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Streamlined, recombinase-free genome editing with CRISPR-Cas9 in *Lactobacillus plantarum* reveals barriers to efficient editing

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Key words: Genome editing, Lactobacilli, Recombineering, Shuttle plasmid

Abbreviations: CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats, Cas - CRISPR-associated, sgRNA - single-guide RNA, RT - recombineering template

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1 **ABSTRACT**

2 Lactic-acid bacteria such as *Lactobacillus plantarum* are commonly used for fermenting foods
3 and as probiotics, where increasingly sophisticated genome-editing tools are currently being
4 employed to elucidate and enhance these microbes' beneficial properties. The most advanced
5 tools to-date require heterologous single-stranded DNA recombinases to integrate short
6 oligonucleotides followed by using CRISPR-Cas9 to eliminate cells harboring unedited
7 sequences. Here, we show that encoding the recombineering template on a replicating plasmid
8 allowed efficient genome editing with CRISPR-Cas9 in multiple *L. plantarum* strains without a
9 recombinase. This strategy accelerated the genome-editing pipeline and could efficiently
10 introduce a stop codon in *ribB*, silent mutations in *ackA*, and a complete deletion of *lacM*. In
11 contrast, oligo-mediated recombineering with CRISPR-Cas9 proved far less efficient in at least
12 one instance. We also observed unexpected outcomes of our recombinase-free method,
13 including an ~1.3-kb genomic deletion when targeting *ribB* in one strain, and reversion of a point
14 mutation in the recombineering template in another strain. Our method therefore can streamline
15 targeted genome editing in different strains of *L. plantarum*, although the best means of
16 achieving efficient editing may vary based on the selected sequence modification, gene, and
17 strain.

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18 **INTRODUCTION**

19 *Lactobacillus* represents a diverse and biotechnologically important genus of bacteria. As they
20 naturally produce lactic acid, many *Lactobacilli* strains are commonly found in yogurts and other
21 food products (Kailasapathy and Chin, 2000; Wang et al., 2004). This widespread usage has
22 also pushed their development as ingestible probiotics to improve gut health. *In vivo* studies
23 have demonstrated that members of the *Lactobacillus plantarum* can combat gut-residing
24 infections in humans (Wullt et al., 2007). Recent work has also highlighted their ability to
25 promote host growth under nutrient-limiting conditions in fruit flies and in mice (Schwarzer et al.,
26 2016; Storelli et al., 2011). A significant part of these successful applications can be attributed to
27 ever-advancing genetic tools. These tools have been used to elucidate how Lactobacilli
28 genetics contribute to their desirable properties (Martino et al., 2018; Matos et al., 2017) and to
29 forward-engineer strains as enhanced probiotics or to produce metabolites in different food
30 products (Bron et al., 2007; Okano et al., 2018).

31 To date, a number of studies have established increasingly sophisticated genetic tools
32 for *Lactobacilli* (Alegre et al., 2004; Aukrust, 1988; Bryan et al., 2000; Kleerebezem et al., 1997;
33 Kullen and Klaenhammer, 2000; Thompson and Collins, 1996) that have created a framework
34 for genome editing in this genus (Bron et al., 2007; Maguin et al., 1996, 1992; Okano et al.,
35 2018, 2009). Recently, a scarless genome-editing system was developed in several Lactobacilli
36 that relies on expressing a phage-derived single-stranded DNA recombinase to integrate
37 oligonucleotides into the genome (van Pijkeren and Britton, 2012). This approach was further
38 enhanced in the model bacterium *L. reuteri* by DNA cleavage with CRISPR-Cas9 (Oh and van
39 Pijkeren, 2014). CRISPR-Cas9 is a two-component system comprising a guide RNA (gRNA)
40 and the RNA-guided Cas9 nuclease from *Streptococcus pyogenes* (SpCas9) (Gasiunas et al.,
41 2012; Jinek et al., 2012). The gRNAs are designed to be complementary to target DNA
42 sequences flanked by a 3' NGG protospacer-adjacent motif (PAM) based on a guide-centric
43 orientation (Jinek et al., 2012; Leenay and Beisel, 2017), leading SpCas9 to cleave the DNA 3

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44 bps upstream of the PAM. The gRNA can be an engineered single-guide RNA (sgRNA) or
45 processed from a transcribed CRISPR array with the help of RNase III and a trans-acting
46 CRISPR RNA (tracrRNA) (Deltcheva et al., 2011; Jinek et al., 2012). In bacteria, the traditional
47 paradigm for oligo-based editing with CRISPR-Cas9 is that the oligonucleotide mutates the
48 genomic site targeted by the sgRNA, and Cas9 only targets cells that did not undergo
49 recombination. Due to the cytotoxicity of cleaving bacterial genomes with a CRISPR nuclease
50 (Gomaa et al., 2014; Jiang et al., 2013; Leenay and Beisel, 2017; Vercoe et al., 2013), Cas9
51 effectively serves as a negative selection against unedited cells, thereby greatly boosting the
52 frequency of successful editing.

53 While promising, these genome-editing tools have rarely been applied outside of model
54 strains of Lactobacilli. One potential bottleneck relates to the recombinase. It normally must be
55 under inducible control to limit unintended recombination, and the associated sensory proteins
56 often must be co-expressed (e.g. NisR and NisK for nisin). Furthermore, the co-transformation
57 of large quantities of the oligos and the CRISPR-Cas9 plasmid can reduce the overall
58 transformation efficiency, thus not all cells will receive both the oligos and the plasmid. Recent
59 work has begun to suggest that the recombinase can be dispensable by taking a different
60 approach to recombineering. For instance, prior work in *E. coli* showed that RecA and the
61 endogenous homologous recombination machinery could drive efficient recombineering
62 between a plasmid harboring a double-stranded recombineering template and the genomic site
63 targeted by SpCas9 (Cui and Bikard, 2016). Similarly, accumulating examples of Cas9-based
64 genome editing in bacteria rely on a plasmid-encoded recombineering template without the use
65 of a heterologous recombinase (Altenbuchner, 2016; Huang et al., 2015; Jiang et al., 2013). In a
66 recent example in the model bacterium *Lactobacillus casei*, a single construct encoding a
67 nicking mutant of SpCas9, a targeting CRISPR array, and a recombineering template achieved
68 editing efficiencies up to 62% (Song et al., 2017). These examples suggested that Cas9-based
69 editing could be achieved in *Lactobacillus plantarum* without the need for a recombinase.

