

# 1 **Inducible Plasmid Self-Destruction (IPSD) assisted genome engineering in lactobacilli** 2 **and bifidobacteria**

3 Fanglei Zuo<sup>1,2,3\*</sup>, Zhu Zeng<sup>1,2,4</sup>, Lennart Hammarström<sup>2</sup> and Harold Marcotte<sup>2\*</sup>

4

5 <sup>1</sup>Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Food Science and Nutritional  
6 Engineering, China Agricultural University, Beijing 100083, P. R. China.

7 <sup>2</sup>Department of Laboratory Medicine, Division of Clinical Immunology and Transfusion Medicine, Karolinska Institutet at  
8 Karolinska University Hospital Huddinge, Stockholm SE-141 86, Sweden.

9 <sup>3</sup>Present address: Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm SE-  
10 10691, Sweden.

11 <sup>4</sup>Present address: College of Biotechnology, Southwest University, Chongqing 400715, P. R. China.

12

## 13 **ABSTRACT**

14 Genome engineering is essential for application of synthetic biology in probiotics including lactobacilli and  
15 bifidobacteria. Several homologous recombination system-based mutagenesis tools have been developed for  
16 these bacteria but still, have many limitations in different species or strains. Here we developed a genome  
17 engineering method based on an inducible self-destruction plasmid delivering homologous DNA into bacteria.  
18 Excision of the replicon by induced recombinase facilitates selection of homologous recombination events. This  
19 new genome editing tool called Inducible Plasmid Self-Destruction (IPSD) was successfully used to perform  
20 gene knock-out and knock-in in lactobacilli and bifidobacteria. Due to its simplicity and universality, the IPSD  
21 strategy may provide a general approach for genetic engineering of various bacterial species.

22

23

## 24 **KEYWORDS**

25 Genome engineering; Homologous recombination; Lactobacilli; Bifidobacteria; Synthetic biology

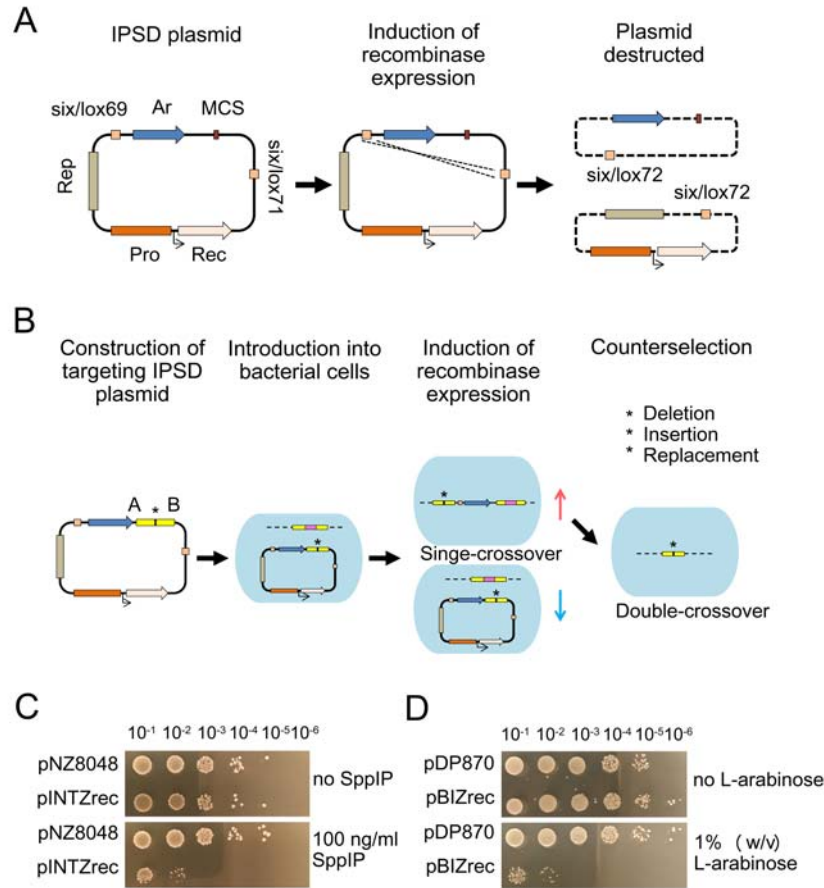
26

27 Lactobacilli and bifidobacteria, have been extensively used as probiotics and have become increasingly studied  
28 as living diagnostics and therapeutics that can probe and improve human health.<sup>1-4</sup> In contrast, the genetic tools  
29 (especially mutagenesis) to investigate and enhanced their probiotic activity are rather poorly developed,  
30 especially for bifidobacteria.<sup>5</sup> The traditional genome engineering methods are largely dependent on the bacterial  
31 transformation efficiency. This defect can be overcome by the conditional replicate plasmid assisted homologous  
32 recombination, such as plasmids containing a thermosensitive replication origin,<sup>6,7</sup> but the latter may have a  
33 limited host range. Some counterselection systems based on *upp* and *pyrE* genes has been developed.<sup>8,9</sup>  
34 However, this strategy needs modification of the parental strains and could not resolve the problem of the low  
35 frequency of single-crossover homologous integration events. Prophage recombinase-assisted double-stranded  
36 (dsDNA) recombineering can be used for marker-free genome engineering, but it is not a seamless strategy since  
37 a “scar” is left at the modification site after excision of the selection marker.<sup>10</sup> ssDNA recombineering allows  
38 high efficiency mutagenesis in lactobacilli and lactococci independent of antibiotic selection, while it still  
39 requires high transformation efficiency (i.e.  $\sim \geq 10^5$  cfu/ $\mu$ g DNA) and inducible expression of recombinase  
40 RecT.<sup>11</sup> Therefore, it might be difficult to be used in strains with low transformation efficiency. More recently,

41 CRISPR-Cas system has been applied for genome editing in various lactic acid bacteria species, including *L.*  
42 *reuteri*,<sup>12</sup> *L. casei*,<sup>13</sup> *L. plantarum*,<sup>14</sup> and *L. lactis*.<sup>15</sup> However, the editing outcomes can vary across strains and  
43 between methods,<sup>14</sup> and have occasional target failure.<sup>16</sup> Furthermore, CRISPR-Cas system has not yet been  
44 successfully used in bifidobacteria.<sup>17</sup> Therefore, more flexible and universal genome engineering strategies need  
45 to be developed for a better understanding and application of these health-promoting microorganisms.

