Fluorescent protein vectors for promoter analysis in lactic acid bacteria and *Escherichia coli*

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Running title: mCherry based vectors for lactic acid bacteria

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Abstract

Fluorescent reporter genes are valuable tools for real-time monitoring of gene expression in living cells. In this study we describe the construction of novel promoter-probe vectors containing a synthetic mCherry fluorescent protein gene, codon-optimized for lactic acid bacteria, divergently linked, or not, to a gene encoding the S65T and F64L variant of the green fluorescent protein. The utility of the transcriptional fusion vectors was demonstrated by the cloning of a single or two divergent promoter regions and by the quantitative evaluation of fluorescence during growth of *Lactococcus lactis*, *Enterococcus faecalis* and *Escherichia coli*.

Keywords: Lactic acid bacteria, mCherry, GFP, divergent promoters, expression vectors.

Introduction

Lactic acid bacteria (LAB) are widely used in the production of fermented foods and beverages (Leroy and de Vuyst 2004). Some LAB strains exert beneficial effects on health, either directly via the live microbial cells or indirectly through the production of secondary metabolites with health-promoting properties (Gerritsen et al. 2011; Stanton et al 2005). *Lactococcus lactis* is commonly used in the manufacture of cheese, and it has been shown recently that this species can be genetically engineered and orally formulated to deliver therapeutic proteins and for use as living oral vaccines (Rottiers et al. 2009; Wells and Mercenier 2008; Wells 2011). LAB strains belonging to the genus *Enterococcus* are commensals of the intestinal tract of humans and other animals, and can produce enterocins active against pathogenic bacteria (Montalbán-López et al. 2011). However, some species, such as *E. faecalis,* can become opportunistic pathogens and can cause bacteraemia, endocarditis and urinary tract infection (Willems et al. 2011).

Due to the increasing scientific interest in LAB, there is a need for a wider range of genetic tools to facilitate their study, and particularly for the analysis of regulation of gene expression. A commonly occurring mechanism for the regulation of gene expression is by means of promoters that transcribe in opposite directions. For example, members of the LysR family of transcriptional regulators, which are representative of the most abundant type of prokaryotic transcriptional regulators, usually are divergently transcribed from their adjacent target genes (Maddocks and Oyston 2008). Analysis of the regulatory characteristics of divergent promoters can be greatly facilitated by fusing them to a pair of divergently oriented genes, which encode two reporter proteins that can be monitored simultaneously.

Fluorescent proteins are versatile *in vivo* reporters that can be used to study gene functionality and to tag proteins by fusion for cellular and subcellular localization in bacteria (Chudakov et al. 2005; Frommer et al. 2009; Fukuda et al. 2000). Vectors expressing variants of the green fluorescent protein (GFP) from jellyfish *Aequorea victoria* are now widely used as reporters in bacteria. Promoter probe vectors based on the streptococcal pMV158 plasmid and carrying the gene encoding the highly fluorescent GFP S65T and F64L mutant have been validated in *E. faecalis* (Ruiz-Cruz et al. 2010) and *L. lactis* (Fernández de Palencia et al. 2000). The use of the red fluorescent protein (RFP) from *Dicosoma* sp. in bacteria was initially hampered due to slow maturation and oligomerization as well as toxicity (Baird et al. 2000). However improved monomeric variants of RFP have been developed with better maturation and with over 10-fold increase in photo-stability. Of these mCherry is considered as one of the better alternatives (Shaner et al. 2004). The combination of mCherry with GFP is ideal for dual assays due to the minimal overlap of the fluorescence emission of each protein (Müller-Taubenberger and Anderson 2007).

In this study we describe the development of two promoter-probe shuttle vectors and derivatives carrying Gram-positive promoters. The vectors were specially designed for carrying out studies on gene expression and regulation in *L. lactis* and *E. faecalis*. We also show the utility of the vectors for real-time monitoring of gene expression and regulation during bacterial growth and for live cell imaging.

Materials and methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmid vectors used in this study are listed in Table 1. *L. lactis* and *E. faecalis* strains were cultured in M17 broth (Pronadisa, Madrid, Spain) containing 0.5% glucose (GM17) at 30°C. *Streptococcus pneumoniae* JNR7/87 was grown in AGCH medium (Lacks 1968) supplemented with 0.25% yeast extract (AGCHY) and 0.8% sucrose at 37ºC. *Lactobacillus plantarum* NCIMB8826 was cultured in MRS broth (Pronadisa) supplemented with 0.05% L-cysteine hydrochloride (Panreac, Barcelona, Spain) at 37ºC. *E. coli* strains were grown in Luria-Bertani broth at 37°C with vigorous shaking. When necessary, erythromycin (Em) and ampicillin (Amp) (Sigma-Aldrich, St Louis, MO, USA) were added to the culture medium: Em was used at a final concentration of 5 µg/ml for *L. lactis* and *E. faecalis*, and 250 µg/ml for *E. coli*; Amp was used at a final concentration of 5 and 150 µg/ml for *L. plantarum* and *E. coli*, respectively. Plate media were prepared by adding agar (Pronadisa) to liquid broth at a final concentration of 1.5%.

