study shows that clean deletion of both *bdhA* and *bdhB* strongly impaired solvent production, and that a triple mutant Δ*bdhA*Δ*bdhB*Δ*bdhC* was even more affected. Complementation experiments confirmed the key role of these enzymes, and the capacity of each *bdh* copy to fully restore an efficient ABE fermentation in the triple deletion strain.

IMPORTANCE

An efficient CRISPR-Cas9 editing tool based on a previous two plasmid system was developed for *C. acetobutylicum* and used to investigate the contribution of chromosome-encoded butanol dehydrogenase genes during solventogenesis. Thanks to the control of *cas9* expression by inducible promoters and of Cas9-gRNA complex activity by an anti-CRISPR protein, this genetic tool allows relatively fast, precise, markerless and iterative modifications in the genome of this bacterium, and potentially of other bacterial species. As an example, scarless mutants in which up to

three genes coding for alcohol dehydrogenases are inactivated were then constructed and characterized through fermentation assays. The results obtained show that in *C. acetobutylicum*, other enzymes than the well-known AdhE1 are crucial for the synthesis of alcohol, and more globally, to perform an efficient solventogenesis.

INTRODUCTION

Clostridium acetobutylicum has been established as the model organism for the study of ABE (Acetone - Butanol - Ethanol) fermentation. The need of sustainable alternatives to fossil-based ways of production of these chemicals has renewed the interest for this microorganism in the last decades. Since *C. acetobutylicum* is mainly producing alcohols, enzymes implicated in their production have been thoroughly studied.

In total, seven genes encoding enzymes with predicted alcohol dehydrogenase activity have been identified in the genome of *C. acetobutylicum* DSM 792 (also known as ATCC 824) (1). Genes CA_P0162 and CA_P0035 code for AdhE1 and AdhE2, respectively. Those enzymes contain both aldehyde dehydrogenase and alcohol dehydrogenase domains, although AdhE1 only retains aldehyde dehydrogenase activity (2). The five other identified genes (CA_C3298, CA_C3299, CA_C3375, CA_C3392, CA_P0059) code for alcohol dehydrogenases (1). The contributions of the different enzymes involved in the butanol flux has been recently characterized in *C. acetobutylicum* (2). During acidogenesis, the low amount of butyraldehyde produced by AdhE2 is reduced in butanol mainly by the BdhB dehydrogenase, but also by AdhE2 and BdhA. During solventogenesis, most of the butanol flux depends on AdhE1 which converts butyryl-CoA in butyraldehyde and on BdhB which converts butyraldehyde in butanol (2).

Out of the six enzymes with alcohol dehydrogenase activity identified, three (BdhA, BdhB and BdhC, encoded by CA_C3299, CA_C3298, and CA_C3392, respectively) have been shown to be NADPH-dependent, unlike AdhE2 which is NADH-dependent (2). Mutants of strains ATCC 824 and DSM 1731 have been constructed previously

in two different studies (3, 4), in which the three corresponding genes have been inactivated separately with ClosTron-based approaches (5), which showed that those genes have no or little contribution to solvent production. However, to date, there has been no study about the concomitant deletion of several copies of *bdh* genes.

In fact, difficulties to genetically modify this bacterium, mainly because of poor transformability and low homologous recombination (HR) efficiencies (6), still limit a full understanding of its metabolism and physiology. To overcome this issue, genetic tools dedicated to the *Clostridium* genus in general and to *C. acetobutylicum* in particular have been developed in the recent years (7). Lately, several research groups working on *Clostridium* have developed tools based on CRISPR-Cas (8–14). Briefly, the Cas nuclease is guided to a target site by a so-called guide RNA (gRNA), where it generates a double strand break (DSB). For most bacteria, including those belonging to the *Clostridium* genus, those DSBs are lethal when occurring in an essential nucleic acid, such as the chromosome, and are repaired through HR when a template is available. Providing a template altering the target site will allow the bacteria to repair its genome and to prevent another DSB. The strength of the strategy is that the bacteria can survive only if the target site is modified through the allele exchange event using the provided editing template.

In some cases (11), endogenous CRISPR-Cas systems have been exploited, but since none of them has been identified in *C. acetobutylicum* strains so far, the heterologous Cas9 from *Streptococcus pyogenes* is, to date, the most commonly used nuclease in this bacterium. Given the high toxicity of Cas9 and the low transformation and HR frequencies in *Clostridium*, the use of constitutive promoters for the expression of *cas9* and gRNA is generally not possible. In fact, only a few publications reported on the successful generation of mutants when using

constitutively expressed nucleases and gRNA in this genus (8, 14). To alleviate this issue, several groups used Cas9n, a variant of Cas9 that catalyze a single strand break (SSB) instead of a DSB, which can be repaired by the cell and is therefore less toxic (9, 15). Ultimately, the cell will be protected against the nickase action only if the target site is modified through HR with the provided editing template. On one hand, it is therefore easier to obtain transformants but, on the other hand, mutants are generally isolated at lower frequencies and after sub-culturing, because of the less selective action of the nickase (7). Another strategy relies on the use of inducible promoters to drive the expression of *cas9* and/or the gRNA (10, 12, 13). Once transformants in which *cas9* expression is repressed have been obtained, it is possible to trigger the synthesis of the ribonucleoprotein complex through the addition of the inducer, and to select edited cells.

Our group recently developed an inducible CRISPR-Cas9 tool for *Clostridium* based on the use of two plasmids (16). In this system, the *cas9* gene is located on a first plasmid, $pCas9_{ind}$, under the control of Pcm-tetO2/1, together with the *tetR* gene whose product represses the transcription of *cas9* in the absence of anhydrotetracycline (aTc) (17). While this first plasmid is unique and modification-independent, the second plasmid, generically designated as pGRNA, is target-specific and contains a gRNA expression cassette and the appropriate editing template. Both $pCas9_{ind}$ and pGRNA plasmids are introduced into the cell sequentially and *cas9* and gRNA expression is induced by aTc in transformants to select genome edited mutants.

Although this system has been successfully used to target several genes in the genome of *C. acetobutylicum*, we report here on the difficulties encountered to obtain transformants containing both pCas9 and pGRNA in some cases, most likely

because of a basal leaky expression in the absence of inducer of both the nuclease and the gRNA. To overcome this problem, we optimized pCas9$_{ind}$ by cloning a gene encoding the AcrIIA4 anti-Cas9 protein (18) under the control of a lactose-inducible promoter (19). Anti-Cas proteins have been discovered recently in CRISPR-Cas antagonists that have evolved inhibitors of those bacterial defense mechanisms (20, 21). The ability to regulate at a post-translational level the action of Cas nucleases opens applications for these proteins in CRISPR-based genetic tools, in particular for organisms with low transformation efficiencies (22). In this work, the use of AcrIIA4 allowed to limit the undesired activity of the CRISPR-Cas system at the electroporation step, resulting in an increased transformation efficiency of pGRNA plasmids.

This refined tool allowed us to investigate the role of the NADPH-dependent alcohol dehydrogenases encoding genes *bdhA*, *bdhB* and *bdhC* genes in the industrially relevant *C. acetobutylicum* DSM 792. Mutants were generated in which one, two, or all of the corresponding genes were deleted, and were further characterized in batch fermentation assays. Subsequently, mutants displaying the stronger effects were complemented with plasmids carrying each *bdh* copy to confirm the phenotypes obtained and investigate the contribution of each gene to alcohol production.

RESULTS

Improvement of a CRISPR-Cas9 genetic tool

In order to determine the contribution of BdhA, BdhB and BdhC for butanol production in *C. acetobutylicum* DSM 792, we first sought to create mutant strains using our CRISPR-Cas9 genetic tool (16). First of all, we implemented a protocol for the rapid replacement of the gRNA sequence in the targeting plasmids. A cassette containing the aTc-inducible promoter Pcm-2tetO1 (17) and the gRNA chimeric sequence (23), separated by a 38 nt cassette containing two BsaI sites was cloned into pEC750C, yielding $pGRNA_{ind}$. Upon restriction with BsaI, overhang extremities were generated, allowing the insertion of two hybridized single-strand oligonucleotides with extremities compatible with the linearized $pGRNA_{ind}$, in order to generate a new target specific plasmid (Fig. 1A). This simple modification allowed us to generate new pGRNA plasmids faster than through the cloning of a synthesized complete gRNA expression cassette, as previously done in our laboratory (16). We used this strategy to construct plasmids targeting *bdhA*, *bdhB* and *bdhC*, designated as pGRNA-bdhA, pGRNA-bdhB and pGRNA-bdhC, respectively.