46 We have designed a novel vector that could be destructed under certain conditions facilitating the selection of  
47 homologous recombination event between the vector and chromosomal DNA. Essentially, the replicon and the  
48 antibiotic resistance gene were separated by two oriented *six* or *lox* fragments.<sup>18,19</sup> Upon addition of the inducer  
49 and expression of the site-specific recombinase ( $\beta$  or Cre),<sup>18,19</sup> the vector can recombine and lose function due to  
50 the excision of the replicon (Figure 1A). This vector termed Inducible Plasmid Self-Destruction (IPSD) could be  
51 used to assist bacterial genome engineering, including gene deletion, insertion, and replacement (Figure 1B).  
52 When the vector delivering homologous DNA into bacteria, homologous recombination naturally occurred,  
53 induce plasmid destruction could facilitate homologous recombination and selective enrichment of single-  
54 crossover integrant precursor (Figure 1B).

55 As a proof-of-concept, an IPSD plasmid pINTZrec was constructed from pNZ8048 based on the  $\beta$ -*six* site  
56 specific recombination system (Figure S1a) in which the  $\beta$  recombinase gene was under the control of the  
57 sakacin-inducible promoter P<sub>orfX</sub>. When the plasmid pINTZrec carrying a chloramphenicol resistance gene was  
58 introduced into the vaginal probiotic strain *L. gasseri* DSM 14869,<sup>20</sup> the pINTZrec transformant showed a  
59 dramatic sensitivity to Sakacin P (SppIP) induction. The viability of SppIP-induced pINTZrec transformant on  
60 MRS agar plates supplemented with chloramphenicol was decreased by three to four orders of magnitude  
61 compared to the non-induced control (Figure 1C), suggesting that the plasmid pINTZrec was destructed  
62 following expression of  $\beta$  recombinase. Such observation was also found in other *Lactobacillus* species, such as  
63 *L. paracasei*, *L. acidophilus*, and *L. plantarum* (Figure S2). We further extended this method to *Bifidobacterium*,  
64 a genus for which genetic tools are poorly developed. An IPSD plasmid pBIZrec was constructed from pDP870  
65 based on the Cre-*lox* site specific recombination system (Figure S1b) and in which the recombinase gene *Cre*  
66 was under the control of the L-arabinose-inducible promoter araC-P<sub>BAD</sub>. The plasmid pBIZrec carrying a  
67 spectinomycin resistance gene was introduced into *B. longum* NCC 2705 and the pBIZrec transformant also  
68 showed decreased viability on agar plates supplemented with spectinomycin following cultivation in the  
69 presence of L-arabinose, by four to five orders of magnitude compared to the non-induced control (Figure 1D).  
70 These results indicated that the IPSD strategy could be used for assisting genome engineering in lactobacilli and  
71 bifidobacteria.



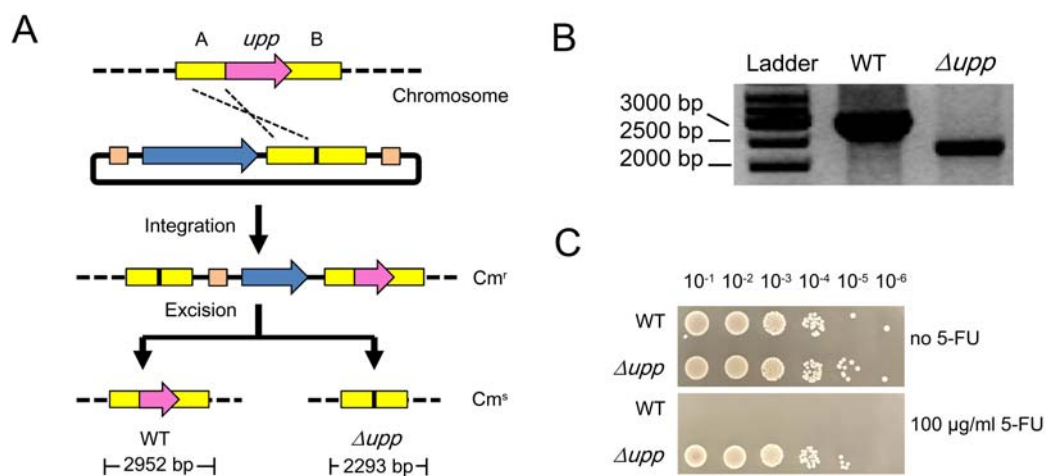
72

73 Figure 1. IPSD strategy for bacterial genome engineering. (A) Schematic illustration of inducible plasmid self-destruction. A  
 74 vector in which the replicon and the antibiotic resistance gene are separated by two oriented *six* or *lox* fragments was  
 75 constructed. Upon addition of the inducer and  $\beta$  or Cre mediated recombination, the vector loses function due to the excision  
 76 of the replicon. Rep, replicon; Pro, controlled expression promoter; Rec, recombinase; *six/lox*: two oriented *six* or *lox* target  
 77 sequence sites; Ar, antibiotic resistance gene; MCS, multiple cloning sites. (B) Schematic illustration of IPSD assisted  
 78 bacterial genome engineering, including gene deletion, insertion, and replacement (indicated by an asterisk). After  
 79 recombination, the ratio of subpopulation harboring the episomal vector decreased (blue arrows) while the ratio of  
 80 subpopulation with integrated DNA fragment through single-crossover increased (red arrows) under antibiotic selection. The  
 81 single-crossover integrant precursor could be screened by PCR and the double-crossover clones could be selected by  
 82 counterselection. The homologous ends flanking the target gene are indicated by A and B. The target gene on the  
 83 chromosome is represented by a pink rectangle. (C) Growth of the indicated *L. gasseri* DSM 14869 strain on MRS agar plate  
 84 supplemented with 10 µg/ml chloramphenicol and in presence or not of 100 ng/ml SppIP. (D) Growth of the indicated *B.*  
 85 *longum* NCC 2705 strain on MRSc agar plate supplemented with 100 µg/ml spectinomycin and in presence or not of 1 %  
 86 (w/v) L-arabinose.

