J Ind Microbiol Biotechnol (2015) 42:247–253
DOI 10.1007/s10295-014-1567-4

BIOTECHNOLOGY METHODS

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

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Received: 29 August 2014 / Accepted: 12 December 2014 / Published online: 23 December 2014
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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 *Escherichia 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 *S. thermophilus* STY-31 inducer bacterium.

Keywords *Lactobacillus* · Broad-host-range vector · pRCR · Bacteriocin · Promoter regulation

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Introduction

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

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

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

Materials and methods

Bacterial strains and culture conditions

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

General DNA manipulation and transformation

The promoter-probe vector and the expression plasmid constructed in this work were based on the pSH71 replicon [7, 12]. Plasmid pRCR was constructed as follows (Fig. 1). Plasmid pNZ8048 [16] was digested with *Bgl*II and *Sac*I to remove the nisin promoter. The resultant 3,168 bp DNA fragment, containing the rolling-circle replicon of the pSH71 plasmid and the chloramphenicol resistance *cat* gene, was purified from a 0.8 % agarose gel using the QIAquick Gel Extraction Kit (Qiagen Iberia, Madrid, Spain). The *mrfp* gene encoding the mCherry protein [11] was amplified from plasmid pTVCherry (National Collections of Industrial and Marine Bacteria, Aberdeen, UK) by using the specific primers mCherryF (5′-GGA AGATCTTCCCGAATTCCCCGGGGATCCTCTAGAGG GATACGCACG AGTTTCAACT-3′) and mCherryR (5′-CGCGAGCTCATTTATATAATTCGTCCATGCCAC CTGT-3′) to obtain a 801 pb amplicon containing the *mrfp* gene preceded by the multicloning site *Bgl*II, *Eco*RI *Sma*I, *Xma*I *Bam*HI, *Xba*I. The PCR product was then digested with *Bgl*II and *Sac*I restriction enzymes and ligated, with T4 DNA ligase (Thermo Fisher Scientific, Waltham, USA), to the 3,168 bp fragment from pNZ8048. The resulting plasmid, named pRCR (3,960 bp), was established in *S. pneumoniae* 708 by transformation as previously described [19]. Transformants were selected for chloramphenicol resistance, and the correct nucleotide sequence of the insert, containing a multicloning site and the *mrfp* gene in pRCR, was confirmed by DNA sequencing at Secugen S.L. (Centro de Investigaciones Biológicas, Madrid, Spain).

The promoter (P_{lbaB}) of the *L. acidophilus* La5 lactacin B gene [22] was cloned upstream of *mrfp* in pRCR, generating the transcriptional fusion P_{lbaB}-*mrfp* in pRCR11. To this end, an amplicon was generated using the chromosomal DNA of *L. acidophilus* La5 as template and the primers LBA1797F (5′-AGGAGATCTGCGTACAAAGAT GTGGTTAA-3′) and LBA1797R (5′-AGGTCTAGATGAG ATTTTTATCTCATTTCAAC-3′) to obtain a 221 pb fragment containing the P_{lbaB} sequence. After digestion with *Bgl*II and *Xba*I, the amplicon was introduced into the multicloning site of pRCR, after digestion with the same restriction enzymes. The resulting plasmid pRCR11 (4,166 bp) was established in *E. coli* DH5α by transformation as described previously by Hanahan [13]. The presence of the transcriptional fusion in pRCR11 was confirmed by DNA sequencing with primers LBA1797F and LBA1797R.

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

Induction assay and determination of fluorescence

Induction of expression of mCherry from the P_{lbaB} promoter in *L. acidophilus* CECT903[pRCR11] cells was assayed by co-culturing *L. acidophilus* CECT903[pRCR11] with the inducer *Streptococcus thermophilus* STY-31 strain [22].

