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1 **Construction and validation of a mCherry protein vector for promoter analysis in**

2 ***Lactobacillus acidophilus***

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20

Abstract

Lactobacilli are widespread in natural environments and are increasingly being investigated as potential health modulators. In this study we have adapted the broad-host range vector pNZ8048 to express the mCherry protein (pRCR) to expand the usage of the mCherry protein for analysis of gene expression in *Lactobacillus*. This vector is also able to replicate in *Streptococcus pneumoniae* and *E. coli*. The usage of pRCR as a promoter probe was validated in *Lactobacillus acidophilus* by characterizing the regulation of lactacin B expression. The results show that the regulation is exerted at the transcriptional level, with *lbaB* gene expression being specifically induced by co-culture of the *L. acidophilus* bacteriocin producer and the *Streptococcus thermophilus* STY-31 inducer bacterium.

Keywords *Lactobacillus*; broad host-range vector; pRCR, bacteriocin; promoter regulation.

37 **Introduction**

38 Lactobacilli belong to the lactic acid bacteria (LAB) group, and traditionally they have
39 been used for fermented food production. They are widespread in natural environments
40 including niches such as the human and animal gastrointestinal tracts, where they can
41 play important roles as health modulators [2, 23]. Thus, they are increasingly used as
42 functional food ingredients (probiotics), and as vectors for live oral vaccines and drugs
43 [3, 24, 25]. Several *Lactobacillus* species have been identified to produce bacteriocins,
44 some of them have a dual role, acting as inhibitors at high concentrations and
45 participating in interspecies communication or bacterial cross talk [8].

46 Due to the increased interest in lactobacilli health effects, it is relevant to develop
47 genetic tools that can detect regulation in living cells of gene expression such as the
48 production of proteins, including enzymes, from these bacteria [9, 15]. One strategy is
49 the construction of fluorescent proteins to study the effect of lactobacilli in the immune
50 system and their localization within the gastrointestinal tract [2, 6]. Fluorescent proteins
51 could also facilitate the monitoring of these bacteria in biofilm formation and interaction
52 with the host. In addition, fluorescent proteins are used as reporters for transcriptional
53 gene expression and regulation. In our previous work we designed a *mrfp* gene codon,
54 optimized for expression in Gram-positive bacteria, from the monomeric variant of the
55 'mCherry' red fluorescent protein (RFP) from *Dicosoma* sp. [11]. Its use as a reporter in
56 *Lactococcus lactis* and *Enterococcus faecalis* was validated by the construction and
57 testing of shuttle vectors based on the pAK80 plasmid [11]. Additional applications of
58 the mCherry-derived vectors in these LAB species have been recently reported [4, 5].

59 In this study we have constructed a vector based on the pSH71 replicon [7, 12], in
60 order to expand the usage of mCherry for analysis of gene expression in *Lactobacillus*
61 and its usage as a promoter probe validated in *L. acidophilus.*

62

63 **Materials and methods**

64 Bacterial strains and culture conditions

65 *L. acidophilus* CECT 903 from the Colección Española de Cultivos Tipo (Paterna,
66 Spain) and *L. acidophilus* La5 (Chr. Hansen, Hørsholm, Denmark) strains were grown
67 in MRS broth (Pronadisa, Madrid, Spain) supplemented with 0.05% L-cysteine
68 hydrochloride (Calbiochem, Merck KGaA, Darmstadt, Germany) and 0.2% Tween 80
69 (Oxoid, Hampshire, UK) (MRSCT) at 37 ºC. *S. thermophilus* STY-31 (Chr. Hansen)
70 was grown in ESTY broth (Pronadisa) supplemented with 0.5% glucose at 37 ºC.
71 *Escherichia coli* DH-5α [21] was grown in Luria-Bertani broth at 37 ºC with vigorous
72 shaking. *Streptococcus pneumoniae* 708 [18] was grown in AGCH medium [17]
73 supplemented with 0.25% yeast extract and 0.8% sucrose at 37 °C without shaking
74 When necessary, chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) was added to
75 the culture medium at a final concentration of 10 μg mL^{-1} for *E. coli* and 5 μg mL^{-1} for
76 *S. pneumoniae* and *L. acidophilus*. Plate media were prepared by adding agar
77 (Pronadisa) to liquid broth at a final concentration of 1.5%.

