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

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18 **ABSTRACT**

19 Although *Clostridium acetobutylicum* is the model organism for the study of Acetone
20 Butanol Ethanol (ABE) fermentation, its characterization has long been impeded by
21 the lack of efficient genome editing tools. In particular, the contribution of alcohol
22 dehydrogenases to solventogenesis in this bacteria has mostly been studied with the
23 generation of single gene deletion strains. In this study, the three butanol
24 dehydrogenases encoding genes located on the chromosome of the DSM 792
25 reference strain were deleted iteratively by using a recently developed CRISPR-Cas9
26 tool improved by using an anti-CRISPR protein encoding gene *acrIIA4*. Although the
27 literature has previously shown that inactivation of either *bdhA*, *bdhB* or *bdhC* had
28 only moderate effects on the strain, this study shows that clean deletion of both *bdhA*
29 and *bdhB* strongly impaired solvent production, and that a triple mutant
30 $\Delta bdhA\Delta bdhB\Delta bdhC$ was even more affected. Complementation experiments
31 confirmed the key role of these enzymes, and the capacity of each *bdh* copy to fully
32 restore an efficient ABE fermentation in the triple deletion strain.

33

34 **IMPORTANCE**

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

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

47 INTRODUCTION

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

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

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

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

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

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

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

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

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

121 because of a basal leaky expression in the absence of inducer of both the nuclease
122 and the gRNA. To overcome this problem, we optimized pCas9_{ind} by cloning a gene
123 encoding the AcrIIA4 anti-Cas9 protein (18) under the control of a lactose-inducible
124 promoter (19). Anti-Cas proteins have been discovered recently in CRISPR-Cas
125 antagonists that have evolved inhibitors of those bacterial defense mechanisms (20,
126 21). The ability to regulate at a post-translational level the action of Cas nucleases
127 opens applications for these proteins in CRISPR-based genetic tools, in particular for
128 organisms with low transformation efficiencies (22). In this work, the use of AcrIIA4
129 allowed to limit the undesired activity of the CRISPR-Cas system at the
130 electroporation step, resulting in an increased transformation efficiency of pGRNA
131 plasmids.

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

139 RESULTS

140 Improvement of a CRISPR-Cas9 genetic tool

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

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

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

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

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

187 **Bdhs mutants construction**

188 Once transformants containing pCas9_{acr} and either pGRNA- Δ *bdhA*, pGRNA- Δ *bdhB*,
189 pGRNA- Δ *bdhC* or pGRNA- Δ *bdhA* Δ *bdhB* were obtained, expression of *cas9* was
190 induced as previously described (16). For each modification, at least three aTc-
191 resistant colonies obtained from independent transformants were analyzed and
192 confirmed by PCR, yielding the corresponding mutants that we stored for further
193 experiments. As for the early version of our tool (16), the editing efficiency for every
194 single modification was 100 % (data not shown). To generate DSM 792
195 Δ *bdhA* Δ *bdhC*, DSM 792 Δ *bdhB* Δ *bdhC* and DSM 792 Δ *bdhA* Δ *bdhB* Δ *bdhC*, one DSM
196 792 Δ *bdhC* mutant was randomly chosen (i.e. the first mutant obtained), streaked on
197 2YTG plates devoid of thiamphenicol until pGRNA- Δ *bdhC* was cured, and further
198 transformed with plasmids pGRNA- Δ *bdhA*, pGRNA- Δ *bdhB* or pGRNA- Δ *bdhA* Δ *bdhB*,
199 respectively. Once transformants were obtained, expression of *cas9* was induced
200 and aTc-resistant colonies obtained from independent transformants were analyzed
201 by PCR, confirmed, and saved for further experiments. For each type of deletion, six
202 independent colonies were tested and the editing efficiency for this new round of
203 modification was again 100 % (data not shown). PCR analyzes of *bdhA*-*bdhB* and
204 *bdhC* loci in each different mutant are shown in Fig. 3.