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70 Here, we developed a recombinase-free method of genome editing with SpCas9 for *L.*
71 *plantarum*. The method relies on a recombineering template encoded on a replicated plasmid to
72 achieve editing. We show that our method efficiently generated a premature stop codon into the
73 riboflavin biosynthetic gene *ribB*, whereas oligo-mediated recombineering fails to generate this
74 same edit. Then, we expand the design of the recombineering template to insert silent
75 mutations in the acetate kinase gene *ackA* and a complete deletion of the β-galactosidase
76 subunit gene *lacM*. Finally, we observed two instances where this editing approach failed--
77 specifically through the recombineering template reverting to the WT sequence in the model *L.*
78 *plantarum* strain WCFS1 and the generation of a consistent deletion within the target gene in
79 the non-model *L. plantarum* strain NIZO2877. Our method therefore offers a streamlined means
80 of genome editing in *L. plantarum*, although the availability of multiple editing methods are still
81 necessary.
82
83 **RESULTS**

84 **Constructs for recombinase-free genome editing use two *E. coli-Lactobacillus* shuttle**
85 **vectors.** We sought to simplify the editing pipeline in *L. plantarum* using a recombinase-free
86 system (**Figure 1A**). To facilitate this pipeline, we generated two *E. coli-Lactobacillus* shuttle
87 vectors to enhance the rate of cloning the final constructs (**Figure S1**). The CRISPR-Ca9
88 targeting plasmid encodes SpCas9, its tracrRNA, and a CRISPR array under the control of the
89 P_{pgm} constitutive promoter (Duong et al., 2011). A CRISPR array was chosen over the standard
90 single-guide RNA (Jinek et al., 2012) to avoid the need for a defined transcriptional start site
91 and to allow multi-spacer arrays for multiplexed editing. New spacers are added by digesting
92 unique cutsites within the processed region of an existing spacer (**Figure 1B**). This construct
93 exhibited potent activity based on a large drop in the transformation efficiency when targeting a
94 conserved site in the *rpoB* gene in both strains (**Figure S2**). The base recombineering-template
95 plasmid was generated by adding *E. coli* replication components into a compatible shuttle vector

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96 (van Pijkeren and Britton, 2012) as well as a multi-cloning site for insertion of the
97 recombineering template. Each plasmid was isolated from the methylation-free *E. coli* strain
98 EC135 to boost the transformation efficiency (Zhang et al., 2012). In some cases, the plasmid
99 was then passaged through the highly-transformable strain WCFS1 prior to transformation into
100 other *L. plantarum* strains.
101
102 **The recombinase-free method efficiently generated a premature stop codon in *ribB* in *L.*
103 *plantarum* WJL.** As a test case for validating our recombinase-free genome editing constructs,
104 we chose the moderately transformable *L. plantarum* strain WJL. This strain has been shown to
105 provide a fitness benefit when added to germ-free fruit fly larvae or pre-weaned mice under
106 nutrient-limiting conditions, and it could offer a chassis for engineered probiotics (Schwarzer et
107 al., 2016; Storelli et al., 2011). We decided to introduce a premature stop codon into the *ribB*
108 gene. This gene is crucial for the production of Vitamin B_2, an essential nutrient that humans
109 must obtain from their diet or their gut microbiota. Certain strains of *L. plantarum*, including the
110 model strain WCFS1, do not contain a complete *ribB* operon (Kleerebezem et al., 2003) and
111 require riboflavin for growth (Burgess et al., 2006). In contrast, the WJL strain contains a
112 complete *ribB* operon (Martino et al., 2015a). We designed a novel spacer to target a site near
113 the 5' end of the *ribB* riboflavin synthase gene, and we designed a recombineering template
114 with 1-kb homology on both sides of a premature stop codon that would incorporate into the 5'
115 end of the *ribB* gene. The stop codon was placed within the targeted PAM, thereby preventing
116 any subsequent cleavage by SpCas9 once recombination occured (**Figure 2A**).
117 Editing was performed by first transforming the cells with the recombineering-template
118 plasmid followed by the *ribB*-targeting plasmid. Cells were then plated on MRS with or without
119 200-μM riboflavin to test whether the mutants required riboflavin for growth (**Figure 2B**). We did
120 not obtain any colonies in the absence of riboflavin, while in the presence of riboflavin we
121 obtained ~28-fold fewer colonies compared to a no-guide control. Survivors were subjected to

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122 colony PCR using primers that bind the genome outside the region used for the recombineering
123 template, thereby preventing any false-positives. Of the 13 sequenced survivors, all contained
124 the intended premature stop codon. After clearing the two plasmids, we found that the survivors
125 surprisingly grew in regular MRS broth, likely due to trace amounts of riboflavin in the undefined
126 media. A chemically defined medium (CDM) was therefore used to assess the *ribB*-deletion
127 phenotype (Hébert et al., 2004). Strains containing the premature stop codon exhibited
128 negligible growth in the CDM medium without riboflavin and modest growth in CDM with trace
129 amounts of riboflavin (**Figure 2C**). This phenotypic result is in line with the essentiality of
130 riboflavin production via the *ribB* operon for cell growth in *L. plantarum* WJL. Overall, we found
131 that efficient genome editing without a recombinase is possible in *L. plantarum* WJL.
132
133 **The oligo-based method failed to generate the premature stop codon in *ribB* in *L.***
134 ***plantarum* WJL.** After successful demonstration of genome editing, we used the *ribB* site to
135 evaluate how our our method compared to the previously established method of oligo-based
136 editing with CRISPR-Cas9 (**Figure 1A**). We first generated a construct containing NisR, NisK,
137 and the P_{nisin} for nisin-inducible expression of RecT (Bryan et al., 2000) (**Figure S1**). The
138 recombineering activity of RecT was confirmed by transforming the *L. plantarum* strains WCFS1
139 and NIZO2877 with an oligonucleotide conferring rifampicin resistance following prior work
140 (**Figure S2B**) (Garibyan et al., 2003; van Pijkeren and Britton, 2012). We then confirmed that
141 oligo-mediated recombineering with CRISPR-Cas9 could select for the same edit in WJL and
142 WCSF1, although WJL showed low editing efficiency (2/23 sequenced survivors contained the
143 intended edit) **(Figure S2C,D**). Next, an 80-nt single-stranded oligonucleotide was designed to
144 incorporate the same premature stop codon used with recombinase-free editing. We then
145 transferred the RecT construct into WJL, induced with Nisin, and co-transformed the oligo and
146 the *ribB*-targeting construct following the previously reported method (Oh and van Pijkeren,
147 2014). The co-transformation yielded only two erythromycin-resistant colonies on riboflavin-