For fluorescence experiments, *L. lactis*, *E. faecalis* and *E. coli* strains were grown in a chemically defined medium (Otto et al. 1983; Poolman and Konings 1988) lacking riboflavin (CDM-riboflavin), and the pH was adjusted to 7.0 with 0.19 M 3-(N-morpholino)propanesulfonic acid (MOPS) (Sigma-Aldrich). The CDM was sterilized by passing through a 0.22µm pore-size filter (Sarstedt, La Roca del Vallès, Spain). For the induction of the expression of the two divergent non-overlapping promoter consensus sequences designated P_{kivD} and $P_{rmaF\text{-}rlrC}$ (De la Plaza et al., 2009), isoleucine was eliminated from the CDM-riboflavin (CDM-Ile). For the promoter repression experiments, 1.5% casitone (tryptic digest of casein) was added to the CDM-riboflavin instead of free amino acids (CDMK).

General DNA manipulation and transformation

Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). DNA restriction and modification enzymes were purchased from Fermentas (Vilnius, Lithuania) or New England BioLabs (Herts, United Kingdom) and used as recommended by the suppliers. The oligonucleotides used during this study were synthesized by Invitrogen (Paisley, United Kingdom) and are listed in Table 2. PCR amplifications (Table 2) were carried out on the PTC-100 Programmable Thermal Controller Thermocycler (MJ Research Inc., Watertown, MA, USA) by using Phusion High-Fidelity PCR DNA polymerase (Finnzymes, Vantaa, Finland). Samples for sequencing were prepared using QIAquick PCR Purification Kit (Qiagen) and analyzed by Secugen S.L. (Centro de Investigaciones Biológicas, Madrid, Spain). Transformation of chemically competent *E. coli* cells was carried out as described previously by Hanahan (1985). Electroporation of *L. lactis* and *E. faecalis* was performed according to the methods described by Holo and Nes (1989) and Fiedler and Wirth (1991), respectively.

Construction of pTL plasmids containing the *mrfp* and *gfp* genes

The promoter-probe vectors and expression plasmids constructed in this work are based on the pAK80 vector. This plasmid has two replication origins, one from the lactococcal plasmid pCT1138 and the other from the *E. coli* p15A plasmid. In addition, pAK80 contains a polylinker sequence and the *ermC* gene for erythromycin resistance (Em^R). A diagram of the plasmid constructions as well as a physical map of the vectors is depicted in Figure 1. All the primers used for amplification during plasmid constructions as well as for DNA sequencing of the inserts are shown in Table 2.

For the construction of pTLR and pTLR1, plasmid pAK80 was digested with both *Sma*I and *Sal*I. The resultant 7 kb DNA fragment, containing the two replication origins as well as the *erm* gene, was purified from a 0.8% agarose gel using the QIAquick Gel Extraction Kit (Qiagen). The *mrfp* gene encoding the mCherry was amplified from plasmid pTV-mCherry by using the specific primers FormRFP and RevmRFP containing the restriction sites *Sma*I and *Sal*I. The *mrfp* gene cloned in pTV-mCherry had been previously synthesized chemically with an optimized codon usage in order to enhance its translational efficiency in *L. lactis* and *L. plantarum* (García-Cayuela et al. 2011). The 0.8 kb amplicon containing the *mrfp* gene preceded by a ribosomal binding site (RBS) was purified, digested with *Sma*I and *Sal*I and ligated with T4 DNA ligase (Fermentas) to the 7 kb fragment from pAK80. The resulting plasmid named pTLR (7.8 kb) was established in chemically competent *E. coli* DH5α by transformation and selection for Em^R. The integrity of the cloned gene *mrfp* cassette was verified by DNA sequencing of pTLR by use of FormRFP and RevmRFP primers.

In order to evaluate the functional expression of mCherry in LAB, the strong P_X promoter of the *malXCD* operon (Nieto et al. 2001) from *S. pneumoniae* JNR7/87 (Bricker and Camilli, 1999) was cloned upstream of *mrfp* in pTLR, generating the transcriptional fusion Px–*mrfp* in pTLR1. A 0.5 kb amplicon was generated by use of chromosomal DNA of *S. pneumoniae* JNR7/87 as template and primers $ForP_X$ and $RevP_X$. After digestion of the amplicon with *Pst*I and *Bam*HI, it was introduced in the multicloning site of pTLR, after digestion with the same restriction enzymes. The ligation mixture was used to transform competent *E. coli* DH5α cells. The presence of the transcriptional fusion in pTLR1 was confirmed by DNA sequencing with primers $ForP_X$ and RevmRFP.