78

79 General DNA manipulation and transformation

80 The promoter-probe vector and the expression plasmid constructed in this work were
81 based on the the pSH71 replicon [7, 12]. Plasmid pRCR was constructed as follows
82 (Fig. 1). Plasmid pNZ8048 [16] was digested with *Bgl*II and *Sac*I to remove the nisin
83 promoter. The resultant 3168 bp DNA fragment, containing the rolling-circle replicon
84 of the pSH71 plasmid and the chloramphenicol resistance *cat* gene, was purified from a
85 0.8% agarose gel using the QIAquick Gel Extraction Kit (Qiagen Iberia, Madrid,
86 Spain). The *mrfp* gene encoding the mCherry protein [11] was amplified from plasmid

87 pTVCherry (National Collections of Industrial and Marine Bacteria, Aberdeen, UK) by
88 using the specific primers mCherryF (5'-
89 GGAAGATCTTCCCGAATTCCCCGGGATCCTCTAGAGGGATACGCACG
90 AGTTTCAACT-3') and mCherryR (5'-
91 CGCGAGCTCATTTATATAATTCGTCCATGCCACCTGT-3') to obtain a 801 pb
92 amplicon containing the *mrfp* gene preceded by the multicloning site *Bgl*II, *Eco*RI
93 *Sma*I, *Xma*I *Bam*HI, *Xba*I. The PCR product was then digested with *Bgl*II and *Sac*I
94 restriction enzymes and ligated, with T4 DNA ligase (Thermo Fisher Scientific,
95 Waltham, USA), to the 3168 bp fragment from pNZ8048. The resulting plasmid, named
96 pRCR (3960 bp), was established in *S. pneumoniae* 708 by transformation as previously
97 described [19]. Transformants were selected for chloramphenicol resistance, and the
98 correct nucleotide sequence of the insert, containing a multicloning site and the *mrfp*
99 gene in pRCR, was confirmed by DNA sequencing at Secugen S.L. (Centro de
100 Investigaciones Biológicas, Madrid, Spain).
101 The promoter (P_{lbaB}) of the *L. acidophilus* La5 lactacin B gene [22] was cloned
102 upstream of *mrfp* in pRCR, generating the transcriptional fusion P_{lbaB}-*mrfp* in pRCR11.
103 To this end, an amplicon was generated using the chromosomal DNA of *L. acidophilus*
104 La5 as template and the primers LBA1797F (5'-
105 AGGAGATCTGCGTACAAAGATGTGGTTAA-3') and LBA1797R (5`-
106 AGGTCTAGATGAGATTTTTATCTCATTTCAAC-3') to obtain a 221 pb fragment
107 containing the P_{lbaB} sequence. After digestion with *Bgl*II and *Xba*I, the amplicon was
108 introduced into the multicloning site of pRCR, after digestion with the same restriction
109 enzymes. The resulting plasmid pRCR11 (4166 bp) was established in *E. coli* DH5α by
110 transformation as described previously by Hanahan [13]. The presence of the
111 transcriptional fusion in pRCR11 was confirmed by DNA sequencing with primers

112 LBA1797F and LBA1797R.

113 Plasmid pRCR11 was transferred to *L. acidophilus* CECT903 cells by
114 electrotransformation as follows. The CECT903 strain was grown under aerobic
115 conditions at 37 ºC, without shaking, during 15 h in MRSCT broth supplemented with
116 1% glycine. Subsequently, 4 mL of the culture were used to inoculate 200 mL of the
117 same fresh medium, and grown until it reached an OD_{600} of 0.3-0.4. Cells were
118 collected by centrifugation at 0 ºC and 8600 $\times g$ for 10 min and washed four times with
119 ice-cold electroporation buffer (HEPES 0.1 mM, sucrose 0.5 M, pH 7.5). Finally, cells
120 were resuspended in 1.6 mL of ice-cold electroporation buffer. 750 µL of cells and 0.5
121 µg of plasmid DNA were used for electrotransformation. The electroporation conditions
122 were 25 µF, 200 Ω and 2.5 kV in a 0.4-cm cuvette, using a Gene Pulser and a Pulse
123 Controller apparatus (Bio-Rad, Richmond, CA, USA). After electroporation, cells were
124 resuspended in 8 mL of MRSCT broth supplemented with 10 mM $CaCl_2$ and 0.5 M
125 sucrose, incubated aerobically at 37 ºC for 3 h, without agitation, and then plated onto
126 MRSCT medium supplemented with 1% agar and chloramphenicol.