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148 supplemented plates compared to one survivor on plates without additional riboflavin (**Figure**
149 **2C**), and these survivors contained the WT sequence. Thus, in this initial example, the
150 recombinase-free method efficiently generated the desired edit while the oligo-based method
151 failed to generate any edits.
152
153 **The recombinase-free method simultaneously introduced multiple point mutations in the**
154 ***ackA* gene in WJL.** We subsequently evaluated whether this method could generate other
155 mutations within different genes of WJL. The acetate kinase gene *ackA* was chosen as its loss
156 of function in *L. plantarum* NIZO2877 has been correlated to a fitness benefit in *Drosophila*
157 models similar to the host fitness benefits mediated by *L. plantarum* WJL (Martino et al., 2018).
158 We designed a spacer to target the *ackA* gene and a recombineering template that generates
159 three unique silent mutations within the the seed region of the target (**Figure 3A**).
160 Transformation of the *ackA*-targeting construct in a strain harboring the recombineering-
161 template construct yielded an ~400-fold drop compared to the no-guide control (**Figure 3B**).
162 This drop was noticeably larger than the ~28-fold drop observed when targeting *ribB* and
163 mutating the PAM sequence (**Figure 2B**), suggesting some differential targeting activity when
164 mutating the guide sequence versus the PAM. Following multiple editing attempts, we screened
165 20 colonies for the intended edit, where 19 contained all three silent mutations, while the
166 remaining colony harbored the WT sequence. The generation of three point mutations in the
167 WJL *ackA* gene demonstrated that the recombinase-free method can be expanded to
168 incorporate multiple mutations in the seed region of the spacer.
169
170 **The recombinase-free method deleted the entire *lacM* open-reading frame in WJL.** We
171 then explored whether our recombineering template-based method could yield a complete gene
172 deletion. We chose the lactose mutase *lacM*, a 960-bp gene that expresses one of the subunits
173 of β-galactosidase in *L. plantarum (Iqbal et al., 2010)*. The enzyme complex is responsible for

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174 breaking down lactose into D-galactose and D-glucose as well as producing galacto-
175 oligosaccharides commonly used as prebiotics. For this reason, β-galactosidase activity is used
176 as a marker to screen Lactobacilli with significant probiotic potential (Mandal and Bagchi, 2018).
177 Importantly, the other subunit encoded by *lacL* cannot produce a fully functional beta-
178 galactosidase enzyme if *lacM* is absent (Nguyen et al., 2007). Therefore, the recombineering
179 template was designed to include 1 kb of homology upstream and downstream of the *lacM*
180 open-reading frame, where recombination would result in a complete deletion from the start
181 codon through the stop codon (**Figure 3C**). Transformation of the *lacM*-targeting construct into
182 the recombination template-containing strain yielded an ~35-fold drop in the number of colonies
183 compared to the no-guide control (**Figure 3D**), comparable to the CFU drop observed when
184 mutating the PAM (**Figure 2B**). Of these 14 screened survivors, 13 harbored the intended *lacM*
185 deletion. To phenotypically confirm successful deletion, we streaked mutants on MRS plates
186 containing Isopropyl β-D-1-thiogalactopyranoside (IPTG), the synthetic inducer of the lac operon
187 in *E. coli*, and X-gal, a compound that β-galactosidase hydrolyzes to generate a blue dye. Thus,
188 cells containing a functional β-galactosidase should be blue, and cells expressing non-functional
189 enzymes should be white. On the IPTG+X-gal plates, the three tested *lacM*-deletion mutants
190 yielded white streaks while the WT strain yielded blue-ish streaks, confirming disruption of *lacM*
191 (**Figure 3E**). The successful editing of *lacM* marks the third distinct gene that was successfully
192 and efficiently edited using our recombinase-free method.
193
194 **The recombinase-free method revealed two potential failure modes.** To investigate how
195 well this recombinase-free genome editing method works in other *L. plantarum* strains, we
196 attempted to generate the same stop codon in *ribB* in the less tractable strain NIZO2877
197 (**Figure S2A**) that generated ~10 fold fewer colonies compared to WJL after transformation with
198 5 µg of no-guide control. Once the recombineering-template plasmid was introduced into
199 NIZO2877, we transformed the previous *ribB*-targeting plasmid and plated cells on MRS with or

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200 without supplemented riboflavin. Surprisingly, transformation of the *ribB*-targeting construct into
201 the recombination template-containing strain yielded only ~1.8-fold fewer colonies compared to
202 the no-guide control, and there were a comparable number of survivors on MRS plates with or
203 without supplemented riboflavin (**Figure 4A**). As the original colony-PCR primers did not yield a
204 band for any of the surviving colonies, we used a primers pair with a larger intervening distance.
205 These wider flanking primers yielded a consistent ~1.3-kb deletion spanning upstream and into
206 the 5' end of *ribB* (**Figure 4A**) and partially overlapping with the recombineering template,
207 including the Cas9 target site. The deletion was not necessarily due to failed editing activity in
208 NIZO2877, as we recently and successfully applied the recombinase-free method in this strain
209 to delete a codon within the *ackA* gene (Martino et al., 2018). That aside, the unintended
210 deletion represents one means by which the Cas9-based editing can fail.

211 The recombinase-free method was also used to target the RNA polymerase *rpoB* gene
212 in the model strain WCFS1, the earlier described test case to demonstrate editing with the oligo-
213 based method (**Figure S2D**). The recombineering template was thus designed to include the
214 same single point mutation conferring rifampicin resistance and flanked by 1-kb homology arms
215 (**Figure 4B**). After transforming the previously used *rpoB*-targeting construct into cells
216 containing this recombineering template, we screened 20 survivors and all contained the WT
217 sequence. Further investigation revealed that the single point mutation within the
218 recombineering template had reverted to the wild-type sequence after it was transferred from *E.*
219 *coli* to WCFS1 (**Figure 4C**). Hypothesizing that recombination was occurring between the
220 naturally occuring genomic sequence and our recombineering template, three additional silent
221 point mutations were added around the rifampicin-conferring mutation to reduce the extent of
222 homology between the template and the WCSF1 genome. Transferring the updated
223 recombineering-template plasmid from EC135 into WCFS1 did not result in reversion of the
224 point mutations, suggesting extensive homology may have been the cause. Subsequent
225 transformation of the *rpoB*-targeting construct yielded surviving colonies, although screening 20