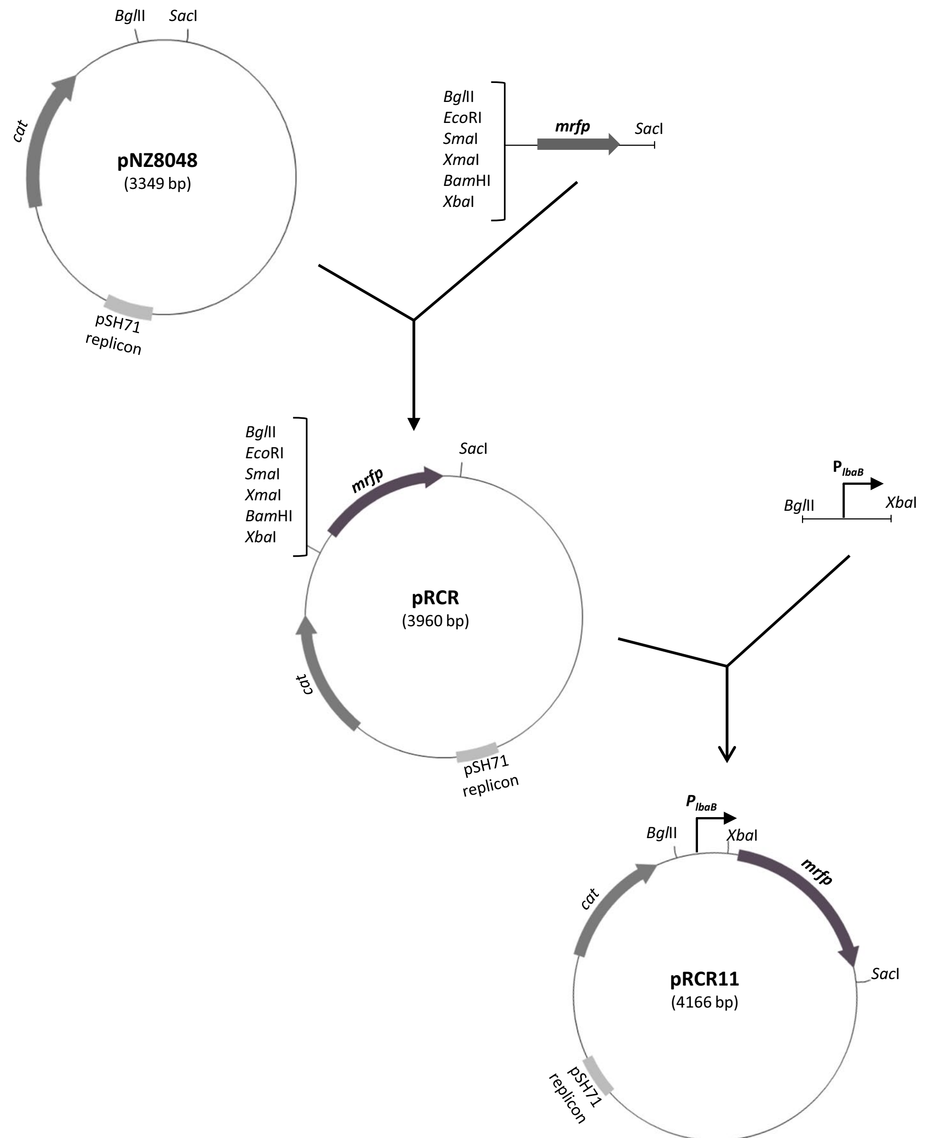

Fig. 1 Schematic diagram showing the construction of pRCR and pRCR11. For details, see "Materials and methods". Relevant restriction sites are shown. Specific genes are: *mrfp* and *cat* that encode mCherry and the protein responsible for the resistance to chloramphenicol, respectively. P_{lbaB}, promoter of the lactacin B structural gene of *Lactobacillus acidophilus* La5

Co-cultures were carried out in MRSCT medium inoculated with 2 % each of *L. acidophilus* CECT903 [pRCR11] and *S. thermophilus* STY-31 overnight cultures.