205 **Phenotypic analysis**

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

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

221 Hypothesizing that the inability of the mutants to efficiently assimilate acids and
222 convert them into solvents led to an irreversible pH decrease, we performed pH-
223 controlled batch fermentations of the wild-type strain as well as $\Delta bdhA\Delta bdhB$ and
224 $\Delta bdhA\Delta bdhB\Delta bdhC$ mutants in bioreactor (Table 2). Although solvent production of
225 the mutants was slightly increased compared to flask fermentations, the final solvent
226 concentrations were still strongly reduced compared to the wild-type strain. Unlike
227 what was observed for flask fermentations, butanol was still the main solvent
228 detected at the end of the fermentation. Interestingly, the major difference observed
229 compared to flask fermentation was a stronger accumulation of lactate, reaching up
230 to 2 g/L in pH-controlled bioreactor instead of 0.2 g/L in flask assays.

231 **Complementation assays**

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

235 native promoter. As a control, the wild-type strain was also transformed with those
236 plasmids. For each complementation (i.e. each plasmid introduced in each strain),
237 three randomly chosen transformants were analyzed through batch fermentation
238 assays performed in flasks as previously done, and final fermentation products are
239 presented in Table 3. In the wild-type strain, overexpression of each gene had no
240 significant effect compared to the presence of the empty control vector. However, the
241 $\Delta bdhA\Delta bdhB$ and $\Delta bdhA\Delta bdhB\Delta bdhC$ mutants assessed were successfully
242 complemented with any of the *bdh* copy, without significant difference in terms of
243 product pattern of selectivity. The two mutants assessed still undergone acid crash
244 when transformed with an empty vector. Interestingly, even the phenotype of the
245 $\Delta bdhA\Delta bdhB$ mutant was reverted by the overexpression of *bdhC*, which led to
246 fermentation performances in the range of the wild-type strain.

247 **DISCUSSION**

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

264 In this study, we intended to use a two-plasmid CRISPR-Cas tool recently developed
265 to inactivate *bdhA*, *bdhB* and *bdhC*, the three genes identified in the genome of *C.*
266 *acetobutylicum* DSM 792 that are coding for NADPH-dependent alcohol
267 dehydrogenases. We first failed in trying to generate DSM 792 $\Delta bdhB$ and DSM 792
268 $\Delta bdhA\Delta bdhB$ mutants because we did not manage to obtain transformants
269 containing both pCas9_{ind} and the corresponding pGRNA plasmids. Although it cannot
270 be ruled out that transformation efficiency of pGRNA plasmids is affected by the
271 editing template, it is intuitively most likely linked to the gRNA strength. In the

272 example reported in this study, only one assay allowed us to obtain three
273 transformants of DSM 792 (pCas9_{ind}) containing pGRNA- $\Delta bdhB$, after dozens of
274 attempts. Similarly, no transformant was ever obtained when introducing pGRNA-
275 $\Delta bdhA\Delta bdhB$ in DSM 792 (pCas9_{ind}). Obviously, an alternative to circumvent this
276 problem would have been to change the gRNA sequence targeting *bdhB*, especially
277 since we facilitated the retargeting of pGRNA plasmids compared to the early version
278 of the tool (Fig. 1A). However, we also faced this toxicity problem with a few other
279 targeting plasmids in our laboratory (data not reported), and we therefore wanted to
280 modify our tool so that it would be possible to generate transformants with any
281 pGRNA plasmid used. Therefore, we added an extra control of the Cas9-gRNA
282 complex nuclease activity through the use of a recently described anti-CRISPR
283 protein (18). Interestingly, the transformation efficiency of pGRNA- $\Delta bdhA$ and
284 pGRNA- $\Delta bdhC$ was not improved, suggesting that placing *cas9* under the control of
285 an inducible promoter was sufficient when using those gRNA sequences. However,
286 transformation efficiencies with pGRNA- $\Delta bdhB$ and pGRNA- $\Delta bdhA\Delta bdhB$ were
287 greatly increased when DSM 792 (pCas9_{acr}) was used, demonstrating that we
288 effectively improved our tool. To the best of our knowledge, the use of anti-CRISPR
289 proteins to realize CRISPR-based genome editing was already documented in
290 human cells (25) and in the archaea *Sulfolobus islandicus* (26). Combining two
291 antagonist activities controlled by inducible promoters, we constructed a fast, reliable
292 and efficient genome editing tool. Although only deletions were performed in this
293 study, the CRISPR/Anti-CRISPR strategy can also be employed to perform insertion
294 and single nucleotide modification as shown with the first version of the tool (16),
295 without the presence of any unwanted scar or antibiotic resistance marker in the
296 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-bdhA459s::CT*
301 and *Cac-bdhB475a::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 $\Delta yqhD$ 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