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226 colonies only yielded the WT sequence (**Figure 4D**). These results revealed a unique failure
227 mode of the recombinase-free editing method, where the template appeared to undergo
228 recombination with the genome to lose the desired mutation. Even addressing this issue did not
229 produce the desired edit, suggesting that other failure modes remain to be identified.
230

231 **DISCUSSION**

232 We developed a method of Cas9-mediated genome editing in *L. plantarum* that relies on a
233 double-stranded recombineering template rather than a heterologous recombinase and a single-
234 stranded oligo. Our method efficiently produced various edits in three different genes, including
235 introduction of a premature stop codon in *ribB*, generation of multiple silent mutations in *ackA*,
236 and deletion of the entire *lacM* open-reading frame (**Figures 2, 3**). In one direct comparison
237 with the oligo-based method editing *ribB* in WJL, we found that the recombinase-free method
238 yielded efficient editing while oligo-mediated recombineering did not yield any edits. However, in
239 another direct comparison editing *rpoB* in WCFS1, the oligo-method was successful whereas
240 the recombinase-free method was not (**Figures 4B, S2**) . Therefore, both methods can be
241 utilized, although their efficiency may depend on the target and the strain.

242 As part of this work, we improved on the previously reported method for oligo-mediated
243 recombineering with CRISPR-Cas9 (Oh and van Pijkeren, 2014). We placed the RecT, Cas9,
244 the tracrRNA, and the CRISPR array into *E. coli*-*Lactobacillus* shuttle vectors to simplify and
245 accelerate cloning. We also equipped the RecT plasmid with *nisR* and *nisK* so the plasmid
246 could be used in strains lacking these genes. These constructs should aid others applying the
247 oligo-based editing method.

248 Despite successful examples of recombinase-free editing, we did encounter two failed
249 attempts at editing while applying this method. In one instance, a recombineering template was
250 designed to generate a premature stop codon into the *ribB* gene of the non-model strain
251 NIZO2877, but instead generated an ~1.3-kb genomic deletion (**Figure 4A**). Similar excisions

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252 have been observed when targeting genomic islands in other bacteria, where surviving cells
253 circumvented to genome targeting by eliminating the targeted region (Selle et al., 2015; Vercoe
254 et al., 2013). In a separate instance, we observed the reversion of a point mutation in the
255 recombineering template to the wild-type sequence after passaging from *E. coli* to *L. plantarum,*
256 and before introducing Cas9 (**Figure 4C**). It may be that introducing a point mutation of this
257 essential gene on a shuttle vector somehow is cytotoxic, as growth of the recombineering
258 template strain was slower compared to WT WCFS1 (data not shown). These failed instances
259 of genome editing should be instructive as others perform genome editing with recombineering
260 templates or explore ways of improving the method.

261 Looking ahead, there is ample room for improving CRISPR-mediated, recombinase-free
262 genome editing in Lactobacilli. Although SpCas9 has been the standard for achieving genome
263 editing in bacteria, the CRISPR nuclease Cas12a/Cpf1 is becoming more prevalent (Hong et al.,
264 2018; Yan et al., 2017; Zetsche et al., 2015) and in once instance yielded efficient editing when
265 Cas9 could not be introduced (Jiang et al., 2017). There are also other emerging approaches to
266 enhance editing that could be incorporated to our method. For instance, the Cas9 and the gRNA
267 can be placed under inducible control to ensure that a large population of cells possess
268 CRISPR-Cas9 and the recombineering template prior to DNA induction (Reisch and Prather,
269 2015). The challenge is finely tuning Cas9 induction to ensure negligible leaky expression in the
270 absence of inducer and sufficient expression to drive editing in the presence of inducer.
271 Separately, using a nicking Cas9 in multiple bacteria including the model bacterium
272 *Lactobacillus casei* was shown to increase editing efficiencies (Li et al., 2018; Song et al., 2017;
273 Xu et al., 2015), presumably based on promoting homologous recombination without introducing
274 a lethal double-stranded break. However, because of the lack of lethality, the technique often
275 leaves unedited cells. These other options provide ways to further enhance editing in *L.*
276 *plantarum* and other lactic-acid bacteria, even as they introduce additional engineering
277 challenges.

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278 Overall, our study simplified the machinery required for efficient genome editing in *L.*
279 *plantarum* WJL, and provided the framework for achieving editing in other non-model strains of
280 Lactobacilli (Martino et al., 2018). The insights we gathered while applying our method highlight
281 that strain-dependent behavior is a major obstacle to streamlining genome editing in this diverse
282 genus, as the best means of editing may vary based on the desired edit, gene, and target strain.
283 Continuously-evolving CRISPR genome editing tools will likely drive enhanced editing
284 capabilities in Lactobacilli, which should in turn expand our understanding of their genomic
285 versaitility and open the door to improved and novel applications.
286

287 **MATERIALS AND METHODS**

288 **Strains, plasmids, oligonucleotides.** Table S1 contains descriptions and locations of every
289 plasmid, strain, and oligonucleotide used for this study. *Lactobacillus plantarum* NIZO2877 and
290 WJL were sent to us by Dr. Francois Leulier (Martino et al., 2015a, 2015b), and *L. plantarum*
291 WCFS1 was sent to us from Dr. Nikhil U. Nair. Plasmids pJP042 and pJP005 were sent to us by
292 the van-Pijkeren lab, and pMSP3545 (CN#46888) and pCas9 (CN#42876) were purchased from
293 Addgene. Plasmids from this work will made available through Addgene.
294

295 **Plasmid generation.** To generate pRecTNisRK, pJP005 was digested with XbaI and HindIII
296 and the *nisR* and *nisK* genes were amplified with oRL9-10 and digested with XbaI and HindIII.
297 The ligation was performed with 200 ng of digested backbone with a 3:1 molar excess of insert
298 and was subsequently ethanol precipitated and transformed into *L. plantarum* WCFS1. Colonies
299 were screened with oligos oRL7-8 by Miniprep (Zymo CN# D4036) of plasmids after cells were
300 lysed with 20 ng/mL of Lysozyme for 30 minutes.