The levels of fluorescence of the mCherry encoded by pRCR11 and bacterial growth were tested simultaneously with the Varioskan Flash system (Thermo Fisher Scientific, Waltham, MA, USA), which provides quantitative data of cell density via measuring OD at 600 nm and in vivo mCherry expression at an excitation wavelength of 587 nm and an emission wavelength of 612 nm. Background fluorescence of the control strain (*L. acidophilus* CECT903 [pRCR11] without *S. thermophilus* STY-31 grown under the same conditions) was used to normalize the fluorescence signals during cultivation. All measurements were performed in sterile 96-well optical bottomed microplates (Nunc, Rochester, NY, USA) with a final assay volume of 300 µL per well by using the microtiter plate assay system Varioskan Flash. The microplates were incubated for 24 h at 37 °C. Measurements were made at 1 h intervals.

Preparation of nucleic acids

For cloning and sequencing experiments, plasmidic DNA was purified from *E. coli* DH-5α by usage of QIAprep Spin Miniprep and Midiprep kits (Qiagen, Hilden, Germany) or from *S. pneumoniae* 708 as previously described [21]. For primer extension analysis, a culture of *L. acidophilus* CECT903[pRCR11] and the co-culture of *L. acidophilus* CECT903 [pRCR11] and *S. thermophilus* STY-31 were grown to an OD_{600} of 1.2 and then used for analysis of *mrfp* mRNA. Total RNA was isolated with a Ribolyser and Recovery kit from Hybaid (Middlesex, UK) as specified by the supplier. The RNAs were checked for the integrity and yield of the rRNAs by Qubit™ fluorometer (Invitrogen,

Madrid, Spain) and by Gel Doc 1000 (Bio-Rad). The patterns of rRNAs were similar in all preparations.

Primer extension analysis

Primer extension analysis was performed by a modification of the method described by Fekete et al. [10]. The start site of *lbaB* mRNA was detected using the LBABP primer (5′-TGAGTTGAAACTCGGTGCGTATCCTCT-3′) labeled with 6-FAM at its 5′-end (Sigma-Aldrich). Two hundred picomoles of primer were annealed to 40 µg of total RNA. Primer extension reactions were performed by incubation of the annealing mixture with 20 nmol each of dNTP (dATP, dGTP, dCTP and dTTP), 200 U of Maxima Reverse Transcriptase (Thermo Fisher Scientific, Madrid, Spain) in 1× reverse transcriptase buffer (Thermo Fisher) in a final volume of 50 µL at 50 °C for 60 min. Then, the reactions were supplemented with 50 µL of TE (10 mM Tris HCl pH 8.0, 1 mM EDTA) and purified by treatment with phenol (vol:vol) for 5 min at room temperature and ethanol precipitation with three volumes of 100 % ethanol in the presence of 0.3 mM Na acetate. After overnight storage at −20 °C, samples were sedimented by centrifugation at 12,000×*g* for 30 min at −10 °C and resuspended in TE (12 µL).

Detection and quantification of the reaction products were carried out in a 8 % polyacrylamide gel containing 7 M urea. Bands labeled with 6-FAM were detected and directly quantified with a FujiFilm Fluorescent Image Analyzer FLA-3000 (Fujifilm, Düsseldorf, Germany).

For determination of the length of the extended products, the primer extension reactions were further purified using Agencourt Clean Seq (Beckam Coulter, Alcobendas, Madrid, Spain) and kept frozen at −20 °C until use. Samples were separated on an Abi 3730 DNA Analyzer (Applied Biosystems, Tres Cantos, Madrid, Spain) capillary electrophoresis instrument using techniques and parameters recommended by the manufacturer. A DNA sequence of pRCR11 determined by the dideoxynucleotide method with unlabeled LBABP primer was included in the same capillary in each run to determine fragment length. The Peak Scanner version v1.0 (Applied Biosystems) was used to screen the data and identify major peaks.