127

128 Induction assay and determination of fluorescence

129 Induction of expression of mCherry from the P_{lbaB} promoter in *L. acidophilus*
130 CECT903[pRCR11] cells was assayed by co-culturing *L. acidophilus*
131 CECT903[pRCR11] with the inducer *Streptococcus thermophilus* STY-31 strain [22].
132 Co-cultures were carried out in MRSCT medium inoculated with 2% each of *L.*
133 *acidophilus* CECT903 [pRCR11] and *S. thermophilus* STY-31 overnight cultures.

134 The levels of fluorescence of the mCherry encoded by pRCR11 and bacterial
135 growth were tested simultaneously with the Varioskan Flash system (Thermo Fisher
136 Scientific, Waltham, MA, USA), which provides quantitative data of cell density via

137 measuring OD at 600 nm and *in vivo* mCherry expression at an excitation wavelength of
138 587 nm and an emission wavelength of 612 nm. Background fluorescence of the control
139 strain (*L. acidophilus* CECT903 [pRCR11] without *S. thermophilus* STY-31 grown
140 under the same conditions) was used to normalize the fluorescence signals during
141 cultivation. All measurements were performed in sterile 96-well optical bottomed
142 microplates (Nunc, Rochester, NY, USA) with a final assay volume of 300 µl per well
143 by using the microtiter plate assay system Varioskan Flash. The microplates were
144 incubated for 24 h at 37 ºC. Measurements were made at 1 h intervals.

145

146 Preparation of nucleic acids

147 For cloning and sequencing experiments, plasmidic DNA was purified from *E. coli* DH-
148 5α by usage of QIAprep Spin Miniprep and Midiprep kits (Qiagen, Hilden, Germany) or
149 from *S. pneumoniae* 708 as previously described (Mohedano et al., 2005). For primer
150 extension analysis, a culture of *L. acidophilus* CECT903[pRCR11] and the co-culture of
151 *L. acidophilus* CECT903 [pRCR11] and *S. thermophilus* STY-31 were grown to an
152 OD_{600} of 1.2 and then used for analysis of *mrfp* mRNA. Total RNA was isolated with a
153 Ribolyser and Recovery kit from Hybaid (Middlesex, United Kingdom) as specified by
154 the supplier. The RNAs were checked for the integrity and yield of the rRNAs by
155 Qubit^{TM} fluorometer (Invitrogen, Madrid, Spain) and by Gel Doc 1000 (Bio-Rad). The
156 patterns of rRNAs were similar in all preparations.

157

158 Primer extension analysis

159 Primer extension analysis was performed by a modification of the method described by
160 Fekete et al. [10]. The start site of *lbaB* mRNA was detected using the LBABP primer
161 (5′-TGAGTTGAAACTCGGTGCGTATCCTCT-3′) labeled with 6-FAM at its 5’-end

162 (Sigma-Aldrich). Two hundred pmol of primer were annealed to 40 µg of total RNA.
163 Primer extension reactions were performed by incubation of the annealing mixture with
164 20 nmol each of dNTP (dATP, dGTP, dCTP and dTTP), 200 U of Maxima Reverse
165 Transcriptase (Thermo Fisher Scientific, Madrid, Spain) in 1× reverse transcriptase
166 buffer (Thermo Fisher) in a final volume of 50 µl at 50 °C for 60 min. Then, the
167 reactions were supplemented with 50 µl of TE (10 mM Tris HCl pH 8.0, 1 mM EDTA)
168 and purified by treatment with phenol (vol:vol) for 5 min at room temperature and
169 ethanol precipitation with three volumes of 100% ethanol in the presence of 0.3 mM Na
170 acetate. After overnight storage at -20 ºC, samples were sedimented by centrifugation at
171 12.000 ×*g*, 30 min at -10 ºC and resuspended in TE (12 µL).
172 Detection and quantification of the reaction products were carried out in a 8%
173 polyacrylamide gel containing 7 M urea. Bands labeled with 6-FAM were detected and
174 directly quantified with a FujiFilm Fluorescent Image Analyzer FLA-3000 (Fujifilm,
175 Düsseldorf, Germany).
176 For determination of the length of the extended products, the primer extension
177 reactions were further purified using Agencourt Clean Seq (Beckam Coulter,
178 Alcobendas, Madrid, Spain) and kept frozen at -20ºC until use. Samples were separated
179 on an Abi 3730 DNA Analyzer (Applied Biosystems, Tres Cantos, Madrid, Spain)
180 capillary electrophoresis instrument using techniques and parameters recommended by
181 the manufacturer. A DNA sequence of pRCR11 determined by the dideoxynucleotide
182 method with unlabeled LBABP primer was included in the same capillary in each run to
183 determine fragment length. The Peak Scanner version V1.0 (Applied Biosystems) was
184 used to screen the data and identify major peaks.
185
186 **Results and discussion**