87

88 Our previous work showed that the transformation efficiency of *L. gasseri* DSM 14869 was very low possibly  
 89 due to the thickness of EPS covering the cell surface or the presence of two resident plasmids, and therefore,  
 90 generating mutation in this strain using existing methods was not possible<sup>20</sup> (unpublished data). In order to  
 91 demonstrate IPSD assisted genome engineering in *L. gasseri* DSM 14869, we targeted *upp*, a non-essential gene  
 92 encoding uracil phosphoribosyltransferase (UPRTases) that is commonly used as a counterselection marker.<sup>8</sup> A

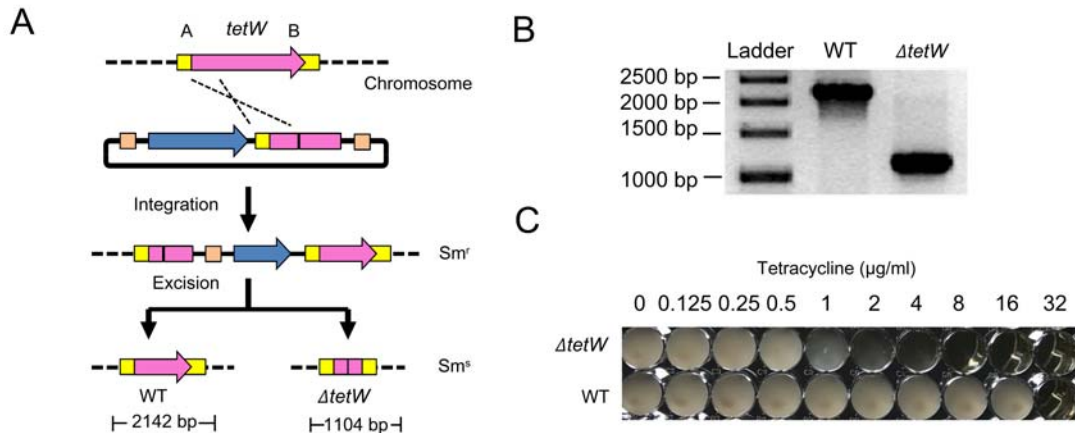
93 recombinant plasmid pINTZrec- $\Delta$ upp containing homologous regions up- and downstream of *upp* gene was  
 94 constructed and introduced into *L. gasseri* DSM 14869. The transformant was induced by SppIP and the single-  
 95 crossover integration event was selected using colony PCR (Figure 2A and Figure S3a). Ten out of randomly  
 96 picked 28 (36%) colonies showed absence of plasmid pINTZrec- $\Delta$ upp, suggesting that the  $\text{Cm}^r$  expression  
 97 cassette was integrated into the chromosome of *L. gasseri* DSM 14869 by a single-crossover event. These results  
 98 were further confirmed by PCR on DNA extracted from isolated clones and six clones showed correct  
 99 integration (Figure S3b). Following growth of the single-crossover clones in the absence of antibiotics, the  
 100 double-crossover *upp* mutant could easily be selected by counterselection of  $\text{Cm}^s$  colonies or by selection of  
 101 colonies resistant to 5-Fluorouracil (5-FU) (Figure 2B and Figure S3c). The *L. gasseri* DSM 14869 *upp* mutant  
 102 showed resistance to 5-FU (100  $\mu\text{g/ml}$ ) in contrast to the parent strain due to the abolished conversion of 5-FU  
 103 into cell toxic 5-fluorodeoxyuridine monophosphate (5-FdUMP) (Figure 2C).<sup>8</sup> We have also generated several  
 104 mutants of cell surface property related genes and integrative expression of broad and potent HIV-1-neutralizing  
 105 antibodies in *L. gasseri* DSM 14869 based on this method (unpublished data). These results suggest that the  
 106 IPSD plasmid could efficiently be used for genome engineering in lactobacilli.  
 107



108  
 109 Figure 2. Generation of *L. gasseri* DSM 14869 *upp* deletion mutant. (A) Schematic representation of a two-steps homologous  
 110 recombination to obtain a *upp* deletion mutant. (B) The *L. gasseri* DSM 14869 *upp* gene was deleted and the deletion mutant  
 111 verified by PCR using primer pairs uppleft-F and upright-R. (C) The *L. gasseri* DSM 14869 *upp* mutant showed resistance  
 112 to 100  $\mu\text{g/ml}$  of 5-FU compared to the WT. Overnight liquid cultures of the *L. gasseri* DSM 14869 WT and *upp* mutant were  
 113 adjusted at the same  $\text{OD}_{600}$  before serial 10-fold dilutions were spotted on plates with or without 5-FU. The plates were  
 114 incubated at 37  $^{\circ}\text{C}$  and photographed the next day.

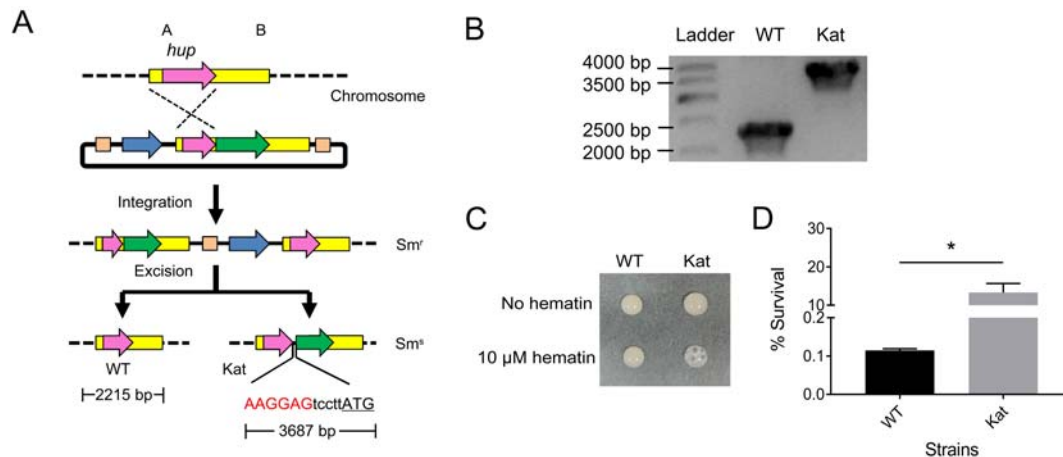
115  
 116 We subsequently tested this method in *Bifidobacterium*. The *tetW* gene provides tetracycline resistance to  
 117 dominant bifidobacterial species from the human gastrointestinal tracts, and it might be horizontally transferred  
 118 to other species.<sup>21</sup> Concerning the safety of probiotics, gene deletion could be the best way to prevent the  
 119 potential horizontal transfer of functional antibiotic resistant genes. The *tetW* gene from different bifidobacterial  
 120 strains shared high identity but with large variation in their flanking regions.<sup>22</sup> Therefore, we inactivated the *tetW*  
 121 gene by in-frame deletion in *B. longum* IF3-53, an infant feces isolate showing tetracycline resistance<sup>23</sup> (Figure