Results and discussion

Plasmids containing the mCherry coding gene

Following the construction and analysis of the pTL family of plasmids designed for using mCherry as a reporter in LAB [11], we were unable to transfer any of these plasmids neither to *L. casei* and *L. acidophilus* strains nor to *S. pneumoniae* strains. These pTL plasmids were derived from pAK80, which carries, in addition to the erythromycin resistance marker, two origins of replication, one from the lactococcal plasmid pCT1138 and the other from the *E. coli* p15A plasmid, and replicates in Gram-positive bacteria by the theta mode mechanism [14]. As an alternative, and with the aim of developing new tools for gene expression analysis in lactobacilli, the use of the replicon of the *L. lactis* pSH71 plasmid [7, 12] was investigated. This plasmid uses the rolling-circle-type mechanism for replication, and is characterized by a broad host-range, which includes Gram-positive bacteria and *E. coli*. Therefore, a promoter-probe vector (pRCR) and an expression plasmid (pRCR11) carrying the synthetic *mrfp* gene optimized for LAB [11] and the chloramphenicol resistance marker were constructed (Fig. 1). pRCR was generated by changing a DNA fragment of pNZ4048 containing the *nisA* promoter, located between the restriction sites *Bgl*II and *Sac*I, by a DNA fragment containing the *mrfp* gene preceded by a polylinker to facilitate further cloning of transcriptional promoters upstream of the mCherry coding gene. The pRCR plasmid was established in *S. pneumoniae* 708 by transformation and selection for chloramphenicol resistance. In addition, as predicted, the plasmid was also able to replicate in *L. sakei* MN1, *L. plantarum* WCFS1 and *E. coli* DH5α (personal communications by M. Nacher, A. Pérez-Ramos and M.L. Mohedano, respectively). Consequently, the resulting vector had kept its broad-host-range attribute and had the potential to be used in various LAB species.

To evaluate the functional expression of mCherry in lactobacilli, the region located upstream of the *lbaB* bacteriocin structural gene from *L. acidophilus* [22] and carrying the putative promoter P_{lbaB} was cloned upstream of *mrfp* in pRCR to generate pRCR11. The expression of lactacin B in *L. acidophilus* has been demonstrated to be inducible by the co-culture with live target bacteria [22]. The *mrfp* gene was used as reporter to monitor the P_{lbaB} activity during the induction of lactacin B production by the transformation of *L. acidophilus* CECT 903 with pRCR11. The induction of bacteriocin expression was assayed by co-culturing this strain with *S. thermophilus* STY-31, a previously identified inducer strain [22]. The functional expression of mCherry under the control of P_{lbaB} and the increase of biomass during cell growth were monitored (Fig. 2). The results revealed that the growth of *L. acidophilus* CECT 903[pRCR11] was very similar in both single and co-cultures. However, the mCherry activity was detected only in the presence of *S. thermophilus* STY-31. Moreover, under co-culture conditions the increase of mCherry fluorescence correlated with the growth pattern. These results indicated that up-regulation of lactacin B expression initiates during exponential growth as previously demonstrated [22]. The maximum fluorescence levels were detected at the