301 The CRISPR-Cas9 plasmids were created by taking the base pMSP3545 shuttle vector
302 and pCas9 and amplifying each fragment with oRL1-oRL4. These PCR fragments were
303 assembled by Gibson assembly kit (NEB CN# E2611S) (Gibson et al., 2009). This created the

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304 non-targeting p3545Cas9 control plasmid containing Cas9 and the tracrRNA. The targeting
305 repeat spacer array was designed as a gBlock (3545_RSR_gBlock) and amplified with oRL5-6.
306 The p3545Cas9 backbone was digested with XbaI and PstI and the fragments were assembled
307 together with Gibson. This created the pCas9_RSR plasmid. Subsequent spacers were cloned
308 in by digesting the backbone with PvuI and NotI, and annealing two oligonucleotides containing
309 these same overhangs (oRL11-oRL12, oRL19-20, oRL27-28, oRL33-34) and ligating the
310 fragments together.

311 The recombineering template shuttle plasmid (RLShut) was generated by amplifying the
312 pJP005 backbone with oRL15-16, amplifying the ColE1 origin and *bla* resistance gene from
313 pBAD18 with oRL13-14, then stitching the two pieces together by Gibson assembly. Functional
314 clones were screened through a chemical transformation assay into *E. coli* DH5-alpha. Double-
315 stranded DNA (dsDNA) recombineering templates were inserted into this shuttle vector by
316 amplifying the desired repair sequence (oRL17-18, oRL21-22, oRL25-26, oRL31-32), digesting
317 the PCR fragment and RLShut backbone with SpeI and SacI, and ligating the fragments
318 together. If the PCR fragment was amplified from the wild-type genome, Q5 site directed
319 mutagenesis (NEB CN# E0554S, oRL23-24) was utilized to create small nucleotide changes.
320 After successful clones were generated in *E. coli* DH5-alpha, the plasmid was passaged
321 through *E. coli* EC135, then *L. plantarum* WCSF1, and then transferred into the intractable
322 *Lactobacillus* strain.

323

324 **Standard growth conditions.** All *L. plantarum* strains were grown on MRS liquid broth (BD
325 CN# 288130) and MRS agar (BD CN# 288210) and incubated at 37°C without shaking in 14-mL
326 polypropylene tubes. Antibiotic concentrations in *L. plantarum* were as follows: rifampicin (25
327 µg/mL), chloramphenicol (10 µg/mL), and erythromycin (10 µg/mL). For the *ribB* knockout, MRS
328 liquid broth was supplemented with and without 200 µM riboflavin. For *lacM* knockout, MRS
329 agar was supplemented with 100 µM IPTG and 3 µL of X-gal per 1 mL of MRS.

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330 All *E. coli* propagation was performed in LB medium (10 g/L NaCl, 5 g/L yeast extract, 10
331 g/L tryptone) while being shaken at 250 rpm at 37°C. Plasmids were maintained at the following
332 antibiotic concentrations: erythromycin (50 μg/mL for liquid cultures, 300 μg/mL for plates),
333 chloramphenicol (34 μg/mL), and ampicillin (50 μg/mL).
334
335 **Electroporation protocol for *L. plantarum*.** Electroporation of *L. plantarum* was adapted from
336 numerous protocols (Alegre et al., 2004; Aukrust, 1988; Spath et al., 2012; Thompson and
337 Collins, 1996). A *L. plantarum* colony was picked from a plate after 48 hours of growth and
338 placed into 3 mL liquid culture containing necessary antibiotics for 18 hours. 1 mL of this culture
339 was back-diluted into 25 mL of MRS containing 0.41 M glycine and any necessary antibiotics.
340 Outgrowth were performed in 50 mL falcon tubes (VWR CN#21008-212) to prevent aeration of
341 the bacteria. These tubes were cultured at 37°C and 250 RPM until the OD_{600} was
342 approximately 0.85 (~3.5 hours). If nisin was used to induce *recT* expression, 2 ng/mL of Nisin
343 was added when the OD_{600} was approximately 0.7 (after ~3 hours of outgrowth), and cultures
344 were shaken until they reached an OD_{600} of 0.85. Then, cells were centrifuged at 5,000 RPM for
345 10 minutes at 4°C to collect the pellet, and washed twice with 5 mL of 10 mM $MgCl_2$. After
346 transferring to a new 50 mL tube, cells were and washed with 5 mL of SacGly (10% glycerol
347 with 0.5M sucrose) (Alegre et al., 2004; Spath et al., 2012). Finally, *L. plantarum* cells were
348 washed in 1 mL of SacGly and centrifuged at 20,000 rpm for 1 minute. The supernatant was
349 removed and the final pellet was resuspended in 500 μL of SacGly. For all transformations, 60
350 μL of this suspension was added to a 1-mm gap cuvette and transformed at 1.8 kV, 200 Ω
351 resistance, and 25 μF capacitance. DNA concentrations used for transformations are reported in
352 the main text. On average, 5 μg dsDNA and 10 μg of ssDNA oligo was transformed into
353 *Lactobacillus* cells. Following electroporation, 1 mL of MRS broth was added to the cuvette and
354 transferred to a sterile tube and incubated at 37°C without shaking for 4 hours. 250 μL of this

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355 recovery was then plated on MRS agar with necessary antibiotics. Any dilutions prior to plating
356 was done in MRS media.
357
358 **Colony PCR.** Colony PCR was performed by picking a single colony into 20 µL of 20 mM NaOH
359 and incubating at 98°C for 20 minutes. These tubes were then microwaved for 1 minute with the
360 cap open, and this mixture was diluted 1:10, and 5µL was added to the reaction. This was
361 combined with NEB OneTaq HotStart 2x MM (CN# M0484).
362
363 **Plasmid clearance.** In order to remove plasmids from mutated *L. plantarum* strains, cells were
364 passed through multiple rounds of inoculation in MRS broth supplemented with appropriate
365 nutrients and without any antibiotics. Once cells exhibited no antibiotic resistances in media and
366 on agar plates, they were screened through colony PCR to ensure mutation remained intact.
367
368 **RibB phenotype assessment.** Mutant and wild-type *L. plantarum* cells were grown on
369 chemically defined media (Hébert et al., 2004) with and without 200-uM riboflavin to assess the
370 effect of the *ribB* knockout. An OD_{600} measurement was taken after 24 and 42 hours on a
371 spectrophotometer (Thermo Fisher CN# ND2000) to assess growth.
372
373 **LacM phenotype assessment.** Strains of *L. plantarum* WJL with a clean *lacM* deletion were
374 grown on MRS agar with 100-µM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 3 µL of
375 ready-to-use X-gal (Thermo CN# R0941) per 1 mL MRS. After 48 hours, colonies were checked
376 for phenotypic color changes compared to wild-type WJL growth on the same plate.
377
378 **ACKNOWLEDGEMENTS**
379 Plasmid pJP005 was a gift from the van Pijkeren lab. Plasmid pMSP3545 was a gift from Gary
380 Dunny (Addgene plasmid # 46888) and plasmid pCas9 was a gift from the Luciano Marraffini

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381 (Addgene plasmid # 42876). This work was support by a CAREER award from the National
382 Science Foundation (MCB-1452902 to C.L.B.), and a European Research Council starting grant
383 (FP7/2007-2013-N°309704 to F.L), and the European Union's Horizon 2020 research and
384 innovation program under the Marie Sklodowska-Curie grant agreement (N°659510 to M.E.M.).