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

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

347 pool, respectively. Protein quantification indicates that BdhB represents 62% of the
348 Bdh pool in acidogenesis, followed by BdhA (27%) and BdhC (11%). Increased
349 transcription of *bdhB* in solventogenesis results in an increase of BdhB in the pool of
350 Bdh enzymes (83%), compared to BdhA (11%) and BdhC (6%). Activities of the
351 purified enzymes were also reported in the same publication and show comparable
352 butanol dehydrogenase activities for all enzymes (2). Taken together, these data
353 suggest that BdhB is the main butanol dehydrogenase. A lower quantity of enzymes
354 is transcribed from the *bdhA* gene, although it represents one quarter of the Bdh
355 enzyme pool during acetogenesis. Our results are in accordance with those data,
356 since the fact that a $\Delta bdhA\Delta bdhB$ mutant cannot perform an efficient
357 solventogenesis while other double mutants can indicates that BdhA and BdhB
358 appear to have a more important role than BdhC. Interestingly, a $\Delta bdhB$ mutant is
359 still capable of performing an efficient fermentation, suggesting that the two
360 remaining genes can compensate the loss of this copy, providing an explanation why
361 so many copies of the same gene can be found in the genome. As intuited, the triple
362 mutant $\Delta bdhA\Delta bdhB\Delta bdhC$ was even more heavily affected than the $\Delta bdhA\Delta bdhB$
363 mutant, although neither butanol nor ethanol production was abolished, suggesting
364 that other enzymes are still capable of compensating the lower alcohol
365 dehydrogenase activity in the mutant, most likely AdhE2 and/or the products of
366 CA_C3375 and CA_P0059. Notably, fermentations performed in pH-controlled
367 bioreactors did not allow to prevent acid accumulation, showing the mutant incapacity
368 to reduce acids. Instead, the triple mutant produced a significant amount of lactate in
369 these conditions, most likely to regenerate its NAD(P)⁺ pool which is not produced by
370 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 $\Delta bdhA\Delta bdhB$ and $\Delta bdhA\Delta bdhB\Delta bdhC$ 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 $\Delta bdhA\Delta bdhB$ and
382 $\Delta bdhA\Delta bdhB\Delta bdhC$ 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 $\Delta bdhA\Delta bdhB\Delta bdhC$ 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.

413 MATERIALS AND METHODS

414 Bacterial strains, plasmids and culture conditions

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

424 DNA manipulation and plasmid construction

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

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

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

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

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

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

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

472 **Transformation, induction of *cas9* expression and mutant confirmation**

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

484 **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 KH₂PO₄, 0.6 g L⁻¹ K₂HPO₄, 1 g L⁻¹ MgSO₄
487 7H₂O, 6.6 mg L⁻¹ FeSO₄ 7H₂O, 0.1 g L⁻¹ 4-aminobenzoic acid, 5.8 g L⁻¹ CH₃COONH₄
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.

518 **ACKNOWLEDGMENTS**

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520 commercial, or not-for-profit sectors. We thank H el ene Velly and Sandra Menir for
521 technical assistance during bioreactor assays.