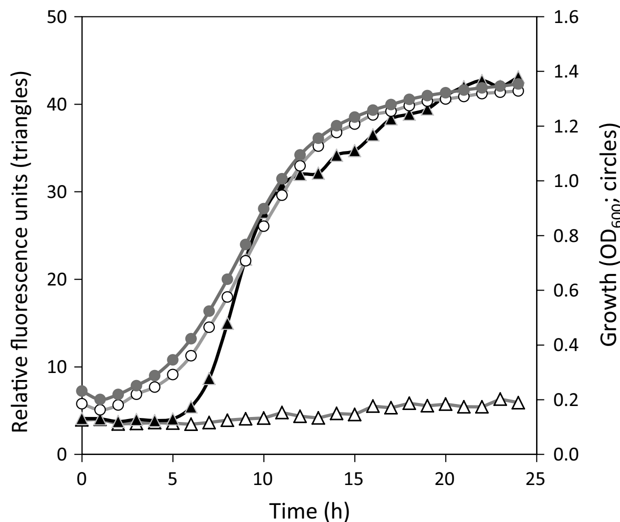

Fig. 2 Detection of induction of expression of mCherry encoded by pRCR11. Fluorescence (relative fluorescence units; *triangles*) and growth (OD_{600}; *circles*) of cultures of *L. acidophilus* CECT 903[pRCR11] (*open symbols*) and co-cultures of *L. acidophilus* CECT 903[pRCR11] and *Streptococcus thermophilus* STY-31 (*closed symbols*) grown in MRSCT are depicted. The growth of cultures was monitored at a wavelength of 600 nm. Fluorescence emission of mCherry was recorded at 612 nm after excitation at a wavelength of 587 nm

stationary phase (OD_{600} = 1.3), consequently growth to this phase was used for further experiments.

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

Our previous quantitative RT-PCR studies of *L. acidophilus* La5 *lbaB* gene expression had shown that in the presence of *S. thermophilus* STY-31 an increase of the *lbaB* transcript takes place [22]. Thus, total RNA was extracted from *L. acidophilus* CECT603[pRCR11] cells grown in the presence or absence of *S. thermophilus* STY-31 to stationary phase and samples were used for primer extension analysis performed with a 5-end 6-FAM labeled LBABP primer. Analysis of the same volume (6 μL) of both reactions in a polyacrylamide gel detected the extended products encoded by pRCR11 complementary to the *lbaB* transcript in *L. acidophilus* CECT603[pRCR11] grown in the presence or absence of *S. thermophilus* STY-31 (Fig. 3b). In the absence of the inducer two bands with similar intensity were observed, whereas in the co-cultures one more prominent and two minor longer extended products were observed. The fluorescence of the bands was quantified with a fluorescent image analyzer, and the results revealed a fivefold induction due to the presence of *S. thermophilus* STY-31 (Fig. 3b).

To determine the length of the extended products and the 5-end of the *lbaB* transcript, the primer extension reactions were also analyzed by capillary electrophoresis in conjunction with a DNA sequence of pRCR11 generated with unlabeled LBABP primer (Fig. 3a). Since we expected low transcript levels of P_{lbaB} in cells grown in mono-culture, we processed 250 nL for the capillary electrophoresis experiments compared to 40 nL derived from cells grown in co-culture. The pattern of the peaks observed (Fig. 3b) correlated with that obtained for the labeled bands in the polyacrylamide gel (Fig. 3a). The two bands detected in cultures of *L. acidophilus* CECT603[pRCR11] corresponded to extended products of 185 and 187 nt, the first being the major band present in the co-cultures (Fig. 3b). This result located the 5′-end of the *lbaB* mRNA at a C and A (Fig. 3c), since 6-FAM labeled DNA extended products run as if they were, on an average, three nucleotides shorter than the dideoxy sequencing products [10]. Upstream of the start sites, a putative promoter was detected composed of a −35 (TTGtAa) and a −10 (aATAAT), these sequences being characteristic for the binding of the vegetative σ factor of the bacterial RNA polymerases with an anomalous (too long) spacing of 24 nt (Fig. 3c). Moreover, the two start sites for transcription are included in one of the arms of the inverted repeat characteristic for binding of transcriptional regulators. This location predicts that the binding of a protein to the inverted repeat will impair initiation of transcription catalyzed by the RNA polymerase. The expression of the lactacin B operon is regulated by the response regulator RR_1798 which is part of a three-component regulatory system composed of the inducing peptide IP_1800, the HK_1799 histidine kinase and the RR_1798 response regulator [1, 22]. Thus, it seems that under uninduced conditions competition between the RNA polymerase and RR_1798 for binding to the upstream region of *lbaB* gene will result in low levels of the transcript starting at the two nucleotides G and C. Then, in the presence of bacteria that compete for the environmental niche, HK_1799 would sense its presence and, by modification of the RR_1798, would impair the repression of transcription of *lbaB* and result in an increase of lactacin B levels. We have previously demonstrated that the production of lactacin B by *L. acidophilus* is controlled by an autoinduction mechanism involving a secreted peptide and by co-culture with live inducer cells [22]. These characteristics of induction of bacteriocin production through autoinduction and co-culture have been recently described to be widespread among bacteriocinogenic *L. plantarum* strains [20]. The use of mCherry as a promoter probe in pRCR11 has allowed us to locate the region where the *lbaB* transcriptional regulation is specifically induced by co-culture of the lactacin B producer with the inducing bacteria.