187 Plasmids containing the mCherry coding gene

188 Following the construction and analysis of the pTL family of plasmids designed for
189 using mCherry as a reporter in LAB [11], we were unable to transfer any of these
190 plasmids to *Lactobacillus casei* and *L. acidophilus* strains nor to *S. pneumoniae* strains.
191 These pTL plasmids were derived from pAK80, which carries, in addition to the
192 erythromycin resistance marker, two origins of replication, one from the lactococcal
193 plasmid pCT1138 and the other from the *E. coli* p15A plasmid, and replicates in Gram-
194 positive bacteria by the theta mode mechanism [14]. As an alternative, and with the aim
195 of developing new tools for gene expression analysis in lactobacilli, the use of the
196 replicon of the *L. lactis* pSH71 plasmid [7, 12] was investigated. This plasmid uses the
197 rolling circle-type mechanism for replication, and is characterized by a broad host-
198 range, which includes Gram-positive bacteria and *E. coli*. Therefore, a promoter-probe
199 vector (pRCR) and an expression plasmid (pRCR11) carrying the synthetic *mrfp* gene
200 optimized for LAB [11] and the chloramphenicol resistance marker were constructed
201 (Fig. 1). pRCR was generated by changing a DNA fragment of pNZ4048 containing the
202 *nisA* promoter, located between the restriction sites *Bgl*II and *Sac*I, by a DNA fragment
203 containing the *mrfp* gene preceded by a polylinker to facilitate further cloning of
204 transcriptional promoters upstream of the mCherry coding gene. The pRCR plasmid
205 was established in *S. pneumoniae* 708 by transformation and selection for
206 chloramphenicol resistance. In addition, as predicted, the plasmid was also able to
207 replicate in *Lactobacillus sakei* MN1, *L. plantarum* WCFS1 and *E. coli* DH5α (personal
208 communications by M. Nacher, A. Pérez-Ramos and M. L. Mohedano, respectively).
209 Consequently, the resulting vector had kept its broad host range attribute and had the
210 potential to be used in various LAB species.

211 To evaluate the functional expression of mCherry in lactobacilli, the region

212 located upstream of the *lbaB* bacteriocin structural gene from *L. acidophilus* [22] and
213 carrying the putative promoter P_{lbaB} was cloned upstream of *mrfp* in pRCR to generate
214 pRCR11. The expression of lactacin B in *L. acidophilus* has been demonstrated to be
215 inducible by the co-culture with live target bacteria [22]. The *mrfp* gene was used as
216 reporter to monitor the P_{lbaB} activity during the induction of lactacin B production by
217 the transformation of *L. acidophilus* CECT 903 with pRCR11. The induction of
218 bacteriocin expression was assayed by co-culturing this strain with *S. thermophilus*
219 STY-31, a previously identified inducer strain [22]. The functional expression of
220 mCherry under the control of P_{lbaB} and the increase of biomass during cell growth was
221 monitored (Fig. 2). The results revealed that the growth of *L. acidophilus* CECT
222 903[pRCR11] was very similar in both single and co-cultures. However, the mCherry
223 activity was detected only in the presence of *S. thermophilus* STY-31. Moreover, under
224 co-culture conditions the increase of mCherry fluorescence correlated with the growth
225 pattern. These results indicated that up-regulation of lactacin B expression initiates
226 during exponential growth as previously demonstrated [22]. The maximum fluorescence
227 levels were detected at the stationary phase (OD_{600}=1.3), consequently growth to this
228 phase was used for further experiments.
229