For the assessment of divergent promoter regions, the promoterless *gfp* gene from pGreenTIR carrying the F64L and S65T mutations was inserted into the vector pTLR for the construction of pTLGR. A 0.77 kb amplicon was generated from pGreenTIR plasmid with ForGFP and RevGFP primers. The amplicon was digested with both *Pst*I and *Xho*I and then ligated to plasmid pTLR cut with the same enzymes. The resulting plasmid was designated pTLGR and the integrity of the *gfp* cassette was verified by use of ForGFP and RevGFP primers for sequencing the plasmid.

In order to obtain simultaneous expression of both GFP and mCherry, a 0.28-kb DNA fragment of the *L. lactis* IFPL730 chromosome carrying the two divergent non-overlapping promoter consensus sequences designated P_{kivD} and $P_{rmaF\text{-}rlrC}$ (De la Plaza et al., 2009) were inserted at the unique *Bam*HI site of pTLGR, Chromosomal DNA of *L. lactis* IFPL730 was amplified with primers ForP_{KivD} and RevP_{KivD}. The presence of the transcriptional fusions $P_{rmaF\text{-}rlrC}$–*gfp* and P_{kivD}–*mrfp* in pTLGR11 and $P_{rmaF\text{-}rlrC}$–*mrfp* and P_{kivD}–*gfp* in pTLGR12 were confirmed by sequencing pTLGR-derivative vectors with primers ForP_{KivD}, RevP_{KivD}, RevmRFP and RevGFP.

Simultaneous determination of cell growth and fluorescence

All measurements were conducted in sterile 96-well optical bottom microplates (Nunc, Rochester, NY, USA) with a final assay volume of 300 µl/well by using the microtiter plate assay system Varioskan Flash (Thermo Fisher Scientific, Waltham, MA, USA). All strains harboring plasmids were grown to an optical density at 480 nm (OD_{480}) of 1.0. Cells were harvested by centrifugation (4,000 ×*g* for 10 min at 4ºC), washed twice with and resuspended in sterile saline solution (0.85% NaCl), and inoculated (10%) into CDM-riboflavin (pH 7.0) medium. The microplates were

incubated for 22 h at 30°C for *L. lactis* and at 37ºC for *E. faecalis* and *E. coli*. Measurements were made at 1 h intervals. During cultivation, the Varioskan Flash simultaneously provided quantitative online data of (i) cell density via measuring OD_{480} and (ii) *in vivo* mCherry and GFP expressions: mCherry fluorescence was measured at an excitation wavelength of 587 nm and an emission wavelength of 612 nm, whereas GFP fluorescence was monitored at 511 nm upon excitation at 488 nm. Background fluorescence of the control strains (harboring plasmids without the fluorescence genes) was used to normalize the fluorescence signals during cultivations.

Detection of the mCherry expression and the binding of bacteria to Caco-2 cells by confocal laser scanning microscopy

To detect the mCherry expression, strains carrying pTLR1 were grown in CDM-riboflavin medium to an OD_{480} of 0.6. Cells were sedimented, washed, and resuspended to half of the original volume in PBS buffer (10 mM Na_2HPO_4, 1mM KH_2PO_4, 140 mM NaCl, 3 mM KCl) at pH 8. Cells were directly analyzed, without fixing, by confocal laser scanning microscopy (CLSM), using a Leica AF6000 LX-DMI6000B model microscope (Leica Microsystems GmbH, Wetzlar, Germany). Confocal illumination was provided with a ×100 objective and numerical aperture of 1.6. Image analysis was performed using FRET and FRAP software (Leica Microsystems GmbH).

The *E. coli* DH5α[pGreenTIRGFP] strain expressing the GFP protein as well as *L. lactis* MG1363[pTLR1] and *E. faecalis* JH2-2[pTLR1] expressing the mCherry protein were detected by their fluorescence in co-cultures with human epithelial cells. The assays were performed as described previously (Fernández de Palencia et al. 2008; Garai-Ibabe et al. 2010). Briefly, bacteria were grown to an OD_{620} of 0.6. Then, 1 ml of

each culture was harvested by centrifugation (12,000 ×*g* for 10 min at 4ºC), washed twice with and resuspended in 0.5 ml of PBS buffer at pH 8. The Caco-2 cells (CIB cell bank, Madrid, Spain) were grown in Men-Alpha Medium (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum, at 37ºC in an atmosphere containing 5% CO_2 to obtain a monolayer of differentiated and polarized cells. Confluent Caco-2 cells were exposed to the indicated bacteria (ratio 1:100) for 1 h at 37ºC. After the incubation time, unbound bacteria were removed by washing three times with PBS at pH 8.0. Samples were inspected after washing by CLSM as indicated above.