522

523 **TABLE 1** Fermentation products of the mutants analyzed in this study. Results shown are average values \pm standard deviations
 524 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 \pm 0.1	9.0 \pm 0.5	12.4 \pm 1.4	22.8 \pm 2.0	2.6 \pm 0.1	2.4 \pm 0.1	ND	5.0 \pm 0.2	69.2 \pm 0.2	5.1 \pm 0.0
$\Delta bdhA$	1.2 \pm 0.1	9.1 \pm 0.4	11.6 \pm 0.4	22.0 \pm 0.1	2.4 \pm 0.1	2.3 \pm 0.1	ND	4.7 \pm 0.0	69.4 \pm 0.2	5.2 \pm 0.0
$\Delta bdhB$	0.9 \pm 0.0	9.2 \pm 0.2	12.1 \pm 0.6	22.2 \pm 0.6	2.2 \pm 0.1	2.7 \pm 0.1	ND	4.9 \pm 0.2	71.0 \pm 2.4	5.3 \pm 0.0
$\Delta bdhC$	1.3 \pm 0.2	9.3 \pm 0.3	12.8 \pm 1.0	23.4 \pm 1.4	2.6 \pm 0.4	2.1 \pm 0.5	ND	4.7 \pm 0.9	71.4 \pm 2.6	5.3 \pm 0.1
$\Delta bdhA\Delta bdhB$	0.5 \pm 0.0	3.8 \pm 0.6	3.2 \pm 0.7	7.4 \pm 1.4	4.5 \pm 0.4	5.0 \pm 0.2	0.2 \pm 0.1	9.7 \pm 0.4	35.9 \pm 2.2	4.8 \pm 0.0
$\Delta bdhA\Delta bdhC$	1.1 \pm 0.2	9.5 \pm 0.5	13.3 \pm 0.1	23.9 \pm 0.4	2.2 \pm 0.2	2.8 \pm 0.4	ND	5.0 \pm 0.2	72.0 \pm 0.6	5.2 \pm 0.0
$\Delta bdhB\Delta bdhC$	1.0 \pm 0.1	9.3 \pm 1.1	11.6 \pm 0.7	21.9 \pm 1.6	2.0 \pm 0.2	2.9 \pm 0.5	ND	4.9 \pm 0.2	73.5 \pm 1.8	5.3 \pm 0.0
$\Delta bdhA\Delta bdhB\Delta bdhC$	0.4 \pm 0.0	2.5 \pm 0.2	1.3 \pm 0.2	4.2 \pm 0.3	5.3 \pm 0.3	5.9 \pm 0.2	0.2 \pm 0.1	11.4 \pm 0.5	26.7 \pm 1.8	4.7 \pm 0.0

^a ND, Not Detected

525

526 **TABLE 2** Fermentation products in pH-controlled bioreactors

Strain	Products (g/L) ^a								Glucose ⁵²⁷ 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
<i>ΔbdhAΔbdhB</i>	0.3	3.1	5.0	8.4	4.3	4.3	1.8	10.4	41.9
<i>ΔbdhAΔbdhBΔbdhC</i>	0.2	1.9	2.8	4.9	4.9	4.9	2.0	11.8	36.8