385

386 **CONFLICTS OF INTEREST STATEMENT**

387 None declared.

388

389 **AUTHOR CONTRIBUTIONS**

390 R.T.L., J.M.V., and C.L.B. designed the experiments. R.T.L, J.M.V., M.S., and C.L.B. analyzed
391 and formatted all data. M.E.M. and F.L. developed riboflavin for editing and phenotypic
392 measurements. R.T.L. generated the figures and J.M.V., M.S., and C.L.B. wrote the manuscript.
393 All authors read and approved the manuscript.

394

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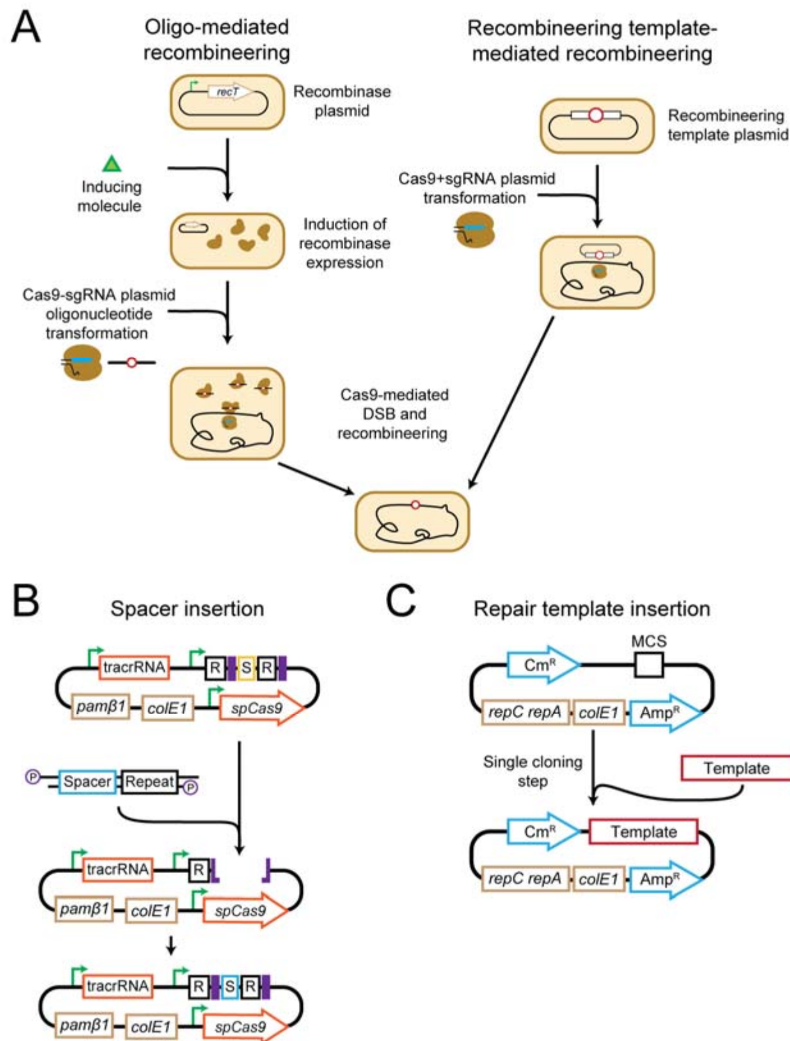
548 **FIGURE LEGENDS**



549

550 **Figure 1: A pipeline for recombinase-free genome editing with CRISPR-Cas9 in *L.***
551 ***plantarum*.** (**A**) Comparison of oligo-mediated recombineering and recombinase-free genome
552 editing methods. (**B**) Cloning scheme to insert a new targeting spacer into the repeat-spacer-
553 repeat array of the CRISPR-Cas9 construct. The base SpCas9 targeting plasmid is first
554 digested with NotI and PvuI enzymes. A new spacer-repeat is designed as two oligonucleotides
555 that are phosphorylated, annealed, and ligated into the digested backbone. All cloning is
556 performed in *E. coli*. (**C**) Cloning scheme to insert a recombineering template into a plasmid that
557 replicates in *E. coli* and *L. plantarum*. Gibson assembly is performed to ligate the amplified

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558 region of the targeted gene into the multiple cloning site of the base plasmid. Site-directed
559 mutagenesis can be used to incorporate the desired change into the recombineering template.
560 All cloning is performed in *E. coli.*