122 3A). An IPSD plasmid pBIZrec- $\Delta tetW$  was constructed and transformed into *B. longum* IF3-53. The  
123 transformant was induced by L-arabinose and the single-crossover integration event was selected using colony  
124 PCR (Figure 3B and Figure S4a). Three out of 16 (19%) randomly picked colonies were shown to contain the  
125 correct insertion (Figure S4b). The double-crossover  $tetW$  mutant was subsequently selected by counterselection  
126 of  $Sm^s$  colonies (Figure 3B). The mutant was sensitive to tetracycline compared to the parent strain, as the  
127 minimum inhibitory concentration (MIC) was reduced from 32  $\mu\text{g/ml}$  to 1  $\mu\text{g/ml}$  (Figure 3C). The results  
128 suggest that the IPSD plasmid could be used for genome engineering in bifidobacteria as well.  
129



130  
131 Figure 3. Generation of *B. longum* IF3-53  $tetW$  in-frame deletion mutant. (A) Schematic representation of a two-step  
132 homologous recombination to obtain a  $tetW$  in-frame deletion mutant. (B) The *B. longum* IF3-53  $tetW$  gene was disrupted and  
133 the mutant verified by PCR using primer pairs tetWleft-F and tetWright-R. (C) The minimum inhibitory concentration (MIC)  
134 of tetracycline was lower for *B. longum* IF3-53  $tetW$  mutant compared to WT. Overnight liquid cultures of the *B. longum*  
135 IF3-53 WT and  $tetW$  mutant were adjusted at the same  $OD_{600}$  before added into 96-well plates containing two-fold serial  
136 dilutions of tetracycline hydrochloride. The plates were incubated at 37 °C under anaerobic conditions and photographed the  
137 next day.

138  
139 We further tested the IPSD vector for stable chromosomal integration and expression of heterologous genes in  
140 *Bifidobacterium*. A gene *LpKatL* encoding catalase from *L. plantarum* was inserted into the chromosome of *B.*  
141 *longum* NCC 2705 under the control of the native  $P_{hup}$  promoter (Figure 4A and 4B). This integration site was  
142 selected based on several criteria as reported previously.<sup>24</sup> First, *LpKatL* gene was integrated downstream of  
143 gene *hup*, encoding the histone-like protein HU, which has high and constitutive expression level.<sup>25</sup> Polycistronic  
144 expression of *LpKatL* was driven by the promoter of the upstream gene *hup*. Second, an optimized ribosome-  
145 binding site (RBS) (AAGGAG) and a 5 nt spacer length was introduced in front of *LpKatL* gene for efficient  
146 translation. Finally, the insertion location between a stop codon and a terminator was selected to minimize the  
147 polar effects. The recombinant strain showed catalase activity in the presence of hematin (Figure 4C), and  
148 improved viability when exposed to  $H_2O_2$  as compared to the parent strain (Figure 4D).  
149



150  
 151 Figure 4. Integrative expression of *Kat* gene in *B. longum* NCC2705. (A) Schematic representation of a two-steps  
 152 homologous recombination to obtain a integrative *LpKatL* expressing mutant. The catalase gene *LpKatL* was inserted into the  
 153 chromosome of *B. longum* NCC 2705 and placed under the control of  $P_{hup}$  by a polycistronic structure, an optimized RBS  
 154 was introduced (marked in red), the start codon is underlined. (B) The *LpKatL* integrative mutant verified by PCR using the  
 155 primer pair Katleft-F and Katright-R. (C) Catalase activity test.  $O_2$  bubble was formed upon addition of  $H_2O_2$  on the cell  
 156 pellet of the *LpKatL* recombinant *B. longum* NCC 2705 strain harvested from liquid culture in MRS broth supplemented with  
 157 hematin. (D) The viability of the *LpKatL* recombinant *B. longum* NCC 2705 strain was dramatically improved compared to  
 158 the WT under  $H_2O_2$  challenge. Values are averages from two independent experiments,  $\pm$ standard deviation.  $p < 0.05$ .

159  
 160 The major advantage of this method is that it does not depend on transformation (or conjugation) efficiency.  
 161 However, it needs two pre-requirements: 1) a functional replicon allowing the plasmid to replicate in the host  
 162 bacteria; 2) a tightly controlled expression element used to drive recombinase gene expression. The plasmid  
 163 pINTZrec contains a pSH71 replicon which is broad-host-range and high-copy-number widely used for  
 164 lactobacilli plasmid construction.<sup>26</sup> The plasmid pBIZrec contains a pNCC293 replicon which could replicate in  
 165 at least seven different bifidobacterial species.<sup>27, 28</sup> So these two IPSD plasmids are expected to be universally  
 166 used for genome engineering in different lactobacilli and bifidobacteria species. However, the inducible  
 167 promoters used in this study are not effective in some strains, either due to strong background expression (*L.*  
 168 *sakei* NC03) (Figure S5a) or low induced expression (*L. rhamnosus* GG, *B. lactis* Bb12) (Figure S5b and S5c).  
 169 Therefore, the development of a universal tightly controlled expression element for lactobacilli or bifidobacteria,  
 170 such as tetracycline-regulated systems, is necessary.<sup>29</sup> Another disadvantage is that the IPSD strategy only  
 171 addressed the selection of single-crossover integration while the double-crossover excision of the antibiotic  
 172 resistant gene was achieved by traditional counterselection of antibiotic sensitive clones. Combination with  
 173 various counterselection methods, especially CRISPR-Cas-assisted negative selection, will further improve the  
 174 genome engineering efficiency in bacteria.<sup>12</sup>

175 In conclusion, we have shown that the IPSD plasmid can be used for genome engineering in lactobacilli and  
 176 bifidobacteria, with the potential to be extended to other bacterial species. The IPSD strategy could be used in a  
 177 range of synthetic biology applications in both the food and pharmaceutical industry such as identification of  
 178 probiotic genes, metabolic engineering, and delivery of therapeutics,<sup>30-32</sup> thus opening new avenues for the  
 179 engineering of biotherapeutic agents with enhanced health-promoting functional features.