^a ND, Not Detected

528

529 **TABLE 3** Complementation assays. Results shown are average values \pm 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 \pm 0.1	8.9 \pm 0.4	13.3 \pm 0.7	24.0 \pm 1.1	2.2 \pm 0.2	2.5 \pm 0.1	ND	4.7 \pm 0.2	64.0 \pm 1.3	5.3 \pm 0.1
	pFW01- <i>bdhA</i>	2.2 \pm 0.1	9.9 \pm 0.2	11.9 \pm 0.6	24.0 \pm 0.9	1.7 \pm 0.3	1.4 \pm 0.4	ND	3.2 \pm 0.7	64.8 \pm 1.5	5.3 \pm 0.1
	pFW01- <i>bdhB</i>	1.6 \pm 0.3	8.4 \pm 1.1	12.1 \pm 1.3	22.1 \pm 2.6	2.3 \pm 0.3	2.7 \pm 0.2	ND	5.0 \pm 0.5	61.5 \pm 2.5	5.1 \pm 0.0
	pFW01- <i>bdhC</i>	1.8 \pm 0.4	9.6 \pm 0.5	13.4 \pm 0.7	24.8 \pm 1.5	2.4 \pm 0.1	2.3 \pm 0.1	ND	4.7 \pm 0.2	64.2 \pm 0.6	5.2 \pm 0.0
<i>ΔbdhAΔbdhB</i>											
	pFW01	0.7 \pm 0.0	2.6 \pm 0.7	2.7 \pm 0.8	6.1 \pm 1.5	5.4 \pm 0.4	4.1 \pm 0.6	0.6 \pm 0.1	10.1 \pm 1.0	21.4 \pm 3.4	4.7 \pm 0.1
	pFW01- <i>bdhA</i>	2.0 \pm 0.1	10.6 \pm 1.1	13.7 \pm 0.8	26.3 \pm 0.9	1.7 \pm 0.5	1.3 \pm 0.1	ND	3.0 \pm 0.5	70.9 \pm 2.2	5.5 \pm 0.1
	pFW01- <i>bdhB</i>	1.8 \pm 0.1	8.8 \pm 0.5	13.6 \pm 0.1	24.2 \pm 0.3	2.1 \pm 0.4	1.6 \pm 0.5	ND	3.7 \pm 0.9	68.7 \pm 0.6	5.4 \pm 0.3
	pFW01- <i>bdhC</i>	1.4 \pm 0.1	10.2 \pm 0.4	13.2 \pm 0.7	24.8 \pm 1.2	1.9 \pm 0.1	2.5 \pm 0.1	ND	4.4 \pm 0.2	67.7 \pm 1.0	5.4 \pm 0.0
<i>ΔbdhAΔbdhBΔbdhC</i>											
	pFW01	0.5 \pm 0.1	2.3 \pm 0.3	2.1 \pm 0.3	4.9 \pm 0.6	5.7 \pm 0.5	4.6 \pm 0.4	0.6 \pm 0.1	10.9 \pm 1.0	18.8 \pm 3.4	4.6 \pm 0.0
	pFW01- <i>bdhA</i>	1.4 \pm 0.1	9.6 \pm 0.3	13.2 \pm 0.3	24.2 \pm 0.6	1.9 \pm 0.0	1.8 \pm 0.1	ND	3.7 \pm 0.2	68.6 \pm 1.1	5.4 \pm 0.1
	pFW01- <i>bdhB</i>	1.7 \pm 0.2	8.7 \pm 0.8	13.8 \pm 0.7	24.2 \pm 1.7	1.9 \pm 0.0	1.7 \pm 0.1	ND	3.6 \pm 0.1	68.6 \pm 1.2	5.5 \pm 0.1
	pFW01- <i>bdhC</i>	1.2 \pm 0.3	10.4 \pm 0.8	12.3 \pm 0.5	23.9 \pm 0.7	1.8 \pm 0.4	2.1 \pm 1.1	ND	3.9 \pm 1.5	67.4 \pm 0.4	5.4 \pm 0.1