230 Transcriptional analysis of the influence of co-culture with *S. thermophilus* on lactacin
231 B expression in *L. acidophilus*

232 Our previous quantitative RT–PCR studies of *L. acidophilus* La5 *lbaB* gene expression
233 had shown that in the presence of *S. thermophilus* STY-31 an increase of the *lbaB*
234 transcript takes place [22]. Thus, total RNA was extracted from *L. acidophilus*
235 CECT603[pRCR11] cells grown in the presence or absence of *S. thermophilus* STY-31
236 to stationary phase and samples were used for primer extension analysis performed with

237 a 5-end 6-FAM labeled LBABP primer. Analysis of the same volume (6 µl) of both
238 reactions in a polyacrylamide gel detected the extended products encoded by pRCR11
239 complementary to the *lbaB* transcript in *L. acidophilus* CECT603[pRCR11] grown in
240 the presence or absence of *S. thermophilus* STY-31 (Fig. 3B). In the absence of the
241 inducer two bands with similar intensity were observed, whereas in the co-cultures one
242 more prominent and two minor longer extended products were observed. The
243 fluorescence of the bands was quantified with a fluorescent image analyzer, and the
244 results revealed a 5-fold induction due to the presence of *S. thermophilus* STY-31 (Fig.
245 3B).

246 To determine the length of the extended products and the 5-end of the *lbaB*
247 transcript, the primer extension reactions were also analyzed by capillary
248 electrophoresis in conjunction with a DNA sequence of pRCR11 generated with
249 unlabelled LBABP primer (Fig. 3A). Since we expected low transcript levels of P_{lbaB} in
250 cells grown in mono-culture, we processed 250 nl for the capillary electrophoresis
251 experiments compared to 40 nl derived from cells grown in co-culture. The pattern of
252 the peaks observed (Fig 3B) correlated with that obtained for the labeled bands in the
253 polyacrylamide gel (Fig. 3A). The two bands detected in cultures of *L. acidophilus*
254 CECT603[pRCR11] corresponded to extended products of 185 and 187 nt, the first
255 being the major band present in the co-cultures (Fig. 3B). This result located the 5´-end
256 of the *lbaB* mRNA at a C and A (Fig. 3C), since 6-FAM labeled DNA extended
257 products run as if they were, on an average, three nucleotides shorter than the dideoxy
258 sequencing products (Fekete et al., 2003). Upstream of the start sites, a putative
259 promoter was detected composed of a -35 (TTGtAa) and a -10 (aATAAT), these
260 sequences being characteristic for the binding of the vegetative σ factor of the bacterial
261 RNA polymerases with an anomalous (too long) spacing of 24 nt (Fig. 3C). Moreover,

262 the two start sites for transcription are included in one of the arms of the inverted repeat
263 characteristic for binding of transcriptional regulators. This location predicts that the
264 binding of a protein to the inverted repeat will impair initiation of transcription
265 catalyzed by the RNA polymerase. The expression of the lactacin B operon is regulated
266 by the response regulator RR_1798 which is part of a three-component regulatory
267 system composed of the inducing peptide IP_1800, the HK_1799 histidine kinase and
268 the RR_1798 response regulator [1, 22]. Thus, it seems that under uninduced conditions
269 competition between the RNA polymerase and RR_1798 for binding to the upstream
270 region of *lbaB* gene will result in low levels of the transcript starting at the two
271 nucleotides G and C. Then, in the presence of bacteria that compete for the
272 environmental niche, HK_1799 would sense its presence and, by modification of the
273 RR_1798, would impair the repression of transcription of *lbaB* and result in an increase
274 of lactacin B levels. We have previously demonstrated that the production of lactacin B
275 by *L. acidophilus* is controlled by an autoinduction mechanism involving a secreted
276 peptide and by co-culture with live inducer cells [22]. These characteristics of induction
277 of bacteriocin production through autoinduction and co-culture have been recently
278 described to be widespread among bacteriocinogenic *L. plantarum* strains [20]. The use
279 of mCherry as a promoter probe in pRCR11 has allowed us to locate the region where
280 the *lbaB* transcriptional regulation is specifically induced by co-culture of the lactacin B
281 producer with the inducing bacteria.
282 In conclusion, the rolling circle-type mechanism for replication of pRCR has
283 broadened the host-range in LAB of the mCherry based vectors pTLR. Indeed, the
284 promoter-probe vector pRCR has demonstrated to be suitable for characterization of
285 complex promoter induction mechanisms such as those related to bacteriocin production
286 by *L. acidtophillus*.