180

## 181 METHODS

182 **Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this study are  
183 listed in Table S1. *Lactobacillus* strains were generally cultured at 37 °C in deMan Rogosa Sharpe (MRS)  
184 medium (Difco, BD BioSciences). For *upp*-based double-crossover event selection, the *Lactobacillus* strain was  
185 grown on semi-defined medium (SDM) agar plates.<sup>33</sup> *Bifidobacterium* strains were cultured in MRS  
186 supplemented with 0.05 % (w/v) L-cysteine HCl (MRSc) at 37 °C under anaerobic conditions. *Escherichia coli*  
187 strains were cultured in Luria-Bertani broth at 37 °C with rotary shaking at 200 rpm, or on LB agar plates. When  
188 needed, antibiotics were supplemented at the following concentrations: 10 µg/ml chloramphenicol for  
189 *Lactobacillus* strains and *E. coli* VE7108 strain, and 100 µg/ml spectinomycin for *Bifidobacterium* strains and *E.*  
190 *coli* DH5α strain.

191 **Plasmid construction.** The primers used in this study are listed in Table S2. For construction of IPSD vector  
192 pINTZrec used for lactobacilli genome engineering, the two *six* DNA fragments were amplified from site-  
193 specific integration vector pEM76 by using the primers pairs SIX-F1&R1 and SIX-F2&R2, respectively, and  
194 inserted into plasmid pNZ8048 on each flank of the Cm<sup>r</sup> expression cassette generating pNZ8048-SIX. The  
195 orientation of the insertion was confirmed by sequencing. Multiple cloning sites was introduced into pNZ8048-  
196 SIX by inserting a linker between *Pst*I and *Bgl*II, resulting in the plasmid pNZmcs-SIX. The β-recombinase gene  
197 was amplified from plasmid pEM94 and inserted into plasmid pVPL3017 downstream of the sakacin-inducible  
198 promoter P<sub>orfX</sub> generating pVPL3017-rec. Subsequently, the P<sub>orfX</sub>-rec expression cassette was digested using *Sal*I  
199 and *Hind*III, and inserted into similarly digested pNZmcs-SIX plasmid to obtain the final plasmid pINTZrec.

200 For construction of the plasmid used for *L. gasseri* DSM 14869 *upp* gene deletion, two 1090 bp DNA  
201 fragments, upstream and downstream of *upp* gene, were amplified from genomic DNA of *L. gasseri* DSM 14869  
202 using the primer pairs *upp*-up-F/*upp*-up-R and *upp*-down-F/*upp*-down-R, respectively. The upstream DNA  
203 fragment was inserted into pMD19-T Simple vector by TA cloning generating pMD19-*upp*-up, then the  
204 downstream DNA fragment was inserted into pMD19-*upp*-up between *Sac*I and *Sph*I, generating pMD19-Δ*upp*.  
205 Δ*upp* was digested with *Apa*I and *Sph*I and inserted into similarly digested pINTZrec generating pINTZrec-Δ*upp*.

206 For construction of IPSD vector pBIZrec used for bifidobacteria genome engineering, the *lox66-Smr-lox71*  
207 DNA fragment was amplified by PCR amplification from pDP870 using primers *lox66*-F and *lox71*-R, followed  
208 by insertion into pDP870 between *Eco*RV and *Eco*RI generating pDP870-*lox*. The L-arabinose inducible  
209 promoter *araC*-P<sub>BAD</sub> was amplified from *E. coli* DH5α genomic DNA using primers *araP*-*Cre*-F1 and *araP*-*Cre*-  
210 R1 and the recombinase gene *Cre* was amplified from plasmid pAdTrack-*Cre* using primers *araP*-*Cre*-F2 and  
211 *araP*-*Cre*-R2. The *araP*-*Cre* DNA fragment was generated by overlap PCR using primers *araP*-*Cre*-F1 and *araP*-  
212 *Cre*-R2, and a mixture of *araC*-P<sub>BAD</sub> and *Cre* PCR products as templates. The *araP*-*Cre* DNA fragment was then  
213 inserted into pDP870-*lox* between *Acc*I and *Hind*III generating pBIZrec.

214 For construction of the plasmid used for *B. longum* IF3-53 *tetW* gene inactivation, two 497 bp DNA fragments,  
215 upstream and downstream of *tetW* gene were amplified from *B. longum* IF3-53 genomic DNA using the primer  
216 pairs *tetW*-up-F/*tetW*-up-R and *tetW*-down-F/*tetW*-down-R, respectively. These two DNA fragments were fused  
217 by overlap PCR using primers *tetW*-up-F and *tetW*-down-R, generating Δ*tetW* and inserted into pBIZrec  
218 between *Apa*I and *Nhe*I resulting in pBIZrec-Δ*tetW*.

219 For construction of the plasmid used for insertion of the *LpKatL* gene into the chromosome of *B. longum* NCC  
220 2705, the  $P_{hup}$ -Kat DNA fragment was generated by overlap PCR. Briefly, two ~1080 bp DNA fragments,  
221 upstream and downstream of *B. longum* NCC 2705 *hup* gene stop codon were amplified using primer pairs hup-  
222 up-F and hup-up-R, and hup-down-F and hup-down-R, respectively. *LpKatL* gene was amplified from plasmid  
223 pDP401-LpKatL using primers Kat-F and Kat-R, also introducing an optimized ribosome binding site (RBS)  
224 (AAGGAG) and a 5 nt spacer length in front of *LpKatL* gene.<sup>34</sup> These three DNA fragments were fused by  
225 overlap PCR using primers hup-up-F and hup-down-R, generating  $P_{hup}$ -Kat and inserted into pBIZrec between  
226 *ApaI* and *NheI* resulting in pBIZrec- $P_{hup}$ -Kat.