The next step consisted in the cloning of SOE PCR products that can be used as editing template for the reparation of the DSB created through the action of the Cas9-gRNA complex (Fig. 1B). Editing templates were constructed in order to delete most of the targeted genes so that only a few nucleotides at the extremities of the coding sequence were still present, leading to the synthesis of small peptides. These templates were cloned into the appropriate vectors, yielding pGRNA-Δ*bdhA,* pGRNA-Δ*bdhB* and pGRNA-Δ*bdhC*. Since *bdhA* and *bdhB* are located next to each other in

the DSM 792 chromosome, one single editing template was designed for the deletion of both genes at once and cloned into pGRNA-bdhB to obtain pGRNA-Δ*bdhA*Δ*bdhB*.

Those plasmids were then introduced in *C. acetobutylicum* DSM 792 containing $pCas9_{ind}$ (further referred to as DSM 792 ($pCas9_{ind}$)). Notably, most of the assays performed to introduce plasmids pGRNA-Δ*bdhB* or pGRNA-Δ*bdhA*Δ*bdhB* into this recombinant strain failed, as no transformants were obtained. We hypothesized that this result might be due to a toxic leaky expression of both the *cas9* gene and the gRNA, despite their control by inducible promoters Pcm-tetO2/1 and Pcm-2tetO1, respectively, which would prevent the generation of transformants. We therefore modified the $pCas9_{ind}$ plasmid by inserting the *acrIIA4* gene from *Listeria monocytogenes* under the control of Pbgal and the associated transcriptional repressor encoding gene *bgaR* (19, 24), yielding $pCas9_{acr}$. This modification aimed at controlling the action of the Cas9-gRNA complex during the transformation step by addition of lactose in the medium. Once transformants have been obtained, the expression of the Cas9-gRNA complex is triggered with aTc on a lactose-free medium. The procedure by which $pCas9_{acr}$ can be used in combination with pGRNA plasmids to create multiple mutations in the genome of DSM 792 is shown in Fig. 1C.

Transformation of pGRNA plasmids into DSM 792 ($pCas9_{ind}$) and DSM 792 ($pCas9_{acr}$) was further assessed, using lactose-supplemented plates for the selection of transformants. Transformation efficiencies are shown in Fig. 2. Although plasmids pGRNA-Δ*bdhA* and pGRNA-Δ*bdhC* were introduced at similar rates in DSM 792 ($pCas9_{ind}$) or DSM 792 ($pCas9_{acr}$), the generation of transformants containing pGRNA-Δ*bdhB* and pGRNA-Δ*bdhA*Δ*bdhB* was greatly improved when using a strain containing $pCas9_{acr}$ instead of $pCas9_{ind}$. Therefore, all further experiments were performed using DSM 792 ($pCas9_{acr}$).

Bdhs mutants construction

Once transformants containing pCas9$_{acr}$ and either pGRNA-Δ*bdhA*, pGRNA-Δ*bdhB*, pGRNA-Δ*bdhC* or pGRNA-Δ*bdhA*Δ*bdhB* were obtained, expression of *cas9* was induced as previously described (16). For each modification, at least three aTc-resistant colonies obtained from independent transformants were analyzed and confirmed by PCR, yielding the corresponding mutants that we stored for further experiments. As for the early version of our tool (16), the editing efficiency for every single modification was 100 % (data not shown). To generate DSM 792 Δ*bdhA*Δ*bdhC*, DSM 792 Δ*bdhB*Δ*bdhC* and DSM 792 Δ*bdhA*Δ*bdhB*Δ*bdhC*, one DSM 792 Δ*bdhC* mutant was randomly chosen (i.e. the first mutant obtained), streaked on 2YTG plates devoid of thiamphenicol until pGRNA-Δ*bdhC* was cured, and further transformed with plasmids pGRNA-Δ*bdhA*, pGRNA-Δ*bdhB* or pGRNA-Δ*bdhA*Δ*bdhB*, respectively. Once transformants were obtained, expression of *cas9* was induced and aTc-resistant colonies obtained from independent transformants were analyzed by PCR, confirmed, and saved for further experiments. For each type of deletion, six independent colonies were tested and the editing efficiency for this new round of modification was again 100 % (data not shown). PCR analyzes of *bdhA-bdhB* and *bdhC* loci in each different mutant are shown in Fig. 3.

Phenotypic analysis

Those mutants were then characterized by batch fermentation performed in flasks on GAPES medium enriched in ammonium acetate. For each type of mutation (i.e. deletion of one, two, or the three butanol dehydrogenase encoding genes), the three randomly chosen independent mutants selected were assessed. Final fermentation products are shown in Table 1. After 72h, the wild-type strain yielded a classical ABE

product pattern composed of 22-23 g/L of solvents and less than 5 g/L of acids. No significant difference was observed for the single deletion mutants compared to the wild-type strain, nor for the double mutants Δ*bdhA*Δ*bdhC* and Δ*bdhB*Δ*bdhC*. On the opposite, final fermentation products concentrations were strongly affected in mutants Δ*bdhA*Δ*bdhB* and Δ*bdhA*Δ*bdhB*Δ*bdhC*. Lower solvent titers (7.4 and 4.2 g/L respectively) and higher acids concentrations (9.7 and 11.4 g/L respectively) were detected, resulting in lower final pH and consistent with a so-called “acid crash” event. Both ethanol and butanol productions were strongly decreased in those mutants, and final titers were lower than that of acetone, which became the main fermentation product in terms of solvents.

Hypothesizing that the inability of the mutants to efficiently assimilate acids and convert them into solvents led to an irreversible pH decrease, we performed pH-controlled batch fermentations of the wild-type strain as well as Δ*bdhA*Δ*bdhB* and Δ*bdhA*Δ*bdhB*Δ*bdhC* mutants in bioreactor (Table 2). Although solvent production of the mutants was slightly increased compared to flask fermentations, the final solvent concentrations were still strongly reduced compared to the wild-type strain. Unlike what was observed for flask fermentations, butanol was still the main solvent detected at the end of the fermentation. Interestingly, the major difference observed compared to flask fermentation was a stronger accumulation of lactate, reaching up to 2 g/L in pH-controlled bioreactor instead of 0.2 g/L in flask assays.

Complementation assays

To confirm that the deletion of *bdh* genes was responsible for the phenotypes observed, we complemented Δ*bdhA*Δ*bdhB* and Δ*bdhA*Δ*bdhB*Δ*bdhC* mutants with plasmids allowing the expression of each gene separately, under the control of its

native promoter. As a control, the wild-type strain was also transformed with those plasmids. For each complementation (i.e. each plasmid introduced in each strain), three randomly chosen transformants were analyzed through batch fermentation assays performed in flasks as previously done, and final fermentation products are presented in Table 3. In the wild-type strain, overexpression of each gene had no significant effect compared to the presence of the empty control vector. However, the Δ*bdhA*Δ*bdhB* and Δ*bdhA*Δ*bdhB*Δ*bdhC* mutants assessed were successfully complemented with any of the *bdh* copy, without significant difference in terms of product pattern of selectivity. The two mutants assessed still undergone acid crash when transformed with an empty vector. Interestingly, even the phenotype of the Δ*bdhA*Δ*bdhB* mutant was reverted by the overexpression of *bdhC*, which led to fermentation performances in the range of the wild-type strain.

DISCUSSION

Although studied for over one century, the ABE metabolism of *C. acetobutylicum* is still only partially understood. At the molecular level, the role of the main solventogenic actors, the alcohol dehydrogenases genes, has only been investigated using single gene deletion approaches due to a lack of efficient genome editing tools. To overcome this issue, several dedicated tools have been developed in the recent years, including those based on CRISPR-Cas approaches which proved their efficiency in other organisms. Although powerful, CRISPR-Cas-based genetic tools also have drawbacks. In *Clostridium*, the main one is the toxicity of the system, which makes it difficult to use because of the low transformation and HR frequencies observed in these bacteria. In some cases, Cas9n has been used instead of Cas9 because of the too high toxicity of the later, even when placing the corresponding gene under the control of an inducible promoter (9, 15). However, the use of nickases can be laborious, for the very same reason, i.e. because of a lowered toxicity that does not allow to eliminate all unmodified cells. In other words, introduction of the CRISPR-Cas machinery is facilitated when Cas9n is employed, but mutant selection is more efficient with the use of a native Cas9.