^a ND, Not Detected

531

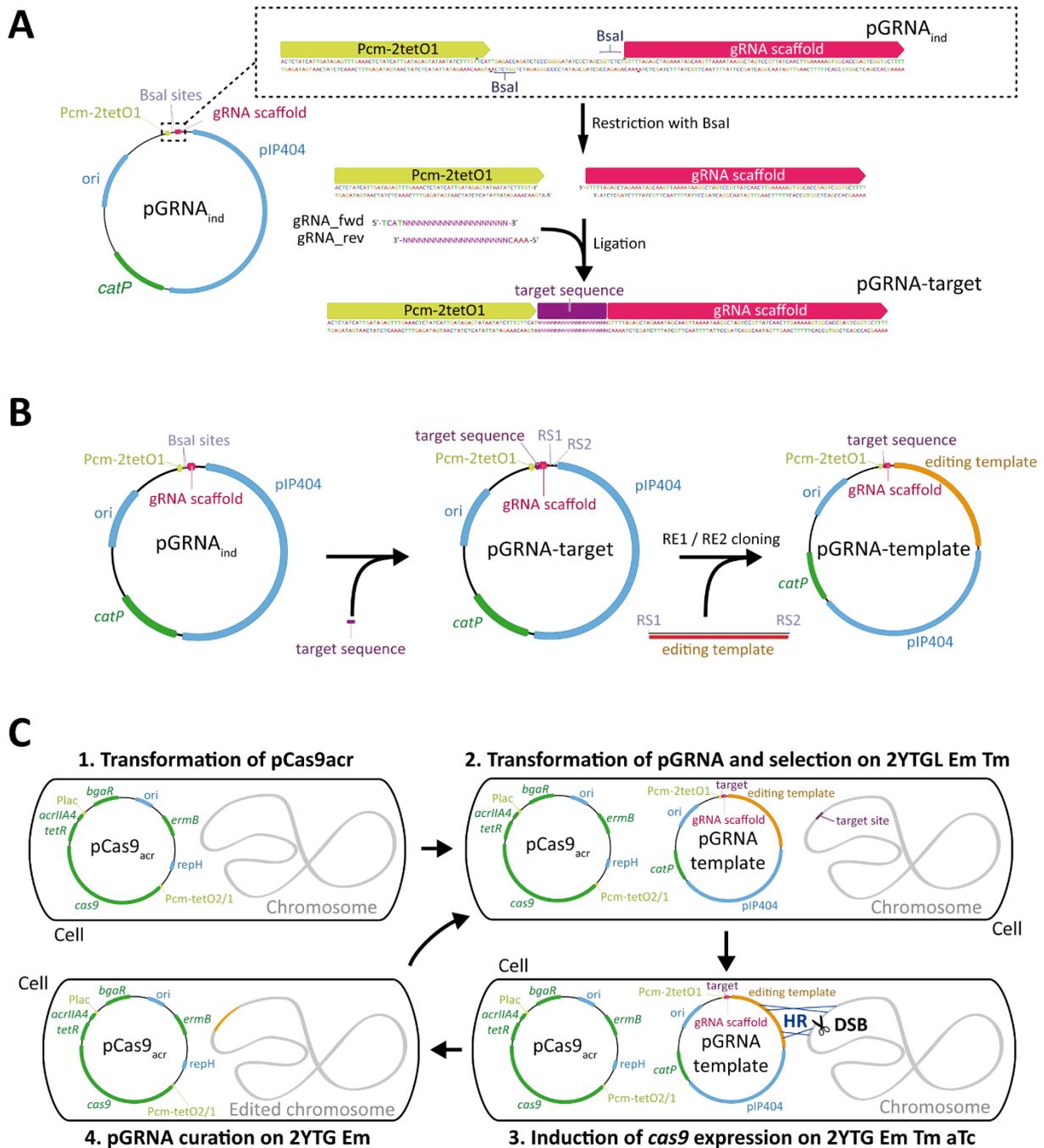
532 **TABLE 4** Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant Characteristics	Source or reference
Strains		
<i>C. acetobutylicum</i> DSM 792	Wild-type	DSMZ
<i>E. coli</i> NEB 10-beta	Cloning strain	NEB
Plasmids		
pAN2	<i>tetA</i> , Φ 3T I gene, p15A origin	(5, 30)
pFW01	<i>ermB</i> , ColE1 origin, pCB102 origin	(16)
pCas9 _{ind}	<i>ermB</i> , ColE1 origin, pCB102 origin, <i>cas9</i> (Pcm-tetO2/1 promoter), <i>tetR</i>	(16)
pCas9 _{acr}	pCas9 _{ind} derivative with <i>acrIIA4</i> (P _{bgal} promoter) and <i>bgaR</i> insertions	This study
pEC750C	<i>catP</i> , 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 <i>bdhA</i>	This study
pGRNA- Δ <i>bdhA</i>	pGRNA-bdhA derivative with Δ <i>bdhA</i> editing template insertion	This study
pGRNA-bdhB	pGRNA _{ind} derivative targeting <i>bdhB</i>	This study
pGRNA- Δ <i>bdhB</i>	pGRNA-bdhB derivative with Δ <i>bdhB</i> editing template insertion	This study
pGRNA- Δ <i>bdhA</i> Δ <i>bdhB</i>	pGRNA-bdhB derivative with Δ <i>bdhA</i> Δ <i>bdhB</i> editing template insertion	This study
pGRNA-bdhC	pGRNA _{ind} derivative targeting <i>bdhC</i>	This study
pGRNA- Δ <i>bdhC</i>	pGRNA-bdhC derivative with Δ <i>bdhC</i> editing template insertion	This study
pFW01- <i>bdhA</i>	pFW01 derivative with <i>bdhA</i> insertion	This study
pFW01- <i>bdhB</i>	pFW01 derivative with <i>bdhB</i> insertion	This study
pFW01- <i>bdhC</i>	pFW01 derivative with <i>bdhC</i> insertion	This study