227 **Transformation.** The plasmids pINTZrec and pINTZrec- $\Delta$ upp were electrotransformed into *L. gasseri* DSM  
228 14869 and other *Lactobacillus* strains according to De Keersmaecker et al.<sup>35</sup> The plasmids pBIZrec, pBIZrec-  
229  $\Delta$ tetW and pBIZrec- $P_{hup}$ -Kat were electrotransformed into *B. longum* NCC 2705 and other *Bifidobacterium*  
230 strains as described previously.<sup>27</sup> The plasmids pNZ8048 and pDP870 were used as controls. The transformants  
231 were confirmed by colony PCR followed by PCR on DNA extracted from pure cultures.

232 **Recombineering.** The single colonies of *L. gasseri* DSM 14869 harboring the plasmid pINTZrec or its  
233 derivatives were grown overnight in MRS broth containing 10  $\mu$ g/ml chloramphenicol. The cultures were  
234 inoculated (1 %, v/v) into MRS broth without antibiotics and grown at 37 °C until OD<sub>600nm</sub> reached ~0.30, then  
235 supplemented with 100 ng/ml sakacin P (SppIP) (Genscript). The cultures were allowed to grow overnight, and  
236 serial dilutions were plated on MRS agar supplemented with 10  $\mu$ g/ml chloramphenicol and 100 ng/ml SppIP.  
237 The single-crossover events were detected by colony PCR followed by PCR on DNA extracted from pure  
238 cultures. For double-crossover selection, the single-crossover clones were grown overnight in MRS broth in  
239 absence of antibiotics, followed by spreading serial dilutions on MRS agar or SDM agar supplement of 100  
240  $\mu$ g/ml 5-Fluorouracil (5-FU) (Sigma). The colonies from MRS agar were replicated to MRS agar containing 10  
241  $\mu$ g/ml chloramphenicol, the Cm<sup>s</sup> colonies were selected and detected by PCR on extracted DNA.

242 The single colonies of *B. longum* NCC 2705 harboring plasmid pBIZrec or its derivatives were grown  
243 overnight in MRSc broth containing 100  $\mu$ g/ml spectinomycin. This overnight culture was used to inoculate  
244 MRSc broth supplemented with 1 % (w/v) L-arabinose (Sigma) without antibiotic followed by overnight culture.  
245 The cultures were serially diluted and plated on MRSc agar supplemented with 100  $\mu$ g/ml spectinomycin and 1 %  
246 L-arabinose. The single-crossover events were detected by colony PCR followed by PCR on extracted DNA. For  
247 double-crossover selection, the single-crossover clones were grown overnight in the MRSc broth in the absence  
248 of antibiotics, followed by spreading serial dilutions on MRSc agar. The colonies from the MRSc agar were  
249 replicated to MRSc agar containing 100  $\mu$ g/ml spectinomycin. Subsequently, the Sm<sup>s</sup> colonies were selected and  
250 the double-crossover events were identified using PCR on extracted DNA.

251 All the mutants generated in this study were confirmed by PCR and sequencing.

252 **MIC assay.** Minimal inhibitory concentration (MIC) was determined by the broth microdilution protocol.<sup>36</sup>  
253 Overnight cultures of *B. longum* IF3-53 and *AtetW* mutant were harvested and re-suspended to an OD<sub>600nm</sub> = 0.8  
254 and further diluted 100-fold in MRSc. Working solutions of tetracycline hydrochloride (Sigma) were prepared in  
255 MRSc and 100  $\mu$ l of two-fold dilution series were distributed in 96-well plates. Subsequently, 100  $\mu$ l of bacterial  
256 suspensions were transferred to the wells. The plates were incubated under anaerobic conditions at 37 °C for 20  
257 h. The MIC was defined as the lowest concentration of the antimicrobial agent that inhibits visible growth of the  
258 tested strains as observed with the naked eye.



259 **Catalase activity and H<sub>2</sub>O<sub>2</sub> stress assay.** *B. longum* NCC 2705 and recombinant *B. longum* NCC 2705  
260 *P<sub>hup</sub>::LpKatL* were grown in MRS broth supplemented with 10 μM hematin (Sigma) for 8 h. Bacteria cells were  
261 then resuspended in TES buffer (50 mM Tris-Cl, 30 mM EDTA, 25% (w/v) sucrose, pH 8.0). Catalase activity  
262 was examined by detecting bubble formation upon addition of 10 % H<sub>2</sub>O<sub>2</sub> to the cell suspension.<sup>37</sup>

263 For H<sub>2</sub>O<sub>2</sub> stress, an aliquot of bacterial cultures were treated with 5 mM H<sub>2</sub>O<sub>2</sub> for 1 h at 37 °C. Then, H<sub>2</sub>O<sub>2</sub>  
264 was removed by washing pellets two times with PBS, and viable cells were counted by plating appropriate  
265 dilutions on MRS agar after two days anaerobic incubation at 37 °C. Cultures not treated with H<sub>2</sub>O<sub>2</sub> were used as  
266 a reference to calculate the survival rate. Statistical analysis was performed by using two-tailed unpaired t-tests.  
267 *p*<0.05 was considered to be statistically significant.

268

## 269 ASSOCIATED CONTENT

### 270 Supporting Information

271 Supplementary Figures S1-S5; Supplementary Tables S1-S2.

272

## 273 AUTHOR INFORMATION

### 274 Corresponding Author

275 \*E-mail: F.Z. ([duroolanzi@gmail.com](mailto:duroolanzi@gmail.com)) and H.M. ([harold.marcotte@ki.se](mailto:harold.marcotte@ki.se)).

### 276 Author Contributions

277 F.Z. conceived the study. F.Z. and H.M. designed the experiments. F.Z. performed all the experiments with  
278 assistance from Z.Z.. F.Z., H.M. and L.H. wrote the manuscript, with contributions from all other authors. All of  
279 the authors read and approved the final manuscript.