In this study, we intended to use a two-plasmid CRISPR-Cas tool recently developed to inactivate *bdhA*, *bdhB* and *bdhC*, the three genes identified in the genome of *C. acetobutylicum* DSM 792 that are coding for NADPH-dependent alcohol dehydrogenases. We first failed in trying to generate DSM 792 Δ*bdhB* and DSM 792 Δ*bdhA*Δ*bdhB* mutants because we did not manage to obtain transformants containing both $pCas9_{ind}$ and the corresponding pGRNA plasmids. Although it cannot be ruled out that transformation efficiency of pGRNA plasmids is affected by the editing template, it is intuitively most likely linked to the gRNA strength. In the

example reported in this study, only one assay allowed us to obtain three transformants of DSM 792 ($pCas9_{ind}$) containing pGRNA-Δ*bdhB*, after dozens of attempts. Similarly, no transformant was ever obtained when introducing pGRNA-Δ*bdhA*Δ*bdhB* in DSM 792 ($pCas9_{ind}$). Obviously, an alternative to circumvent this problem would have been to change the gRNA sequence targeting *bdhB*, especially since we facilitated the retargeting of pGRNA plasmids compared to the early version of the tool (Fig. 1A). However, we also faced this toxicity problem with a few other targeting plasmids in our laboratory (data not reported), and we therefore wanted to modify our tool so that it would be possible to generate transformants with any pGRNA plasmid used. Therefore, we added an extra control of the Cas9-gRNA complex nuclease activity through the use of a recently described anti-CRISPR protein (18). Interestingly, the transformation efficiency of pGRNA-Δ*bdhA* and pGRNA-Δ*bdhC* was not improved, suggesting that placing *cas9* under the control of an inducible promoter was sufficient when using those gRNA sequences. However, transformation efficiencies with pGRNA-Δ*bdhB* and pGRNA-Δ*bdhA*Δ*bdhB* were greatly increased when DSM 792 ($pCas9_{acr}$) was used, demonstrating that we effectively improved our tool. To the best of our knowledge, the use of anti-CRISPR proteins to realize CRISPR-based genome editing was already documented in human cells (25) and in the archaea *Sulfolobus islandicus* (26). Combining two antagonist activities controlled by inducible promoters, we constructed a fast, reliable and efficient genome editing tool. Although only deletions were performed in this study, the CRISPR/Anti-CRISPR strategy can also be employed to perform insertion and single nucleotide modification as shown with the first version of the tool (16), without the presence of any unwanted scar or antibiotic resistance marker in the genome, and can therefore be used to perform several rounds of modification.

297 Two studies previously analyzed the role of the genes investigated in this study,
298 through the construction of single gene deletion mutants obtained with the ClosTron
299 (5), yielding slightly different outcomes (3, 4). In the first of these studies, *bdhA* and
300 *bdhB* were successfully inactivated but the corresponding strains Cac-*bdhA*459s::CT
301 and Cac-*bdhB*475a::CT were not significantly different from the wild-type strain, in
302 accordance with our results. The deletion of *bdhC* was not investigated in this work
303 (3). In a more recent study (4), BdhB was shown to be relevant for alcohol
304 production, since the corresponding mutant had lower titers in butanol and ethanol.
305 Moreover, BdhC (referred to as YqhD) was shown to be involved in ethanol
306 production, following the observation that a Δ*yqhD* mutant had lower final ethanol
307 titer, but was not affected in terms of butanol production (4). Those results contrast
308 with ours, since we did not observe any significant effect of either *bdhB* or *bdhC*
309 deletions on ethanol and butanol production. Similarly, the deletion of *bdhA* had no
310 significant effect, as reported in both previous studies (3, 4).

311 Despite the fact that the ClosTron has revolutionized the genetics of *Clostridium* by
312 allowing fast inactivation of genes in bacteria which were difficult to modify, it suffers
313 from some limitations. First, it has been shown that the disruption of a gene rather
314 than its clean deletion can trigger polar effects, which could explain the differences
315 observed between those two studies concerning the importance of BdhB. Moreover,
316 another limitation of the ClosTron is that the inactivation of a gene requires its
317 disruption with an intron that contains an antibiotic resistance marker, which therefore
318 limits the number of modifications than can be done in the recombinant strain. For
319 this last reason, the two studies cited above (3, 4) focused on the creation of single
320 gene deletion strains. Using our improved CRISPR-Cas9 genetic tool, single, double
321 and triple marker-free, insertion-free mutants in which targeted genes were cleanly

deleted were obtained. The high editing efficiency of the tool allowed to generate numerous independent mutants, providing a higher confidence in the fermentation results obtained.

Results obtained in this study suggest that none of the *bdh* gene is essential for solvent production. Indeed, every single deletion mutant retained the ability to efficiently produce solvents in the conditions tested, without clear difference in the final products pattern. Therefore, it can be hypothesized that the two remaining *bdh* copies allow the synthesis of a sufficient Bdh pool in the cell for the efficient production of solvents. This result is in accordance with most of the conclusions drawn from previous studies, although the lower ethanol production in Δ*bdhC* and the diminished production of both ethanol and butanol in Δ*bdhB* were not observed, unlike what was shown in one of those studies (4). Interestingly, we observed that the deletion of several *bdh* copies could have strong effects on the solvent production capacities of *C. acetobutylicum*. In particular, *bdhA* and *bdhB* apparently have a greater role in solventogenesis than *bdhC*, since Δ*bdhA*Δ*bdhB* is the only double mutant with significantly affected fermentation products pattern compared to the wild-type strain. These results are in accordance with a recent study in which transcriptomic and proteomic data were obtained from phosphate-limited chemostat cultures of *C. acetobutylicum* maintained in different metabolic states (2). Interestingly, the data collected indicate that all three genes are expressed throughout the fermentation, unlike genes from the *sol* operon that are mainly expressed during solventogenesis. Transcription of *bdhA* does not vary between acidogenic and solventogenic phase, unlike transcription of *bdhC* which slightly increases and transcription of *bdhB* which is 73% higher. During solventogenesis, transcripts of *bdhA*, *bdhB* and *bdhC* represent 17, 59 and 24% of the *bdh* mRNA

pool, respectively. Protein quantification indicates that BdhB represents 62% of the Bdh pool in acidogenesis, followed by BdhA (27%) and BdhC (11%). Increased transcription of *bdhB* in solventogenesis results in an increase of BdhB in the pool of Bdh enzymes (83%), compared to BdhA (11%) and BdhC (6%). Activities of the purified enzymes were also reported in the same publication and show comparable butanol dehydrogenase activities for all enzymes (2). Taken together, these data suggest that BdhB is the main butanol dehydrogenase. A lower quantity of enzymes is transcribed from the *bdhA* gene, although it represents one quarter of the Bdh enzyme pool during acetogenesis. Our results are in accordance with those data, since the fact that a Δ*bdhA*Δ*bdhB* mutant cannot perform an efficient solventogenesis while other double mutants can indicates that BdhA and BdhB appear to have a more important role than BdhC. Interestingly, a Δ*bdhB* mutant is still capable of performing an efficient fermentation, suggesting that the two remaining genes can compensate the loss of this copy, providing an explanation why so many copies of the same gene can be found in the genome. As intuited, the triple mutant Δ*bdhA*Δ*bdhB*Δ*bdhC* was even more heavily affected than the Δ*bdhA*Δ*bdhB* mutant, although neither butanol nor ethanol production was abolished, suggesting that other enzymes are still capable of compensating the lower alcohol dehydrogenase activity in the mutant, most likely AdhE2 and/or the products of CA_C3375 and CA_P0059. Notably, fermentations performed in pH-controlled bioreactors did not allow to prevent acid accumulation, showing the mutant incapacity to reduce acids. Instead, the triple mutant produced a significant amount of lactate in these conditions, most likely to regenerate its $NAD(P)^+$ pool which is not produced by Bdh enzymes anymore. Interestingly, although final solvent titers were lower than for

371 the wild-type strain, a classical ratio ethanol:acetone:butanol was obtained, unlike
372 what was observed in flask fermentation assays.