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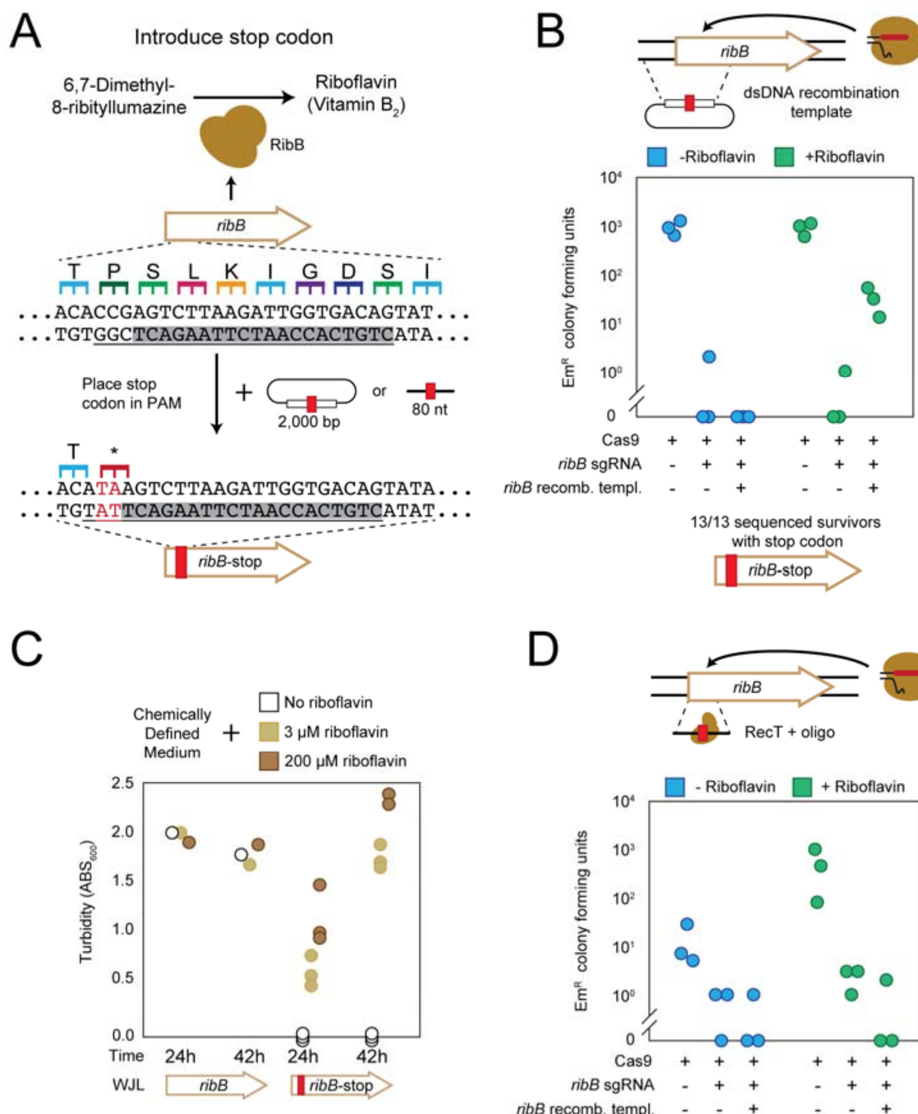

Figure 2: Enhanced editing of *ribB* by recombinase-free editing than by oligo-mediated editing in *L. plantarum* WJL. (**A**) A double-stranded repair template was designed along with a Cas9 targeting construct to generate an early stop codon in the *ribB* gene of *L. plantarum* WJL. Transformed cells were plated on Erythromycin MRS agar with or without supplementation of 200 μM riboflavin. (**B**) Surviving colonies were subject to Sanger sequencing for validation of the premature stop codon. Three confirmed mutants were grown on chemically defined medium (CDM) with the specified concentration of supplemented riboflavin to asses the phenotype of the riboflavin synthase knockout. (**C**) A single-stranded oligo was transformed along with the Cas9

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570 targeting construct into *L. plantarum* WJL containing RecT recombinase induced with nisin.
571 Cells transformed with the indicated plasmids were plated on Erythromycin MRS agar
572 with/without supplementation of 200 μM riboflavin. Replicates represent independent
573 experiments starting from separately validated mutant strains.

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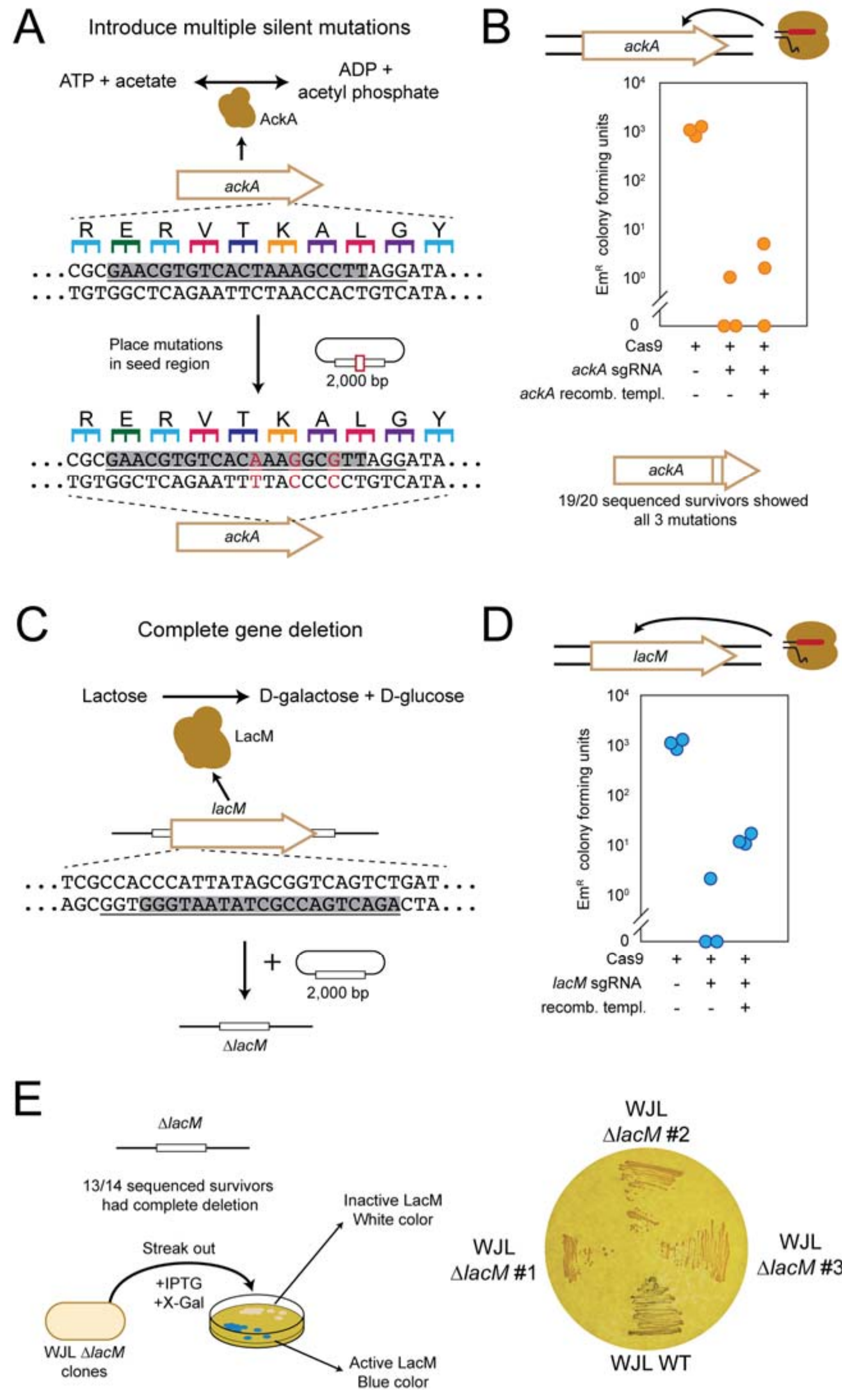