### 280 Notes

281 The authors declare the following competing financial interest: a provisional patent application covering some  
282 parts of the information contained in this article has been filed.

283

## 284 ACKNOWLEDGEMENT

285 *E. coli* VE7108 strain was a gift from D. Mora (University of Milan, Milan, Italy). Plasmid pDP870 and *B.*  
286 *longum* NCC 2705 were a gift from S. Duboux (Nestec Ltd., Nestlé Research Center Lausanne, Lausanne,  
287 Switzerland). Plasmid pAdTrack-Cre was a gift from Z. Zukowska (Georgetown University Medical Center,  
288 Washington, DC, USA). This work was supported by the Swedish Research Council (Vetenskapsrådet) [U-Forsk  
289 grant 348-2013-6609 to L.H.]; and the Stiftelsen Läkare mot AIDS Forskningsfond [Fob2016-0008 to F.Z.].

290

## 291 REFERENCES

- 292 1. Turrone, F.; Ventura, M.; Buttó, L. F.; Duranti, S.; O'Toole, P. W.; Motherway, M. O. C.;  
293 van Sinderen, D., Molecular dialogue between the human gut microbiota and the host: a *Lactobacillus* and  
294 *Bifidobacterium* perspective. *Cellular and Molecular Life Sciences* **2014**, *71* (2), 183-203.
- 295 2. Cano-Garrido, O.; Seras-Franzoso, J.; Garcia-Fruitos, E., Lactic acid bacteria: reviewing the  
296 potential of a promising delivery live vector for biomedical purposes. *Microbial cell factories* **2015**, *14* (1), 137.

- 297 3. Bober, J. R.; Beisel, C. L.; Nair, N. U., Synthetic Biology Approaches to Engineer Probiotics  
298 and Members of the Human Microbiota for Biomedical Applications. *Annu Rev Biomed Eng* **2018**, *20*, 277-300.
- 299 4. Mays, Z. J.; Nair, N. U., Synthetic biology in probiotic lactic acid bacteria: At the frontier of  
300 living therapeutics. *Curr Opin Biotechnol* **2018**, *53*, 224-231.
- 301 5. Fukiya, S.; Sakanaka, M.; Yokota, A., Genetic Manipulation and Gene Modification  
302 Technologies in Bifidobacteria. In *The Bifidobacteria and Related Organisms*, Elsevier: 2018; pp 243-259.
- 303 6. Biswas, I.; Gruss, A.; Ehrlich, S. D.; Maguin, E., High-efficiency gene inactivation and  
304 replacement system for gram-positive bacteria. *Journal of bacteriology* **1993**, *175* (11), 3628-3635.
- 305 7. Sakaguchi, K.; He, J.; Tani, S.; Kano, Y.; Suzuki, T., A targeted gene knockout method using  
306 a newly constructed temperature-sensitive plasmid mediated homologous recombination in *Bifidobacterium*  
307 *longum*. *Applied Microbiology & Biotechnology* **2012**, *95* (2), 499-509.
- 308 8. Goh, Y. J.; Azcárate-Peril, M. A.; O'Flaherty, S.; Durmaz, E.; Valence, F.; Jardin, J.; Lortal,  
309 S.; Klaenhammer, T. R., Development and application of a upp-based counterselective gene replacement system  
310 for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* **2009**,  
311 *75* (10), 3093-3105.
- 312 9. SAKAGUCHI, K.; FUNAOKA, N.; TANI, S.; HOB0, A.; MITSUNAGA, T.; KANO, Y.;  
313 SUZUKI, T., The pyrE gene as a bidirectional selection marker in *Bifidobacterium longum* 105-A. *Bioscience of*  
314 *microbiota, food and health* **2013**, *32* (2), 59-68.
- 315 10. Yang, P.; Wang, J.; Qi, Q., Prophage recombinases-mediated genome engineering in  
316 *Lactobacillus plantarum*. *Microbial cell factories* **2015**, *14* (1), 154.
- 317 11. van Pijkeren, J.-P.; Britton, R. A., High efficiency recombineering in lactic acid bacteria.  
318 *Nucleic Acids Research* **2012**, *40* (10), e76-e76.
- 319 12. Jee-Hwan, O.; Jan-Peter, V. P., CRISPR-Cas9-assisted recombineering in *Lactobacillus reuteri*.  
320 *Nucleic Acids Research* **2014**, *42* (17), e131.
- 321 13. Song, X.; Huang, H.; Xiong, Z.; Ai, L.; Yang, S., CRISPR-Cas9(D10A) Nickase-Assisted  
322 Genome Editing in *Lactobacillus casei*. *Appl Environ Microbiol* **2017**, *83* (22).
- 323 14. Leenay, R. T.; Vento, J. M.; Shah, M.; Martino, M. E.; Leulier, F.; Beisel, C. L., Genome  
324 Editing with CRISPR-Cas9 in *Lactobacillus plantarum* Revealed That Editing Outcomes Can Vary Across  
325 Strains and Between Methods. *Biotechnol J* **2019**, *14* (3), e1700583.
- 326 15. van der Els, S.; James, J. K.; Kleerebezem, M.; Bron, P. A., Versatile Cas9-driven  
327 subpopulation selection toolbox for *Lactococcus lactis*. *Appl. Environ. Microbiol.* **2018**, *84* (8), e02752-17.
- 328 16. Stout, E. A.; Sanozky-Dawes, R.; Goh, Y. J.; Crawley, A. B.; Klaenhammer, T. R.; Barrangou,  
329 R., Deletion-based escape of CRISPR-Cas9 targeting in *Lactobacillus gasseri*. *Microbiology* **2018**, *164* (9),  
330 1098-1111.
- 331 17. Hidalgo-Cantabrana, C.; O'Flaherty, S.; Barrangou, R., CRISPR-based engineering of next-  
332 generation lactic acid bacteria. *Curr Opin Microbiol* **2017**, *37*, 79-87.
- 333 18. Abremski, K.; Hoess, R.; Sternberg, N., Studies on the properties of P1 site-specific  
334 recombination: evidence for topologically unlinked products following recombination. *Cell* **1983**, *32* (4), 1301-  
335 1311.