373 Complementation assays showed that any *bdh* copy is capable of reverting the acid
374 crash phenotype of Δ*bdhA*Δ*bdhB* and Δ*bdhA*Δ*bdhB*Δ*bdhC* mutants in non pH-
375 controlled flasks fermentations. Although those genes have been introduced in the
376 cells under the control of their native promoter, the plasmid copy number most
377 probably allows their overexpression. In fact, previous complementation assays
378 performed with the same plasmid in *Clostridium beijerinckii* DSM 6423 resulted in a
379 80-90 fold overexpression compared to expression from the gene located on the
380 chromosome of the bacterium (27). Interestingly, while a chromosomal *bdhC* is not
381 sufficient to prevent acid crash, its overexpression in Δ*bdhA*Δ*bdhB* and
382 Δ*bdhA*Δ*bdhB*Δ*bdhC* mutants results in an efficient fermentation and highlights the
383 crucial influence of the level of expression of a particular gene for the whole
384 metabolism of the microorganism. It is also interesting to point out that expression of
385 either *bdhA*, *bdhB* or *bdhC* in the Δ*bdhA*Δ*bdhB*Δ*bdhC* mutant had no strong impact
386 on the ethanol/butanol ratio, suggesting that the corresponding enzymes are all three
387 capable of reducing acetaldehyde in ethanol and butyraldehyde in butanol efficiently.
388 A better strategy to optimize the ethanol/butanol ratio could be to modify the
389 selectivity of AdhE1 and AdhE2 for acetaldehyde and butyraldehyde, as recently
390 attempted (28).

391 In summary, the improvement of our CRISPR-Cas9 genetic tool dedicated to *C.*
392 *acetobutylicum* through the control of the Cas9/gRNA activity using the anti-CRISPR
393 protein AcrIIA4 will allow to step up the construction of clean mutants in order to
394 enhance our understanding of the molecular physiology of this industrially relevant
395 microorganism, and to other microorganisms belonging to the *Clostridium* genus. As

396 an example, it allowed us to investigate the importance of the main butanol
397 dehydrogenases of *C. acetobutylicum* DSM 792. In fact, while previous studies only
398 focused on single gene deletion that did not allow to figure out the major role of these
399 enzymes, our tool allowed us to construct multiple deletion mutants and to highlight
400 the major role of BdhA and BdhB in solvent production, as well as the capacity of
401 BdhC to compensate the absence of the others Bdhs in a triple mutant strain. The
402 results obtained indicate that an efficient alcohol production in *C. acetobutylicum*
403 DSM 792 requires a pool of enzymes with butanol dehydrogenase activity, which can
404 be synthesized from any of the three genes investigated in this study. Butanol
405 dehydrogenase activity is still observed in the triple deletion mutant, suggesting that
406 the products of other genes not investigated in this study, most probably AdhE2,
407 CA_C3375 and CA_P0059, are also part of this pool, although not in quantities
408 sufficient to avoid an acid crash. This observation might explain why solventogenic
409 strains carry so many alcohol dehydrogenase encoding genes on their chromosome.
410 In conclusion, efficient metabolic engineering in *Clostridium* in order to modify the
411 fermentation products pattern requires numerous genome editing that can be
412 achieved with iterative and precise genetic tools like the one described in this study.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

Relevant characteristics of the bacterial strains and plasmids used in this study are listed in Table 4. *C. acetobutylicum* was grown anaerobically at 34 °C in liquid 2YTG medium (16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 4 g L^{-1} NaCl and 5 g L^{-1} glucose) or on solid 2YTG with 1.5% agar, supplemented with 40 µg mL^{-1} erythromycin and/or 15 µg mL^{-1} thiamphenicol if necessary. *Escherichia coli* was grown aerobically at 37 °C and 200 rpm in liquid LB medium or solid LB with 1.5% agar supplemented with erythromycin (500 µg mL^{-1} for solid media and 100 µg mL^{-1} for liquid media), chloramphenicol (25 µg mL^{-1} for solid media and 12.5 µg mL^{-1} for liquid media), or tetracycline (20 µg mL^{-1}) if necessary.

DNA manipulation and plasmid construction

All enzymes used for DNA modification were purchased from New England Biolabs (NEB). The Q5® High-Fidelity Polymerase (NEB) was used for all PCR amplifications, except for colony PCRs, which were performed using OneTaq® DNA polymerase (NEB). Genomic DNA (gDNA) was extracted from *C. acetobutylicum* using the ChargeSwitch gDNA Mini Bacteria Kit (ThermoFisher Scientific). Plasmids and PCR product purification kits were purchased from Qiagen. All primers used in this study are listed in Table 5.

A fragment containing *bgaR* and a codon optimized *acrIIA4* gene from *L. monocytogenes* under the control of Pbgal was synthesized and cloned into the pEX-K4 vector (Eurofins Genomics). The whole cassette was amplified with primers P01 and P02, digested with SacI and cloned into $pCas9_{ind}$ linearized with the same

restriction enzyme, yielding $pCas9_{acr}$. A fragment containing the Pcm-2tetO1 promoter fused with the chimeric gRNA sequence, separated by a 38 nt spacer containing two BsaI restriction sites, was synthesized and cloned into the pEX-K4 vector (Eurofins Genomics). This cassette was amplified with primers P01 and P02, digested with SacI and cloned into pEC750C, yielding $pGRNA_{ind}$. Sequences of the synthesized fragments are listed in the Supplementary Information.

The gRNA target sites were chosen using Geneious R10.2 (https://www.geneious.com), which provides for each CRISPR-Cas9 potential target site activity scores (On-target activity, ranging from 0 to 1) predicted with an algorithm (29), and specificity scoring, i.e. the probability that the gRNA will not target another location in the genome (Off-target activity, ranging from 0% to 100%, with a higher score meaning better specificity and less off-target activity). Target sites were chosen so that the On-target score was superior to 0.2 and the Off-target score was superior to 98% when up to five mismatches including one insertion or deletion were allowed to detect off-targets. The pGRNA-bdhA, pGRNA-bdhB and pGRNA-bdhC plasmids were constructed by ligating into the dephosphorylated BsaI-digested $pGRNA_{ind}$ the hybridization product of 5'P-modified oligonucleotides couples P03-P04, P05-P06 and P07-P08, respectively. Briefly, hybridization was performed in a thermocycler according to the following protocol: 500 nmole of each primer were mixed in a 10 µl reaction containing 1 mM Tris-HCl, denatured at 95°C during 30 s, and further hybridized lowering the temperature 1°C every 5 s 70 times.

The pGRNA-Δ*bdhA* plasmid was constructed by cloning the amplicon obtained using a splicing by overhang extension (SOE) PCR with primer pairs P09/P10 and P11/P12 on DSM 792 gDNA, into pGRNA-bdhA digested with BamHI and SalI. The pGRNA-Δ*bdhB* plasmid was constructed by cloning the SOE PCR product obtained using

primer pairs P13/P14 and P15/P16 on DSM 792 gDNA, into pGRNA-bdhB digested with BamHI and SalI. Similarly, the pGRNA-Δ*bdhA*Δ*bdhB* plasmid was constructed by cloning the SOE PCR product obtained using primer pairs P13/P17 and P18/P19 on DSM 792 gDNA, into the same BamHI/SalI double-digested pGRNA-bdhB. The pGRNA-Δ*bdhC* plasmid was constructed by cloning the SOE PCR product obtained using primer pairs P20/P21 and P22/P23 on DSM 792 gDNA, into pGRNA-bdhC digested with XhoI and XbaI.

For complementation assays, plasmids pFW01-*bdhA*, pFW01-*bdhB* and pFW01-*bdhC* were constructed by cloning the PCR products obtained with primer pairs P24/P25, P26/P27 and P28/P29, respectively, into pFW01 using XhoI and SalI restriction enzymes.

Transformation, induction of *cas9* expression and mutant confirmation

Plasmids were transformed into chemically competent NEB 10-beta competent *E. coli* cells containing pAN2 (5) for DNA methylation. The vectors were then isolated and 1 µg was used for electroporation of *C. acetobutylicum* DSM 792 as previously described (30). Cells of DSM 792 containing either $pCas9_{ind}$ or $pCas9_{acr}$ were further transformed with pEC750C and derivative plasmids (i.e. plasmids containing a gRNA expression cassette with or without editing template) and selected on 2YTG solid media supplemented with erythromycin, thiamphenicol and 40 mM lactose. Expression of *cas9* was induced in transformants by exposure to aTc as described before (16), except that inducer concentration was 1 µg mL^{-1}. Mutants were confirmed by amplification of the *bdhA-bdhB* and *bdhC* loci performed on gDNA using primers pairs P30/P31 and P32/P33, respectively.