533

TABLE 5 Oligonucleotides used in this study

Oligonucleotide	Sequence (5'-3')
P1	CAGATTGTA CTGAGAGTGCACC
P2	GTGAGCGGATAACAATTCACAC
P3	TCATGCACTTAACTCGTGTTCCAT
P4	AAACATGGAACACGAGTTAAGTGC
P5	TCATGCTTATTACGACATAACACA
P6	AAACTGTGTTATGTCGTAATAAGC
P7	TCATGCTCTTGTATCATAGTCCGT
P8	AAACACGGACTATGATACAAGAGC
P9	AAAAAAGGATCCTTAGGAGCCATATCTGGATG
P10	TATGCTAAGTTTTAAATCTTATTAATAGAAACTGTAGAGG
P11	TTAATAAGATTTAAAACCTTAGCATACTTCTTACC
P12	AAAAAAGTCGACCTTCTAATCTCCTCTACTATTTTAG
P13	ATGCATGGATCCAAACGAACCCAAAAAGAAAGTTTC
P14	GGTTGATTTCAAATCTGTGTAAACCTACCG
P15	ACACAGATTTGAAATCAACCACTTTAACCC
P16	ATGCATGTCGACTCTTAAGAACATGTATAAAGTATGG
P17	GCTAAGTTTTAAATCTGTGTAAACCTACCG
P18	ACACAGATTTAAAACCTTAGCATACTTCTTACC
P19	ATGCATGTCGACCTTCTAATCTCCTCTACTATTTTAG
P20	AAAAAACTCGAGCTATAAATAATATTACCCCCATAACTG
P21	ATAATTTTGATGAGGGTGCAATGTAAGTTTG
P22	TTGCACCCTCATCAAATTATACATAAGATTATCCC
P23	AAAAAATCTAGAGCAAAGGAATTAGTAGAAATTAC
P24	AAAAAACTCGAGTTTAACCCCTCCTGTTTAGATTAT
P25	AAAAAAGTCGACCGAAAAATTCACCCCTCAA
P26	AAAAAACTCGAGAAAATACTCCCAAGATATTAATGCT
P27	AAAAAAGTCGACTAGAACTGTAGAGGTATTTTTATAATTTAAAAG
P28	AAAAAACTCGAGTTTCTCCACCTTTAATACTAAAATATTTTCA
P29	AAAAAAGTCGACAAAAATACCCCACTATTCTAATTTTATTCA
P30	AGTACCTCCTAAGCCTTTTTATGA
P31	TATGGATTGCCCTACAGCCC
P32	CCACTGCCACCATTTTCTAGC
P33	CAAGGACAGATAGGTGGGGC



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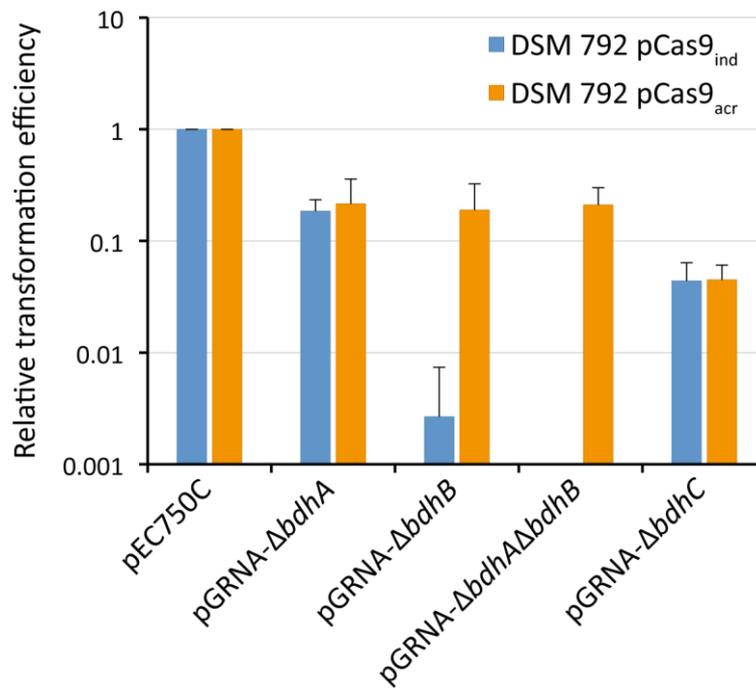
539 **FIG 1** Easy reprogramming of the two plasmid CRISPR-Cas9 genetic tool for multiple