- 336 19. Canosa, I.; Lurz, R.; Rojo, F.; Alonso, J. C.,  $\beta$  Recombinase catalyzes inversion and resolution  
337 between two inversely oriented six sites on a supercoiled DNA substrate and only inversion on relaxed or linear  
338 substrates. *Journal of Biological Chemistry* **1998**, *273* (22), 13886-13891.
- 339 20. Marcotte, H.; Andersen, K. K.; Lin, Y.; Zuo, F.; Zeng, Z.; Larsson, P. G.; Brandsborg, E.;  
340 Brønstad, G.; Hammarström, L., Characterization and complete genome sequences of *L. rhamnosus* DSM 14870  
341 and *L. gasseri* DSM 14869 contained in the EcoVag® probiotic vaginal capsules. *Microbiological research*  
342 **2017**, *205*, 88-98.
- 343 21. Kazimierczak, K. A.; Flint HJScott, K. P., Comparative analysis of sequences flanking tet(W)  
344 resistance genes in multiple species of gut bacteria. *Antimicrobial Agents & Chemotherapy* **2006**, *50* (8), 2632-  
345 2639.
- 346 22. Mohammed Salim, A.; Ana Belén, F.; Pablo, A. M.; Abelardo, M.; Baltasar, M., Analysis of  
347 tetracycline resistance tet(W) genes and their flanking sequences in intestinal *Bifidobacterium* species. *Journal*  
348 *of Antimicrobial Chemotherapy* **2008**, *62* (4), 688-93.
- 349 23. Zuo, F.; Rui, Y.; Feng, X.; Chen, L.; Zhu, Z.; Khaskheli, G. B.; Ma, H.; Chen, S.,  
350 Characterization and in vitro properties of potential probiotic *Bifidobacterium* strains isolated from breast-fed  
351 infant feces. *Annals of Microbiology* **2016**, *66* (3), 1027-1037.
- 352 24. Douglas, G. L.; Klaenhammer, T. R., Directed chromosomal integration and expression of the  
353 reporter gene *gusA3* in *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol* **2011**, *77* (20), 7365-7371.
- 354 25. Zhongke, S.; Annika, B.; Daria, Z.; Jing, Y.; Riedel, C. U., Accessing the inaccessible:  
355 molecular tools for bifidobacteria. *Appl Environ Microbiol* **2012**, *78* (15), 5035-5042.
- 356 26. Vos, D.; Willem, M., Gene cloning and expression in lactic streptococci. *Fems Microbiology*  
357 *Letters* **1987**, *46* (3), 281-295.
- 358 27. Klijn, A.; Moine, D.; Delley, M.; Mercenier, A.; Arigoni, F.; Pridmore, R. D., Construction of  
359 a reporter vector for the analysis of *Bifidobacterium longum* promoters. *Appl Environ Microbiol* **2006**, *72* (11),  
360 7401-5.
- 361 28. Grimm, V.; Gleinser, M.; Neu, C.; Zhurina, D.; Riedel, C. U., Expression of fluorescent  
362 proteins in bifidobacteria for analysis of host-microbe interactions. *Appl Environ Microbiol* **2014**, *80* (9), 2842-  
363 50.
- 364 29. Lim, B.; Zimmermann, M.; Barry, N. A.; Goodman, A. L., Engineered Regulatory Systems  
365 Modulate Gene Expression of Human Commensals in the Gut. *Cell* **2017**, *169* (3), 547-558.
- 366 30. Claudio, H. C.; Borja, S.; Pablo, Á. M.; Patricia, L.; Noelia, M. Á.; Michele, D.; Marc, M.;  
367 Encarna, V.; Ana, S.; María, A., A Single Mutation in the Gene Responsible for the Mucoïd Phenotype of  
368 *Bifidobacterium animalis* subsp. *lactis* Confers Surface and Functional Characteristics. *Applied & Environmental*  
369 *Microbiology* **2015**, *81* (23), 7960-8.
- 370 31. Okano, K.; Uematsu, G.; Hama, S.; Tanaka, T.; Noda, H.; Kondo, A.; Honda, K., [Metabolic  
371 engineering of *Lactobacillus plantarum* for direct L-lactic acid production from raw corn starch]. *Biotechnology*  
372 *Journal* **2018**, *13* (5), 1700517.
- 373 32. Carina, K.; Yanzhong, H.; Qiang, P.; Harold, M.; Anna, H.; Dipu, D.; Dalen, P. J., Van;  
374 Pouwels, P. H.; Leer, R. J.; Kelly, C. G., In situ delivery of passive immunity by lactobacilli producing single-  
375 chain antibodies. *Nature Biotechnology* **2002**, *20* (7), 702-706.

- 376 33. Kimmel, S. A.; Roberts, R. F.; Ziegler, G. R., Optimization of Exopolysaccharide Production by  
377 *Lactobacillus delbrueckii* subsp. *bulgaricus* RR Grown in a Semidefined Medium. *Appl. Environ. Microbiol.*  
378 **1998**, *64* (2), 659-664.
- 379 34. He, J.; Sakaguchi, K.; Suzuki, T., Determination of the ribosome-binding sequence and spacer  
380 length between binding site and initiation codon for efficient protein expression in *Bifidobacterium longum* 105-  
381 A. *Journal of bioscience and bioengineering* **2012**, *113* (4), 442-444.
- 382 35. De Keersmaecker, S. C.; Braeken, K.; Verhoeven, T. L.; Vélez, M. P.; Lebeer, S.;  
383 Vanderleyden, J.; Hols, P., Flow cytometric testing of green fluorescent protein-tagged *Lactobacillus rhamnosus*  
384 GG for response to defensins. *Appl. Environ. Microbiol.* **2006**, *72* (7), 4923-4930.
- 385 36. Wiegand, I.; Hilpert, K.; Hancock, R. E., Agar and broth dilution methods to determine the  
386 minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc* **2008**, *3* (2), 163-75.
- 387 37. Zuo, F.; Yu, R.; Feng, X.; Khaskheli, G. B.; Chen, L.; Ma, H.; Chen, S., Combination of  
388 heterogeneous catalase and superoxide dismutase protects *Bifidobacterium longum* strain NCC2705 from  
389 oxidative stress. *Applied microbiology and biotechnology* **2014**, *98* (17), 7523-7534.

390