Fermentation assays

485 Flasks batch fermentations were performed in modified Gapes medium (31)
486 containing 2.5 g L^{-1} yeast extract, 1 g L^{-1} KH_2PO_4, 0.6 g L^{-1} K_2HPO_4, 1 g L^{-1} $MgSO_4$
487 $7H_2O$, 6.6 mg L^{-1} $FeSO^4$ $7H_20$, 0.1 g L^{-1} 4-aminobenzoic acid, 5.8 g L^{-1} CH_3COONH_4
488 and 80 g L^{-1} glucose. Erythromycin (40 µg mL^{-1}) was used for complementation
489 assays. Fresh colonies were used to inoculate 5 mL Gapes media. After 72 h of
490 growth in an anaerobic chamber at 34°C without agitation, 1 mL of these precultures
491 was inoculated into 100-mL flasks containing 19 mL of the same liquid medium. The
492 flasks were sealed with rubber stoppers, and a pressure relief valve system was
493 punctured through the rubber stoppers to prevent overpressure. Flasks were further
494 incubated outside of the anaerobic chamber for 72 h at 34°C with agitation at 120
495 rpm.

496 For bioreactor batch fermentations, fresh colonies were used to inoculate 5 mL
497 modified Gapes media (31). After 72 h of growth in an anaerobic chamber at 34°C
498 without agitation, 1 mL of these precultures was inoculated into a 100-mL Erlenmeyer
499 containing 40 mL of the same liquid medium and incubated for another 24 h in the
500 anaerobic chamber. This second preculture was then used to inoculate a Biostat Q
501 bioreactor (Sartorius) containing 360 mL of the same medium. Throughout the
502 fermentation, the pH was controlled not to decrease below 5.0 by the addition of 5M
503 KOH.

504 The fermentation samples were centrifuged at 5,000 g for 5 min and the supernatant
505 was diluted with an internal standard (0.5 g L^{-1} propanol). The concentrations of the
506 solvents produced were determined by gas chromatography on a PoraBOND-Q
507 column (25 m length, 0.32 mm internal diameter, 0.5 µm film thickness; Agilent
508 Technologies) equipped with a flame ionization detector. Helium was used as carrier

509 gas, at a flow rate of 1.6 mL min^{-1}. Column was gradually heated from 50°C to 250°C
510 in a 30 min run.

511 Acids concentrations were quantified by HPLC (Aminex HPX-87H from Biorad
512 coupled to a Spectra System RI 150 refractometer and a Waters 2487 Dual λ UV
513 detector set at 210 nm). 0.01 M sulfuric acid mobile phase was used at a flowrate of
514 0.6 mL min^{-1}. Column temperature was set to 60°C.

515 Residual sugar quantities were determined by HPLC using an Aminex HXP-87P (Bio-
516 Rad) coupled to a Varian 350 RI refractometer for detection. Water was used as a
517 mobile phase at a flowrate of 0.4 mL/min.

ACKNOWLEDGMENTS

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. We thank Hélène Velly and Sandra Menir for technical assistance during bioreactor assays.

TABLE 1 Fermentation products of the mutants analyzed in this study. Results shown are average values ± standard deviations from at least three independent technical replicates. For mutants assays, values are from biological replicates.

Strain	Products (g/L)[a]								Glucose consumption (g/L)	Final pH
	Solvents				Acids					
	Ethanol	Acetone	Butanol	Total	Acetate	Butyrate	Lactate	Total		
WT	1.3 ± 0.1	9.0 ± 0.5	12.4 ± 1.4	22.8 ± 2.0	2.6 ± 0.1	2.4 ± 0.1	ND	5.0 ± 0.2	69.2 ± 0.2	5.1 ± 0.0
Δ*bdhA*	1.2 ± 0.1	9.1 ± 0.4	11.6 ± 0.4	22.0 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	ND	4.7 ± 0.0	69.4 ± 0.2	5.2 ± 0.0
Δ*bdhB*	0.9 ± 0.0	9.2 ± 0.2	12.1 ± 0.6	22.2 ± 0.6	2.2 ± 0.1	2.7 ± 0.1	ND	4.9 ± 0.2	71.0 ± 2.4	5.3 ± 0.0
Δ*bdhC*	1.3 ± 0.2	9.3 ± 0.3	12.8 ± 1.0	23.4 ± 1.4	2.6 ± 0.4	2.1 ± 0.5	ND	4.7 ± 0.9	71.4 ± 2.6	5.3 ± 0.1
Δ*bdhA*Δ*bdhB*	**0.5 ± 0.0**	**3.8 ± 0.6**	**3.2 ± 0.7**	**7.4 ± 1.4**	**4.5 ± 0.4**	**5.0 ± 0.2**	**0.2 ± 0.1**	**9.7 ± 0.4**	**35.9 ± 2.2**	**4.8 ± 0.0**
Δ*bdhA*Δ*bdhC*	1.1 ± 0.2	9.5 ± 0.5	13.3 ± 0.1	23.9 ± 0.4	2.2 ± 0.2	2.8 ± 0.4	ND	5.0 ± 0.2	72.0 ± 0.6	5.2 ± 0.0
Δ*bdhB*Δ*bdhC*	1.0 ± 0.1	9.3 ± 1.1	11.6 ± 0.7	21.9 ± 1.6	2.0 ± 0.2	2.9 ± 0.5	ND	4.9 ± 0.2	73.5 ± 1.8	5.3 ± 0.0
Δ*bdhA*Δ*bdhB*Δ*bdhC*	**0.4 ± 0.0**	**2.5 ± 0.2**	**1.3 ± 0.2**	**4.2 ± 0.3**	**5.3 ± 0.3**	**5.9 ± 0.2**	**0.2 ± 0.1**	**11.4 ± 0.5**	**26.7 ± 1.8**	**4.7 ± 0.0**

[a] ND, Not Detected

TABLE 2 Fermentation products in pH-controlled bioreactors

Strain	Products (g/L)[a]								Glucose consumption (g/L)
	Solvents				Acids				
	Ethanol	Acetone	Butanol	Total	Acetate	Butyrate	Lactate	Total	
WT	1.4	8.0	15.7	25.2	2.0	1.8	ND	3.8	72.5
Δ*bdhA*Δ*bdhB*	0.3	3.1	5.0	8.4	4.3	4.3	1.8	10.4	41.9
Δ*bdhA*Δ*bdhB*Δ*bdhC*	0.2	1.9	2.8	4.9	4.9	4.9	2.0	11.8	36.8

[a] ND, Not Detected

529 **TABLE 3** Complementation assays. Results shown are average values ± standard deviations from at least three independent
530 biological replicates.

Strain	Plasmid	Products (g/L)[a]								Glucose consumption (g/L)	Final pH
		Solvents				Acids					
		Ethanol	Acetone	Butanol	Total	Acetate	Butyrate	Lactate	Total		
WT											
	pFW01	1.8 ± 0.1	8.9 ± 0.4	13.3 ± 0.7	24.0 ± 1.1	2.2 ± 0.2	2.5 ± 0.1	ND	4.7 ± 0.2	64.0 ± 1.3	5.3 ± 0.1
	pFW01-*bdhA*	2.2 ± 0.1	9.9 ± 0.2	11.9 ± 0.6	24.0 ± 0.9	1.7 ± 0.3	1.4 ± 0.4	ND	3.2 ± 0.7	64.8 ± 1.5	5.3 ± 0.1
	pFW01-*bdhB*	1.6 ± 0.3	8.4 ± 1.1	12.1 ± 1.3	22.1 ± 2.6	2.3 ± 0.3	2.7 ± 0.2	ND	5.0 ± 0.5	61.5 ± 2.5	5.1 ± 0.0
	pFW01-*bdhC*	1.8 ± 0.4	9.6 ± 0.5	13.4 ± 0.7	24.8 ± 1.5	2.4 ± 0.1	2.3 ± 0.1	ND	4.7 ± 0.2	64.2 ± 0.6	5.2 ± 0.0
Δ*bdhA*Δ*bdhB*											
	pFW01	**0.7 ± 0.0**	**2.6 ± 0.7**	**2.7 ± 0.8**	**6.1 ± 1.5**	**5.4 ± 0.4**	**4.1 ± 0.6**	**0.6 ± 0.1**	**10.1 ± 1.0**	**21.4 ± 3.4**	**4.7 ± 0.1**
	pFW01-*bdhA*	2.0 ± 0.1	10.6 ± 1.1	13.7 ± 0.8	26.3 ± 0.9	1.7 ± 0.5	1.3 ± 0.1	ND	3.0 ± 0.5	70.9 ± 2.2	5.5 ± 0.1
	pFW01-*bdhB*	1.8 ± 0.1	8.8 ± 0.5	13.6 ± 0.1	24.2 ± 0.3	2.1 ± 0.4	1.6 ± 0.5	ND	3.7 ± 0.9	68.7 ± 0.6	5.4 ± 0.3
	pFW01-*bdhC*	1.4 ± 0.1	10.2 ± 0.4	13.2 ± 0.7	24.8 ± 1.2	1.9 ± 0.1	2.5 ± 0.1	ND	4.4 ± 0.2	67.7 ± 1.0	5.4 ± 0.0
Δ*bdhA*Δ*bdhB*Δ*bdhC*											
	pFW01	**0.5 ± 0.1**	**2.3 ± 0.3**	**2.1 ± 0.3**	**4.9 ± 0.6**	**5.7 ± 0.5**	**4.6 ± 0.4**	**0.6 ± 0.1**	**10.9 ± 1.0**	**18.8 ± 3.4**	**4.6 ± 0.0**
	pFW01-*bdhA*	1.4 ± 0.1	9.6 ± 0.3	13.2 ± 0.3	24.2 ± 0.6	1.9 ± 0.0	1.8 ± 0.1	ND	3.7 ± 0.2	68.6 ± 1.1	5.4 ± 0.1
	pFW01-*bdhB*	1.7 ± 0.2	8.7 ± 0.8	13.8 ± 0.7	24.2 ± 1.7	1.9 ± 0.0	1.7 ± 0.1	ND	3.6 ± 0.1	68.6 ± 1.2	5.5 ± 0.1
	pFW01-*bdhC*	1.2 ± 0.3	10.4 ± 0.8	12.3 ± 0.5	23.9 ± 0.7	1.8 ± 0.4	2.1 ± 1.1	ND	3.9 ± 1.5	67.4 ± 0.4	5.4 ± 0.1