287

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294

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374

375

376 **Legend to the Figures**

377 Figure 1. Schematic diagram showing the construction of pRCR and pRCR11. For

378 details, see “Materials and methods”. Relevant restriction sites are shown. Specific

379 genes are: *mrfp* and *cat* that encode mCherry and the protein responsible for the

380 resistance to chloramphenicol, respectively. P_{lbaB}, promoter of the lactacin B structural

381 gene of *Lactobacillus acidophilus* La5.

382

383 Figure 2. Detection of induction of expression of mCherry encoded by pRCR11.

384 Fluorescence (relative fluorescence units, RFU; triangles) and growth (OD_{600}; circles)

385 of cultures of *L. acidophilus* CECT 903[pRCR11] (open symbols) and co-cultures of *L.*

386 *acidophilus* CECT 903[pRCR11] and *Streptococcus thermophilus* STY-31 (closed

387 symbols) grown in MRSCT are depicted. The growth of cultures was monitored at a

388 wavelength of 600 nm. Fluorescence emission of mCherry was recorded at 612 nm after

389 excitation at a wavelength of 587 nm.

390

391 Figure 3. Detection of the start site of the *lbaB* transcript by primer extension. Reactions

392 containing total RNA isolated from cultures of *L. acidophilus* CECT 903[pRCR11]

393 alone or in co-culture with *S. thermophilus* STY-31 were analyzed by capillary

394 electrophoresis in conjunction with DNA sequence of pRCR11 (A) or by 8%

395 denaturating polyacrylamide gel electrophoresis (B). For primer extension and DNA

396 sequence analysis were used, respectively, primers fluorescently labeled at the 5´-end

397 with 6-FAM, or unlabelled (both having the same DNA sequence). Extended products

398 ran as if they were, on an average, three nucleotides shorter than the dideoxy sequencing

399 products. The length of the extended products determined by the analysis depicted in

400 (A) is indicated in the analysis showed in (B).The DNA region surrounding the start site
401 of the mRNA is also depicted (C). The start sites of the transcript detected in (A) are
402 indicated by stars. The -35 and -10 regions of the P_{lbaB} promoter are shown. The
403 inverted repeat, putative binding site of the RR_1798 response regulator, is indicated by
404 arrows.