574

575 **Figure 3: Recombinase-free genome editing can generate different types of edits in *L.***
576 ***plantarum* WJL (A)** The recombineering template was designed to incorporate three silent
577 mutations into the acetate kinase *ackA* gene of WJL. The mutations fall within the seed region
578 of the gRNA target site. (**B**) Cells transformed with the indicated plasmids were plated on

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579 erythromycin MRS agar, and survivors were subjected to Sanger sequencing to confirm the
580 edited sequence. Replicates represent independent experiments starting from separate
581 colonies. **(C)** The recombineering template was designed with 1-kb homology arms upstream
582 and downstream from the start and stop codons, respectively, to generate a complete deletion
583 of the *lacM* open-reading frame. The gRNA target site was within the deleted region. **(D)** Cells
584 transformed with the indicated plasmids were plated on Erythromycin MRS agar. Replicates
585 represent independent experiments starting from separate colonies. **(E)** Survivors were
586 screened by colony PCR and gel electrophoresis and Sanger sequencing to to validate the
587 960-bp deletion. Three *lacM*-deletion mutants along with wild-type WJL were streaked on MRS
588 agar containing Isopropyl β-D-1-thiogalactopyranoside (IPTG) and X-gal to check for a change
589 in the colony color.

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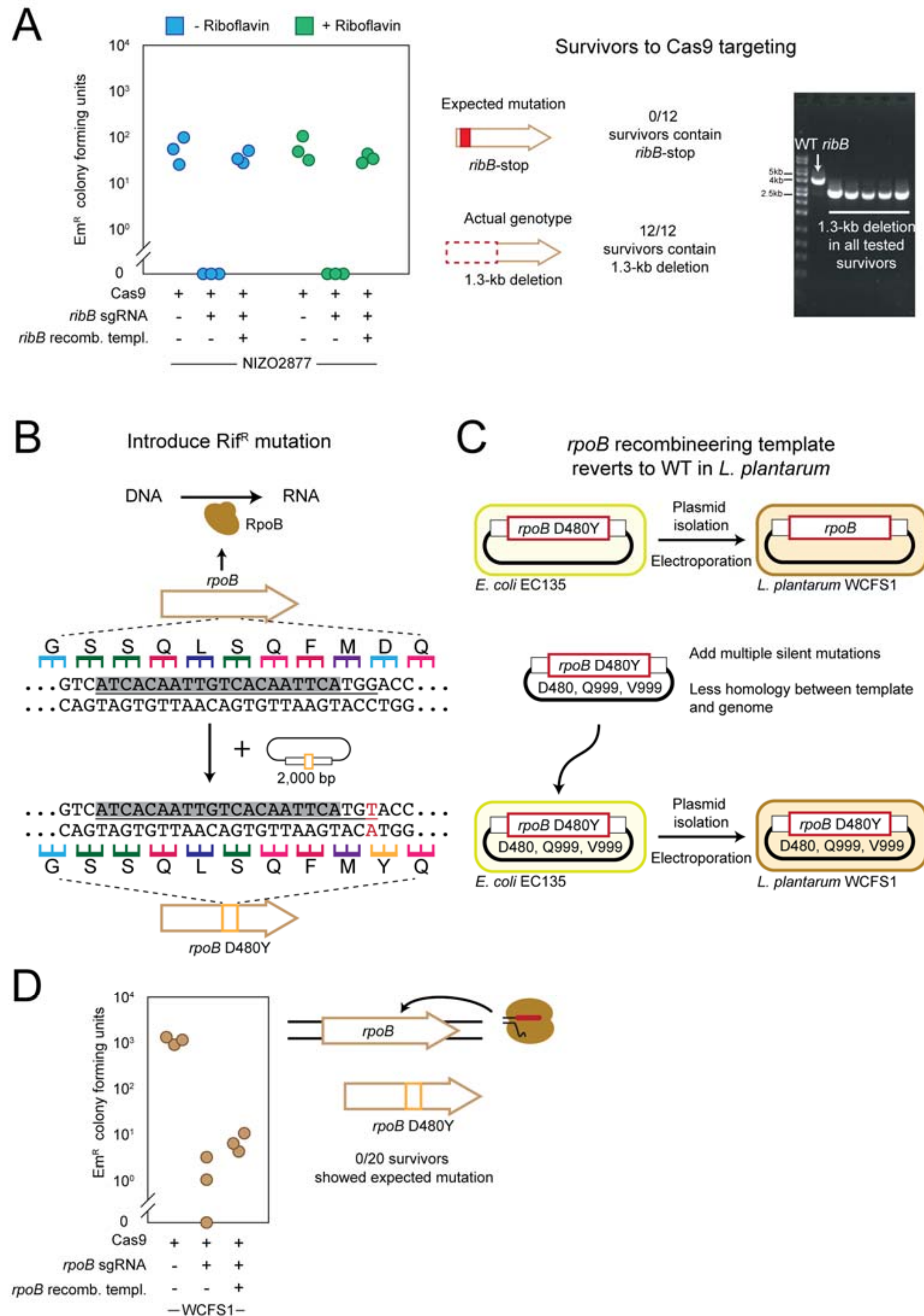

590

591 **Figure 4: Instances of failed recombinase-free editing in *L. plantarum*. (A)** The

592 recombineering template and the *ribB*-targeting construct from Figure 2A was used to generate

593 the premature stop codon in *ribB* in *L. plantarum* NIZO2877. Cells transformed with the

594 indicated plasmids were plated on erythromycin MRS agar with or without supplemented 200-

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595 μM riboflavin. Lane 2 of the gel from colony PCR is from wild-type NIZO2877, and lanes 3-7
596 represent\ different survivor colonies. Replicates represent independent experiments starting
597 from separate colonies. Sanger sequencing confirmed the cPCR results. **(B)** A recombineering
598 template was designed to insert a point mutation conferring resistance to rifampicin into the
599 *rpoB* gene of WCFS1. The mutation also disrupts the PAM in the gRNA target site. **(C)** The
600 recombineering template reverted to the WT sequence when transformed into *L. plantarum*
601 WCFS1 (right). Three silent mutations were subsequently cloned alongside the desired point
602 mutation to prevent reversion of the recombineering template upon electroporation into WCFS1.
603 **(D)** Cells transformed with the indicated plasmids were plated on erythromycin MRS agar.
604 Survivors were subjected to Sanger sequencing to confirm the desired mutated sequence.
605 Replicates represent independent experiments starting from separate colonies.