[a] ND, Not Detected

531

532 **TABLE 4** Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant Characteristics	Source or reference
Strains		
C. acetobutylicum DSM 792	Wild-type	DSMZ
E. coli NEB 10-beta	Cloning strain	NEB
Plasmids		
pAN2	*tetA*, Φ3T I gene, p15A origin	(5, 30)
pFW01	*ermB*, ColE1 origin, pCB102 origin	(16)
$pCas9_{ind}$	*ermB*, ColE1 origin, pCB102 origin, *cas9* (Pcm-tetO2/1 promoter), *tetR*	(16)
$pCas9_{acr}$	$pCas9_{ind}$ derivative with *acrIIA4* (Pbgal promoter) and *bgaR* insertions	This study
pEC750C	*catP*, ColE1 origin, pIP404 origin	(16)
$pGRNA_{ind}$	pEC750C derivative with gRNA expression cassette (Pcm-2tetO1 promoter) insertion	This study
pGRNA-bdhA	$pGRNA_{ind}$ derivative targeting *bdhA*	This study
pGRNA-Δ*bdhA*	pGRNA-bdhA derivative with Δ*bdhA* editing template insertion	This study
pGRNA-bdhB	$pGRNA_{ind}$ derivative targeting *bdhB*	This study
pGRNA-Δ*bdhB*	pGRNA-bdhB derivative with Δ*bdhB* editing template insertion	This study
pGRNA-Δ*bdhA*Δ*bdhB*	pGRNA-bdhB derivative with Δ*bdhA*Δ*bdhB* editing template insertion	This study
pGRNA-bdhC	$pGRNA_{ind}$ derivative targeting *bdhC*	This study
pGRNA-Δ*bdhC*	pGRNA-bdhC derivative with Δ*bdhC* editing template insertion	This study
pFW01-*bdhA*	pFW01 derivative with *bdhA* insertion	This study
pFW01-*bdhB*	pFW01 derivative with *bdhB* insertion	This study
pFW01-*bdhC*	pFW01 derivative with *bdhC* insertion	This study

533

534 **TABLE 5** Oligonucleotides used in this study

Oligonucleotide	Sequence (5'-3')
P1	CAGATTGTACTGAGAGTGCACC
P2	GTGAGCGGATAACAATTTCACAC
P3	TCATGCACTTAACTCGTGTTCCAT
P4	AAACATGGAACACGAGTTAAGTGC
P5	TCATGCTTATTACGACATAACACA
P6	AAACTGTGTTATGTCGTAATAAGC
P7	TCATGCTCTTGTATCATAGTCCGT
P8	AAACACGGACTATGATACAAGAGC
P9	AAAAAAGGATCCTTAGGAGCCATATCTGGATG
P10	TATGCTAAGTTTTAAATCTTATTAATAGAAACTGTAGAGG
P11	TTAATAAGATTTAAAACTTAGCATACTTCTTACC
P12	AAAAAAGTCGACCTTCTAATCTCCTCTACTATTTTAG
P13	ATGCATGGATCCAAACGAACCCAAAAAGAAAGTTTC
P14	GGTTGATTTCAAATCTGTGTAAACCTACCG
P15	ACACAGATTTGAAATCAACCACTTTAACCC
P16	ATGCATGTCGACTCTTAAGAACATGTATAAAGTATGG
P17	GCTAAGTTTTAAATCTGTGTAAACCTACCG
P18	ACACAGATTTAAAACTTAGCATACTTCTTACC
P19	ATGCATGTCGACCTTCTAATCTCCTCTACTATTTTAG
P20	AAAAAACTCGAGCTATAAATAATATTACCCCCATAACTG
P21	ATAATTTTGATGAGGGTGCAATGTAAGTTTG
P22	TTGCACCCTCATCAAAATTATACATAAGATTATCCC
P23	AAAAAATCTAGAGCAAAGGAATTAGTAGAAATTAC
P24	AAAAAACTCGAGTTTAACCCCTCCTGTTTAGATTAT
P25	AAAAAAGTCGACCGAAAAATTCACCCCCTCAA
P26	AAAAAACTCGAGAAAATACTCCCCAAGATATTAATGCT
P27	AAAAAAGTCGACTAGAAACTGTAGAGGTATTTTTATAATTTAAAAG
P28	AAAAAACTCGAGTTTCTCCACCTTTAATACTAAAATATTTTTCA
P29	AAAAAAGTCGACAAAAATACCCCACTATTCTAATTTTATTCA
P30	AGTACCTCCTAAGCCTTTTTATGA
P31	TATGGATTGCCCTACAGCCC
P32	CCACTGCCACCATTTTCTAGC
P33	CAAGGACAGATAGGTGGGGC