Figure 1

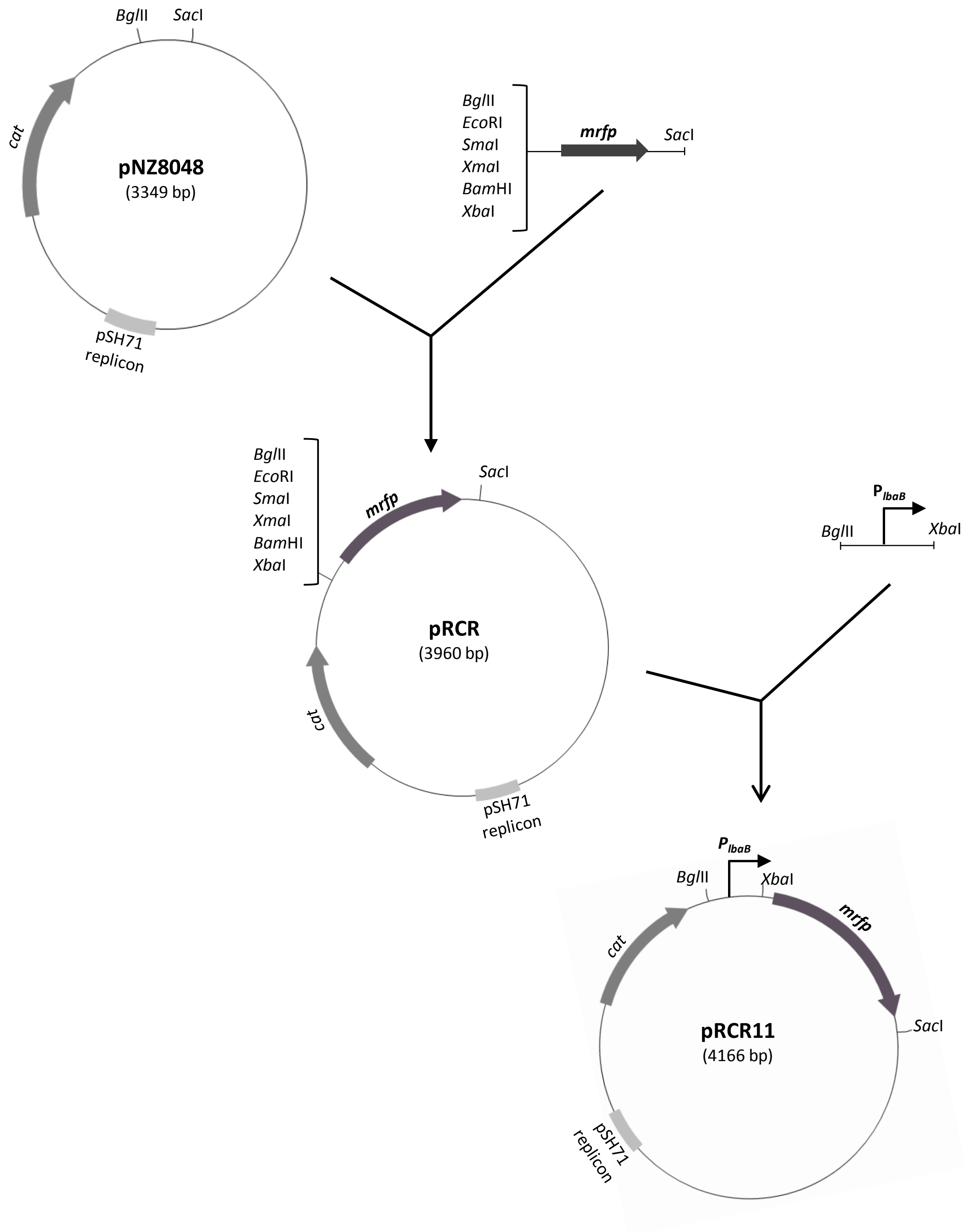

Figure 1

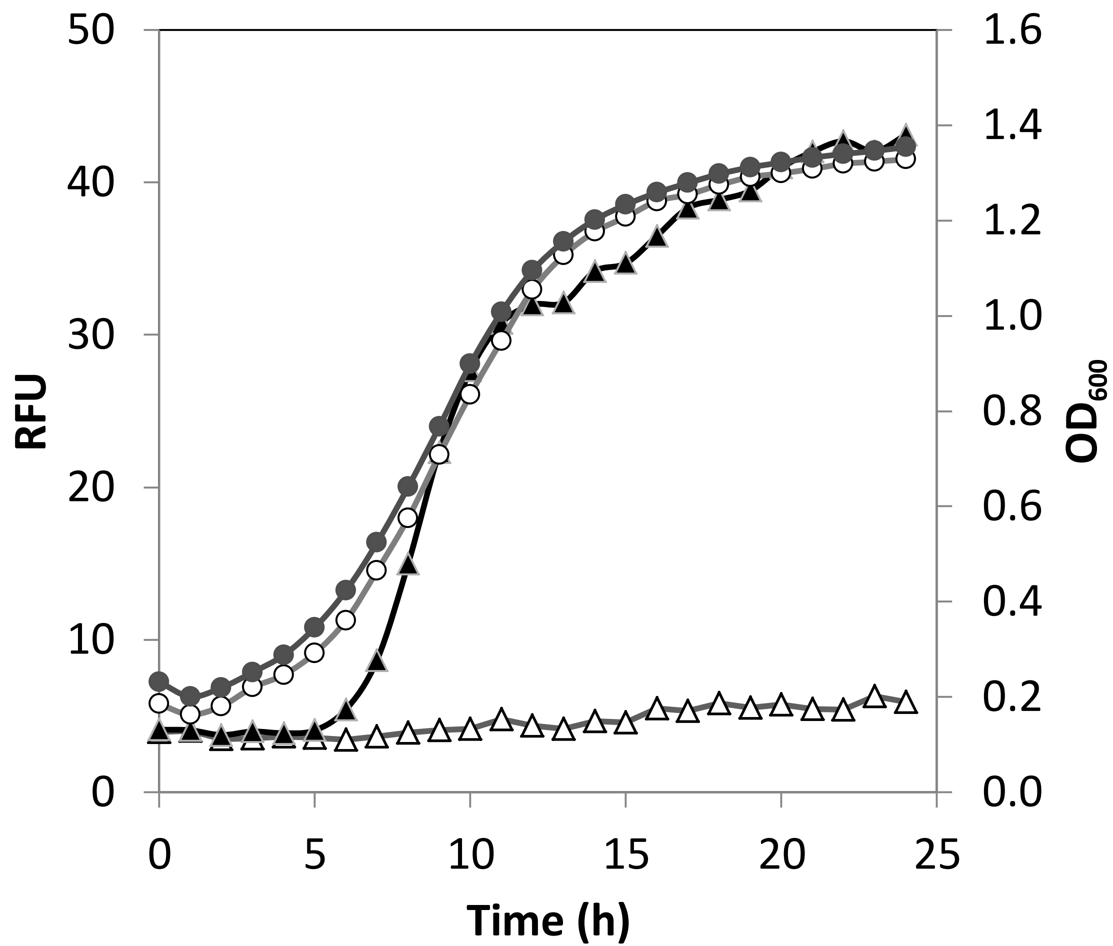

Figure 2

Figure 3

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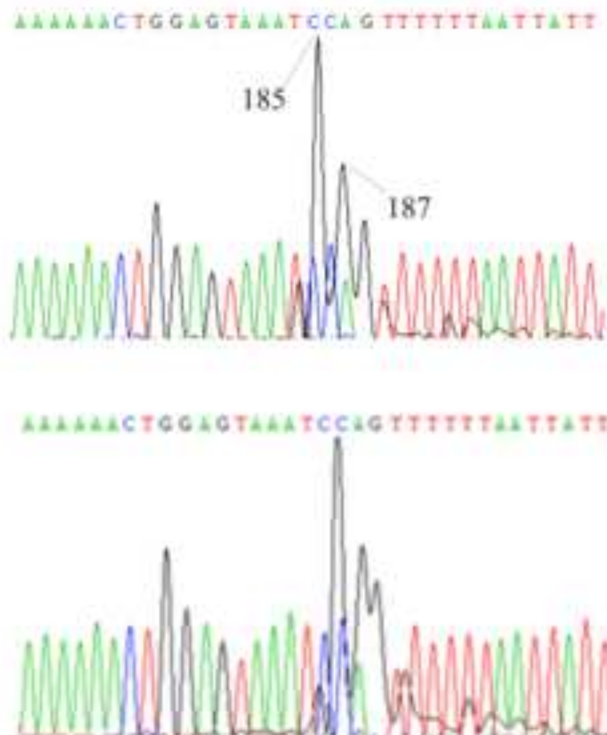
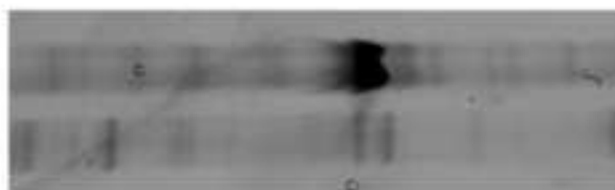
A



L. acidophilus CECT903 [pRCR11] and
S. thermophilus STY-31

L. acidophilus CECT903 [pRCR11]

B



L. acidophilus CECT903 [pRCR11] and
S. thermophilus STY-31

L. acidophilus CECT903 [pRCR11]

185 187

C

```
 -35              -10        *  *
TTGTAA- 24 nt -AATAATTAAAAAACTGGATTTACTCCAGTTTTTT
AACATT- 24 nt -TTATTAATTTTTTGACCTAAATGAGGTCAAAAAA
```