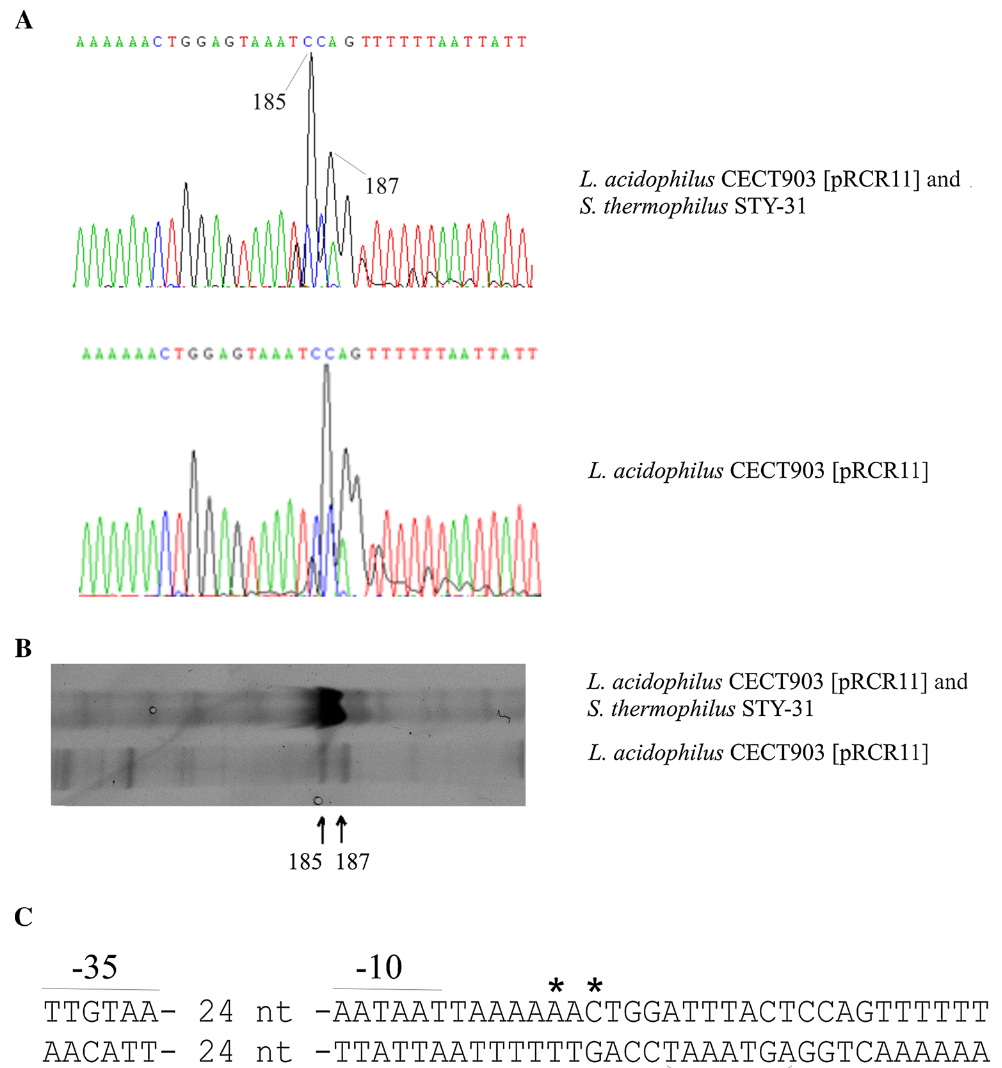

Fig. 3 Detection of the start site of the *lbaB* transcript by primer extension. Reactions containing total RNA isolated from cultures of *L. acidophilus* CECT 903[pRCR11] alone or in co-culture with *S. thermophilus* STY-31 were analyzed by capillary electrophoresis in conjunction with DNA sequence of pRCR11 (**a**) or by 8 % denaturating polyacrylamide gel electrophoresis (**b**). For primer extension and DNA sequence analysis, primers fluorescently labeled at the 5′-end with 6-FAM, or unlabeled (both having the same DNA sequence) were used, respectively. Extended products ran as if they were, on an average, three nucleotides shorter than the dideoxy sequencing products. The length of the extended products determined by the analysis depicted in **a** is indicated in the analysis showed in **b**. The DNA region surrounding the start site of the mRNA is also depicted (**c**). The start sites of the transcript detected in **a** are indicated by *stars*. The −35 and −10 regions of the P_{lbaB} promoter are shown. The inverted repeat, putative binding site of the RR_1798 response regulator, is indicated by *arrows*

In conclusion, the rolling-circle-type mechanism for replication of pRCR has broadened the host-range in LAB of the mCherry-based vectors pTLR. Indeed, the promoter-probe vector pRCR has demonstrated to be suitable for characterization of complex promoter induction mechanisms such as those related to bacteriocin production by *L. acidophilus*.