535

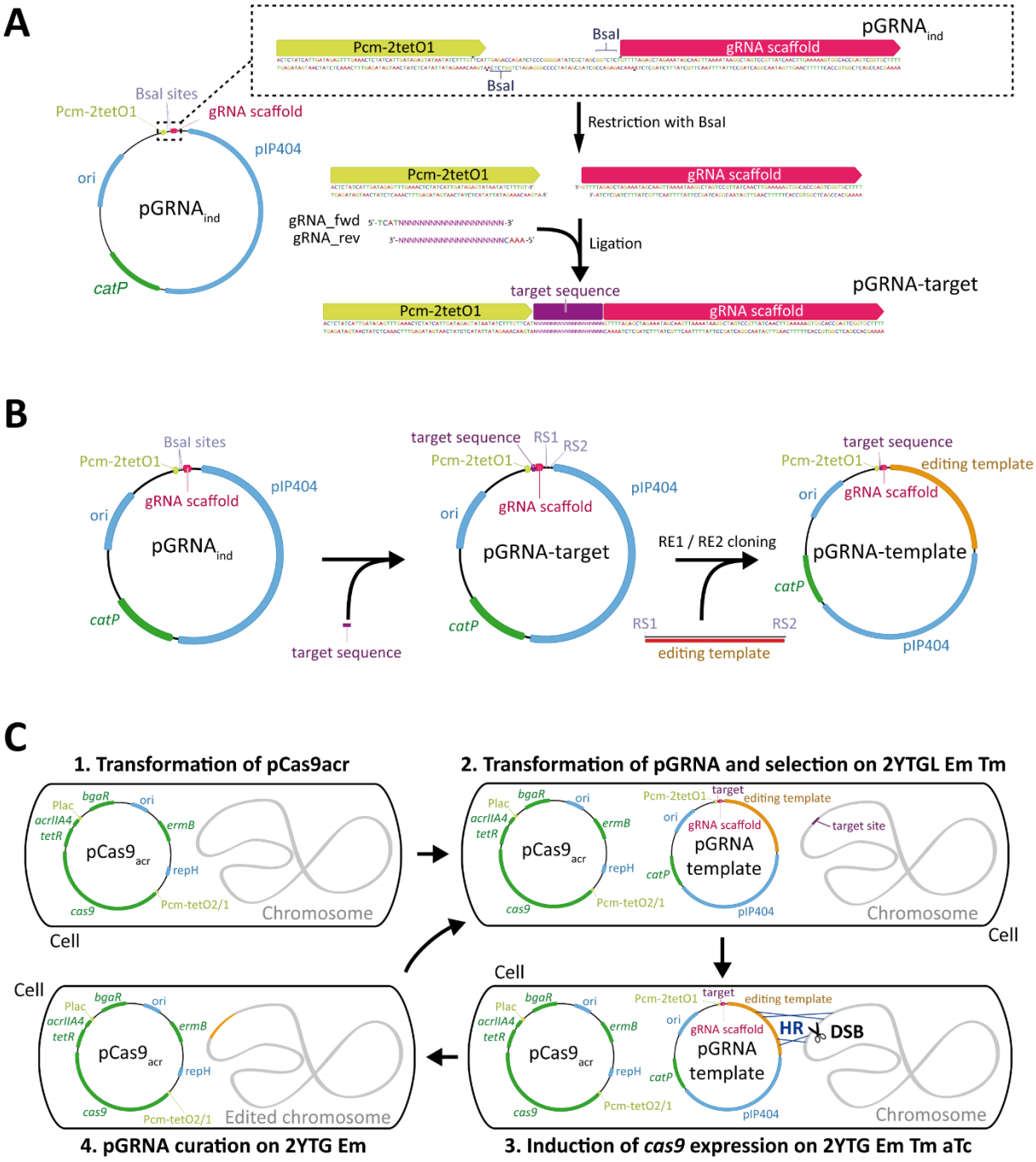
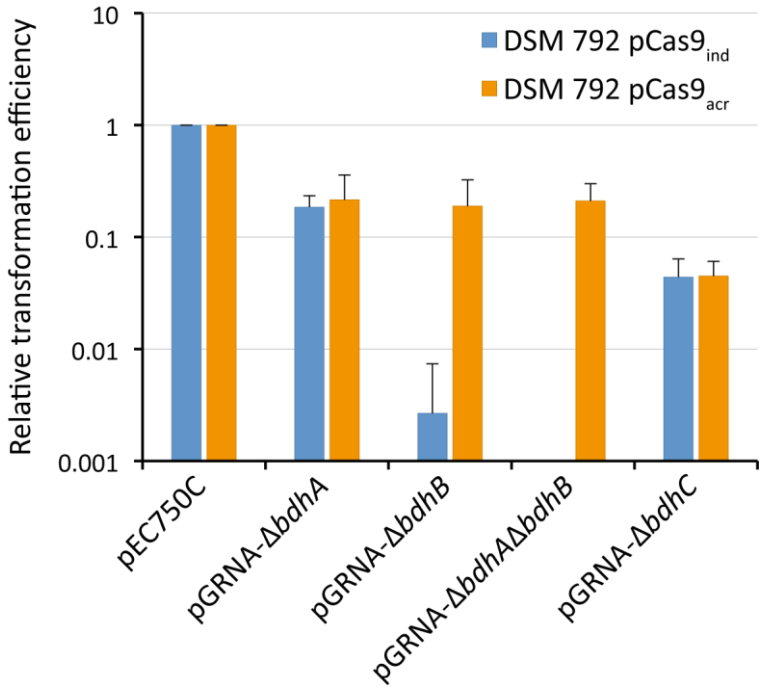


FIG 1 Easy reprogramming of the two plasmid CRISPR-Cas9 genetic tool for multiple genome edition. (A) Retargeting of the pGRNA plasmid by cloning two hybridized primers between the aTc-inducible promoter Pcm-2tetO1 and the gRNA scaffold. (B) Insertion of the editing template within the pGRNA plasmid by restriction enzyme-

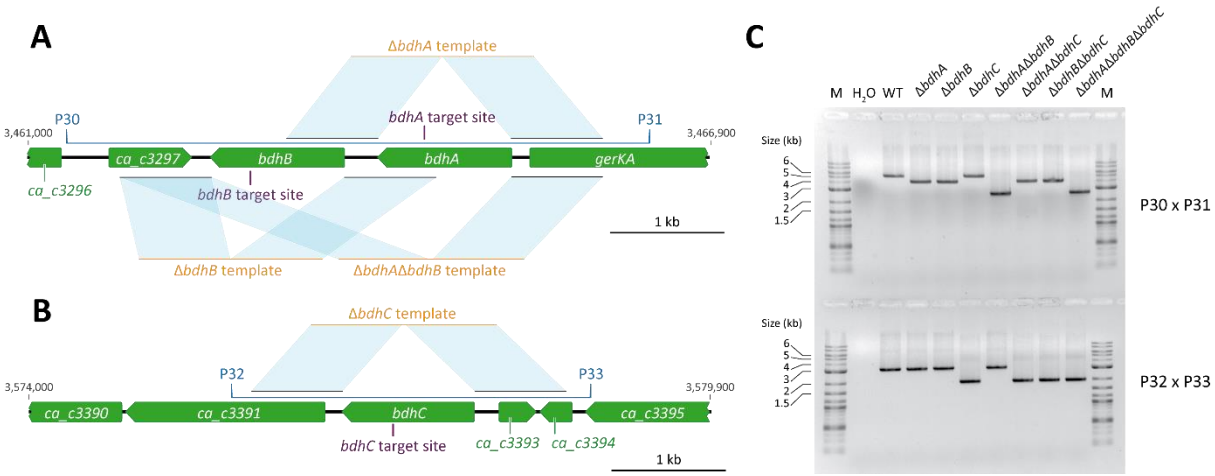
based cloning. (C) Iterative genome modification. Step 1, the strain is transformed with $pCas9_{acr}$ and transformants are selected on erythromycin-containing media. Step 2, the resulting strain is transformed with a pGRNA editing plasmid and transformants are selected on media containing erythromycin, thiamphenicol and lactose to activate the transcription of *acrIIA4*. Step 3, expression of *cas9* is triggered on media containing erythromycin and thiamphenicol and devoid of lactose so that transcription of *acrIIA4* is not induced anymore. The medium contains aTc to trigger the transcription of *cas9* to select mutants in which the editing event occurred. Step 4, once aTc-resistant colonies have been confirmed, pGRNA is curated on media containing erythromycin and no thiamphenicol, yielding cells ready for a new round of modification. ori, replication origin for *E. coli*; pip404 and repH, compatible replication origins for *C. acetobutylicum*; *catP*, thiamphenicol/chloramphenicol-resistance gene; *ermB*, erythromycin-resistance gene; RS, restriction site; RE, restriction enzyme; DSB, double strand break, HR, homologous recombination.



557

558

559 **FIG 2** Relative transformation efficiencies of pGRNA-template plasmids in DSM 792
560 containing pCas9ind or pCas9acr. Results shown are average values ± standard
561 deviations from at least four independent replicates for each transformation. Absolute
562 transformation efficiencies for pEC750C in DSM 792 ($pCas9_{ind}$) and DSM 792
563 ($pCas9_{acr}$) were 148 ± 37 CFU μg^{-1} and 121 ± 92 CFU μg^{-1}, respectively.



564

565

566 **FIG 3** CRISPR-CAS strategy and PCR analyses of representative deletion mutants
567 constructed in this study. (A and B) *bdh* loci from DSM 792. Genes are shown in
568 green, gRNA target sites are indicated in violet, homology sequenced between gDNA
569 and the editing templates are figured in blue. (A) *bdhA-bdhB* locus (B) *bdhC* locus.
570 (C) Amplification of the *bdhA-bdhB* and *bdhC* loci. Amplification with primers pair
571 P30-P31 yields a PCR product of 5173, 4027, 4024 or 2578 bp product in WT,
572 Δ*bdhA*, Δ*bdhB* or Δ*bdhA*Δ*bdhB* strains, respectively. Amplification with primers pair
573 P32-P33 yields a PCR product of 3124 bp or 1993 bp product in WT or Δ*bdhC*
574 strains, respectively. Lane M, 2-log DNA ladder (0.1 to 10 kb; NEB); Lane H_20,
575 amplification on water.