540 genome edition. (A) Retargeting of the pGRNA plasmid by cloning two hybridized

541 primers between the aTc-inducible promoter Pcm-2tetO1 and the gRNA scaffold. (B)

542 Insertion of the editing template within the pGRNA plasmid by restriction enzyme-

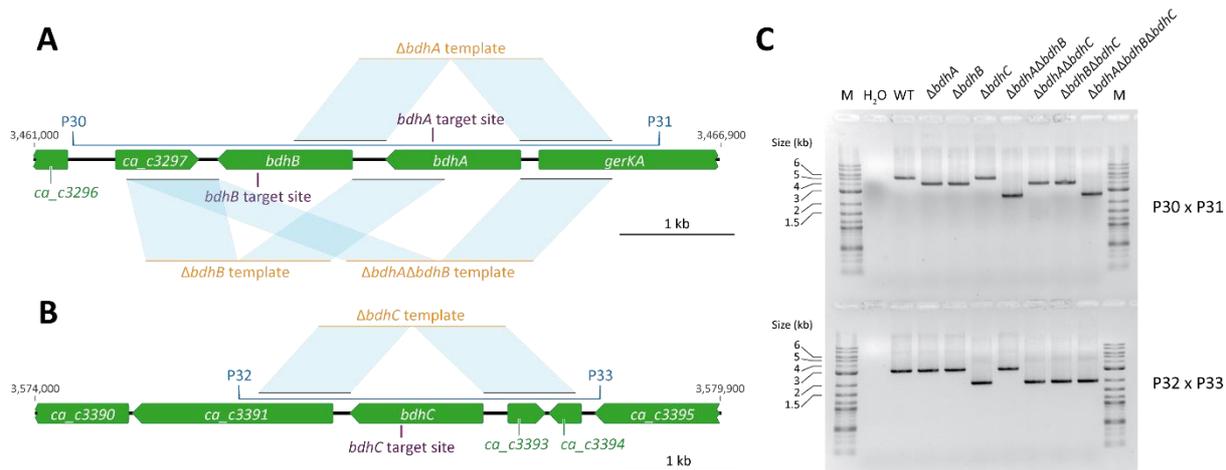
543 based cloning. (C) Iterative genome modification. Step 1, the strain is transformed
544 with pCas9_{acr} and transformants are selected on erythromycin-containing media.
545 Step 2, the resulting strain is transformed with a pGRNA editing plasmid and
546 transformants are selected on media containing erythromycin, thiamphenicol and
547 lactose to activate the transcription of *acrIIA4*. Step 3, expression of *cas9* is triggered
548 on media containing erythromycin and thiamphenicol and devoid of lactose so that
549 transcription of *acrIIA4* is not induced anymore. The medium contains aTc to trigger
550 the transcription of *cas9* to select mutants in which the editing event occurred. Step
551 4, once aTc-resistant colonies have been confirmed, pGRNA is curated on media
552 containing erythromycin and no thiamphenicol, yielding cells ready for a new round of
553 modification. ori, replication origin for *E. coli*; pip404 and repH, compatible replication
554 origins for *C. acetobutylicum*; *catP*, thiamphenicol/chloramphenicol-resistance gene;
555 *ermB*, erythromycin-resistance gene; RS, restriction site; RE, restriction enzyme;
556 DSB, double strand break, HR, homologous recombination.



557

558

559 **FIG 2** Relative transformation efficiencies of pGRNA-template plasmids in DSM 792
 560 containing pCas9_{ind} or pCas9_{acr}. 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 $\Delta bdhA$, $\Delta bdhB$ or $\Delta bdhA\Delta bdhB$ strains, respectively. Amplification with primers pair

573 P32-P33 yields a PCR product of 3124 bp or 1993 bp product in WT or $\Delta bdhC$

574 strains, respectively. Lane M, 2-log DNA ladder (0.1 to 10 kb; NEB); Lane H₂O,

575 amplification on water.

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