Acknowledgments This study was supported by Grants AGL2012-40084-C03-01, AGL2012-35814 and RM2011-00003-00-00 from the Spanish Ministry of Economics and Competitiveness and by European Commission FP7 Initial Training Network (contract 238490). We thank Dr. Stephen Elson for the critical reading of the manuscript. We also thank M. Angeles Corrales for technical assistance.

References

1. Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T, Callanan M, Lick S, Hamrick A, Cano R, Klaenhammer TR (2004) Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc Natl Acad Sci USA 102:3906–3912
2. Bao S, Zhu L, Zhuang Q, Wang L, Xu PX, Itoh K, Holzman IR, Lin J (2013) Distribution dynamics of recombinant *Lactobacillus* in the gastrointestinal tract of neonatal rats. PLoS One 8:e60007
3. Bull M, Plummer S, Marchesi J, Mahenthiralingam E (2013) The life history of *Lactobacillus acidophilus* as a probiotic, a tale of revisionary taxonomy, misidentification and commercial success. FEMS Microbiol Lett 349:77–87
4. Campelo AB, Roces C, Mohedano ML, López P, Rodríguez A, Martínez B (2014) A bacteriocin gene cluster able to enhance plasmid maintenance in *Lactococcus lactis*. Microb Cell Fact 13:77
5. Cebrián R, Rodríguez-Ruano S, Martínez-Bueno M, Valdivia E, Maqueda M, Montalbán-López M (2014) Analysis of the promoters involved in enterocin AS-48 expression. PLoS One 9:e90603
6. Chen Z, Lin J, Ma C, Zhao S, She Q, Liang Y (2014) Characterization of pMC11, a plasmid with dual origins of replication isolated from *Lactobacillus casei* MCJ and construction of shuttle vector with each replicon. Appl Microbiol Biotechnol 98:5977–5989