REFERENCES

1. Nölling J, Breton G, Omelchenko MV, Makarova KS, Zeng Q, Gibson R, Lee HM, Dubois J, Qiu D, Hitti J, Wolf YI, Tatusov RL, Sabathe F, Doucette-Stamm L, Soucaille P, Daly MJ, Bennett GN, Koonin EV, Smith DR. 2001. Genome Sequence and Comparative Analysis of the Solvent-Producing Bacterium *Clostridium acetobutylicum*. J. Bacteriol. 183:4823–4838. doi:10.1128/JB.183.16.4823-4838.2001.
2. Yoo M, Bestel-Corre G, Croux C, Riviere A, Meynial-Salles I, Soucaille P, Papoutsakis E, Lee SY. 2015. A Quantitative System-Scale Characterization of the Metabolism of *Clostridium acetobutylicum*. mBio 6:125. doi:10.1128/mBio.01808-15.
3. Cooksley CM, Zhang Y, Wang H, Redl S, Winzer K, Minton NP. 2012. Targeted mutagenesis of the *Clostridium acetobutylicum* acetone–butanol–ethanol fermentation pathway. Metabolic Engineering 14:630–641. doi:10.1016/j.ymben.2012.09.001.
4. Dai Z, Dong H, Zhang Y, Li Y. 2016. Elucidating the contributions of multiple aldehyde/alcohol dehydrogenases to butanol and ethanol production in *Clostridium acetobutylicum*. Sci Rep 6:651. doi:10.1038/srep28189.
5. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. 2007. The ClosTron. A universal gene knock-out system for the genus *Clostridium*. Journal of Microbiological Methods 70:452–464. doi:10.1016/j.mimet.2007.05.021.
6. McAllister KN, Sorg JA, Margolin W. 2019. CRISPR Genome Editing Systems in the Genus *Clostridium*. A Timely Advancement. J Bacteriol 201:283. doi:10.1128/JB.00219-19.

7. Joseph RC, Kim NM, Sandoval NR. 2018. Recent Developments of the Synthetic Biology Toolkit for *Clostridium*. Front. Microbiol. 9:4382. doi:10.3389/fmicb.2018.00154.
8. Wang Y, Zhang Z-T, Seo S-O, Choi K, Lu T, Jin Y-S, Blaschek HP. 2015. Markerless chromosomal gene deletion in *Clostridium beijerinckii* using CRISPR/Cas9 system. Journal of Biotechnology 200:1–5. doi:10.1016/j.jbiotec.2015.02.005.
9. Xu T, Li Y, Shi Z, Hemme CL, Li Y, Zhu Y, van Nostrand JD, He Z, Zhou J, Spormann AM. 2015. Efficient Genome Editing in *Clostridium cellulolyticum* via CRISPR-Cas9 Nickase. Appl. Environ. Microbiol. 81:4423–4431. doi:10.1128/AEM.00873-15.
10. Nagaraju S, Davies NK, Walker DJF, Köpke M, Simpson SD. 2016. Genome editing of *Clostridium autoethanogenum* using CRISPR/Cas9. Biotechnol Biofuels 9:187. doi:10.1186/s13068-016-0638-3.
11. Pyne ME, Bruder MR, Moo-Young M, Chung DA, Chou CP. 2016. Harnessing heterologous and endogenous CRISPR-Cas machineries for efficient markerless genome editing in *Clostridium*. Sci Rep 6:234. doi:10.1038/srep25666.
12. Wang Y, Zhang Z-T, Seo S-O, Lynn P, Lu T, Jin Y-S, Blaschek HP. 2016. Bacterial Genome Editing with CRISPR-Cas9. Deletion, Integration, Single Nucleotide Modification, and Desirable “Clean” Mutant Selection in *Clostridium beijerinckii* as an Example. ACS Synth. Biol. 5:721–732. doi:10.1021/acssynbio.6b00060.
13. Wang S, Dong S, Wang P, Tao Y, Wang Y, Kelly RM. 2017. Genome Editing in *Clostridium saccharoperbutylacetonicum* N1-4 with the CRISPR-Cas9 System. Appl Environ Microbiol 83:186. doi:10.1128/AEM.00233-17.

14. Huang H, Chai C, Li N, Rowe P, Minton NP, Yang S, Jiang W, Gu Y. 2016. CRISPR/Cas9-Based Efficient Genome Editing in *Clostridium ljungdahlii* an Autotrophic Gas-Fermenting Bacterium. ACS Synth. Biol. 5:1355–1361. doi:10.1021/acssynbio.6b00044.
15. Li Q, Chen J, Minton NP, Zhang Y, Wen Z, Liu J, Yang H, Zeng Z, Ren X, Yang J, Gu Y, Jiang W, Jiang Y, Yang S. 2016. CRISPR-based genome editing and expression control systems in *Clostridium acetobutylicum* and *Clostridium beijerinckii*. Biotechnology Journal 11:961–972. doi:10.1002/biot.201600053.
16. Wasels F, Jean-Marie J, Collas F, López-Contreras AM, Lopes Ferreira N. 2017. A two-plasmid inducible CRISPR/Cas9 genome editing tool for *Clostridium acetobutylicum*. Journal of Microbiological Methods 140:5–11. doi:10.1016/j.mimet.2017.06.010.
17. Dong H, Tao W, Zhang Y, Li Y. 2012. Development of an anhydrotetracycline-inducible gene expression system for solvent-producing *Clostridium acetobutylicum*. A useful tool for strain engineering. Metabolic Engineering 14:59–67. doi:10.1016/j.ymben.2011.10.004.
18. Rauch BJ, Silvis MR, Hultquist JF, Waters CS, McGregor MJ, Krogan NJ, Bondy-Denomy J. 2017. Inhibition of CRISPR-Cas9 with Bacteriophage Proteins. Cell 168:150-158.e10. doi:10.1016/j.cell.2016.12.009.
19. Hartman AH, Liu H, Melville SB. 2011. Construction and Characterization of a Lactose-Inducible Promoter System for Controlled Gene Expression in *Clostridium perfringens*. Appl. Environ. Microbiol. 77:471–478. doi:10.1128/AEM.01536-10.

20. Borges AL, Davidson AR, Bondy-Denomy J. 2017. The Discovery, Mechanisms, and Evolutionary Impact of Anti-CRISPRs. Annu. Rev. Virol. 4:37–59. doi:10.1146/annurev-virology-101416-041616.
21. Stanley SY, Maxwell KL. 2018. Phage-Encoded Anti-CRISPR Defenses. Annu. Rev. Genet. 52:445–464. doi:10.1146/annurev-genet-120417-031321.
22. Marino ND, Pinilla-Redondo R, Csörgő B, Bondy-Denomy J. 2020. Anti-CRISPR protein applications. Natural brakes for CRISPR-Cas technologies. Nat Methods. doi:10.1038/s41592-020-0771-6.
23. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337:816–821. doi:10.1126/science.1225829.
24. Al-Hinai MA, Fast AG, Papoutsakis ET. 2012. Novel System for Efficient Isolation of Clostridium Double-Crossover Allelic Exchange Mutants Enabling Markerless Chromosomal Gene Deletions and DNA Integration. Appl. Environ. Microbiol. 78:8112–8121. doi:10.1128/AEM.02214-12.
25. Shin J, Jiang F, Liu J-J, Bray NL, Rauch BJ, Baik SH, Nogales E, Bondy-Denomy J, Corn JE, Doudna JA. 2017. Disabling Cas9 by an anti-CRISPR DNA mimic. Sci. Adv. 3:e1701620. doi:10.1126/sciadv.1701620.
26. Mayo-Muñoz D, He F, Jørgensen J, Madsen P, Bhoobalan-Chitty Y, Peng X. 2018. Anti-CRISPR-Based and CRISPR-Based Genome Editing of *Sulfolobus islandicus* Rod-Shaped Virus 2. Viruses 10:695. doi:10.3390/v10120695.
27. Hocq R, Bouilloux-Lafont M, Lopes Ferreira N, Wasels F. 2019. σ54 (σL) plays a central role in carbon metabolism in the industrially relevant *Clostridium beijerinckii*. Sci Rep 9:578. doi:10.1038/s41598-019-43822-2.

672 28. Cho C, Hong S, Moon HG, Jang Y-S, Kim D, Lee SY, Lovley DR. 2019.
673 Engineering Clostridial Aldehyde/Alcohol Dehydrogenase for Selective Butanol
674 Production. mBio 10:653. doi:10.1128/mBio.02683-18.
675 29. Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, Sullender
676 M, Ebert BL, Xavier RJ, Root DE. 2014. Rational design of highly active sgRNAs
677 for CRISPR-Cas9–mediated gene inactivation. Nat Biotechnol 32:1262–1267.
678 doi:10.1038/nbt.3026.
679 30. Mermelstein LD, Papoutsakis ET. 1993. In vivo methylation in *Escherichia coli* by
680 the *Bacillus subtilis* phage phi 3T I methyltransferase to protect plasmids from
681 restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. Appl
682 Environ Microbiol 59:1077–1081.
683 31. Gapes JR, Nimcevic D, Friedl A. 1996. Long-Term Continuous Cultivation of
684 *Clostridium beijerinckii* in a Two-Stage Chemostat with On-Line Solvent
685 Removal. Appl Environ Microbiol 62:3210–3219.
686