7. De Vos WM (1987) Gene cloning and expression in lactic streptococci. FEMS Microbiol Rev 46:281–295
8. Dobson A, Cotter PD, Ross RP, Hill C (2012) Bacteriocin production, a probiotic trait? Appl Environ Microbiol 78:1–6
9. Duong T, Miller MJ, Barrangou R, Azcarate-Peril MA, Klaenhammer TR (2010) Construction of vectors for inducible and constitutive gene expression in *Lactobacillus*. Microb Biotech 4:357–367
10. Fekete RA, Miller MJ, Chattoraj DK (2003) Fluorescently labeled oligonucleotide extension, a rapid and quantitative protocol for primer extension. Biotechniques 35:90–98
11. García-Cayuela T, Gómez de Cadiñanos LP, Mohedano ML, Fernández de Palencia P, Boden D, Wells J, Peláez C, López P, Requena T (2012) Fluorescent protein vectors for promoter analysis in lactic acid bacteria and *Escherichia coli*. Appl Microbiol Biotechnol 96:171–181
12. Gasson MJ (1983) Plasmid complements of *Streptococcus lactis* and other lactic streptococci after protoplast-induced curing. J Bacteriol 154:1–9
13. Hanahan D (1985) Techniques for transformation of *E. coli*. In: Glover DM (ed) DNA cloning, a practical approach, vol 1. IRL, Oxford, pp 109–135
14. Israelsen H, Madsen SM, Vrang A, Hansen EB, Johansen E (1995) Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn917-lacZ integrants with the new promoter probe vector, pAK80. Appl Environ Microbiol 61:2540–2547
15. Kleerebezem M, Beerthuyzen MM, Vaughan EE, De Vos WM, Kuipers O (1997) Controlled gene expression systems for lactic acid bacteria, transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. Appl Environ Microbiol 63:4581–4584
16. Kuipers OP, De Rayter PG, Kleerebezem M, De Vos WM (1998) Quorum sensing-controlled gene expression in lactic acid bacteria. J Biotechnol 64:15–21
17. Lacks S (1968) Genetic regulation of maltosaccharide utilization in *Pneumococcus*. Genetics 60:685–706
18. Lacks SA, Greenberg B (2001) Constitutive competence for genetic transformation in *Streptococcus pneumoniae* caused by mutation of a transmembrane histidine kinase. Mol Microbiol 42:1035–1045
19. Lacks SA, López P, Greenberg B, Espinosa M (1986) Identification and analysis of genes for tetracycline resistance and replication functions in the broadhost-range plasmid pLS1. J Mol Biol 192:753–765
20. Maldonado-Barragán A, Caballero-Guerrero B, Lucena-Padrós H, Ruiz-Barba JL (2013) Induction of bacteriocin production by coculture is widespread among plantaricin-producing *Lactobacillus plantarum* strains with different regulatory operons. Food Microbiol 33:40–47
21. Mohedano ML, Overweg K, De la Fuente A, Reuter M, Altabe S, Mulholland F, De Mendoza D, López P, Wells J (2005) Inducible expression of the essential response regulator YycF in *Streptococcus pneumoniae* modulates expression of genes involved in fatty acid biosynthesis and affects membrane composition. J Bacteriol 178:2357–2367
22. Sambrook J, Russell DW (2001) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor
23. Tabasco R, García-Cayuela T, Peláez C, Requena T (2009) *Lactobacillus acidophilus* La-5 increases lactacin B production when it senses live target bacteria. Int J Food Microbiol 132:109–116
24. Van Baarlen P, Wells JM, Kleerebezem M (2013) Regulation of intestinal homeostasis and immunity with probiotic lactobacilli. Trends Immunol 34:208–215
25. Wang A, Yu H, Gao X, Li X, Qiao S (2009) Influence of *Lactobacillus fermentum* I5007 on the intestinal and systemic immune responses of healthy and *E. coli* challenged piglets. Antonie Van Leeuwenhoek 96:89–98
26. Wells JM (2011) Mucosal vaccination and therapy with genetically modified lactic acid bacteria. Ann Rev Food Sci Technol 2:423–445