Results

Vectors containing the monomeric red autofluorescent protein mCherry coding gene

A promoter-probe (pTLR) and an expression (pTLR1) shuttle vector carrying the *mrfp* gene were constructed (Fig. 1) and tested. The vectors also contained an erythromycin resistance gene (*erm*) for plasmid maintenance and selection of the transformants in Gram-positive and Gram-negative bacteria. In order to use the mCherry protein as a promoter-probe marker, the β-galactosidase (*lacL* and *lacM*) genes of pAK80 were replaced in pTLR by a promoterless, synthetic *mrfp* gene (García-Cayuela et al. 2011), that had been codon optimized to increase expression of mCherry in LAB. In addition, a polylinker sequence including *Bgl*II, *Xho*I, *Pst*I, *Bam*HI and *Sma*I located upstream of the *mrfp* gene, was available in pTLR for cloning of DNA fragments containing transcriptional promoters (Fig. 1). The vector pTLR was established in *E. coli* DH5α by transformation and selected by erythromycin resistance.

In order to evaluate the functional expression of mCherry in LAB, the strong P_X promoter of the *malXCD* operon from *S. pneumoniae* (Nieto et al. 2001) was cloned upstream of *mrfp* in pTLR to generate pTLR1. P_X drives constitutive gene expression in the absence of the pneumococcal MalR regulator in heterologous bacterial hosts (Nieto et al. 2001). The plasmid was established in *E. coli* DH5α and then used to transform *L. lactis* MG1363 and *E. faecalis* JH2-2 by electroporation and selected by erythromycin. Bacteria harboring pTLR1 were easily detected on agar plates, since they generated colonies with distinctive colors, bright purple for *E. coli*, pale pink for *L. lactis* and pink for *E. faecalis* (Fig. 2A). The functional expression of mCherry under control of the P_X promoter and the increase of biomass during cell growth was monitored in real time for the three bacterial species harboring pTLR1. Detection of fluorescence during growth of the strains was aided by the lack of intrinsic fluorescence of the CDM-riboflavin medium, (riboflavin was found not to be essential for their growth). The results revealed that the mCherry fluorescence increased in parallel with OD_{480} during the exponential phase of growth of both LAB strains, whereas for *E. coli* levels of fluorescence mainly increased during stationary phase (Fig. 2B). The expression of mCherry during the logarithmic phase of bacterial growth was also examined by confocal laser scanning microscopy (CLSM). All bacteria were visualized as bright red cells (Fig. 2C).

Assays of bacterial capability to adhere to Caco-2 cells were performed in order to visualize by CLSM the expression of mCherry in adhered bacterial cells and to differentiate them in mixed cultures from bacteria expressing GFP. CLSM images (Fig. S1 in the supplemental material) showed that both GFP and mCherry fluorescences were expressed in the Caco-2 co-cultures and it was possible to distinguish between cells of *E. coli* expressing GFP and *L. lactis* and *E. faecalis* carrying pTLR1.

Vectors for expression of the mCherry and the GFP S65T F64L fluorescent proteins

A promoter-probe (pTLGR) and two expression (pTLGR11 and pTLGR12) shuttle vectors carrying the genes encoding mCherry and the GFP S65T F64L were constructed (Fig. 1) and tested. The promoterless *gfp* gene from pGreenTIR with the F64L and S65T mutations (Miller and Lindow 1997) was inserted into the vector pTLR in a divergent orientation to the mCherry coding gene for the construction of pTLGR (Fig. 1). The plasmid retained a polylinker sequence with *Pst*I, *Bam*HI and *Sma*I as available cloning sites. Simultaneous expression of both GFP and mCherry was evaluated by inserting, at the unique *Bam*HI site of pTLGR, the *L. lactis* IFPL730 chromosomal region located between the *kivD* gene (De la Plaza et al. 2009) and the upstream divergent *rmaF* and *rlrC* gene cluster, which encode two putative transcriptional regulators. This region contains two divergent non-overlapping promoter consensus sequences designated P_{kivD} and $P_{rmaF\text{-}rlrC}$ (De la Plaza et al. 2009). Plasmids pTLGR11 and pTLGR12, carrying, respectively, $P_{rmaF\text{-}rlrC}$–*gfp*, P_{kivD}–*mrfp* and $P_{rmaF\text{-}rlrC}$–*mrfp*, P_{kivD}-*gfp* transcriptional fusions (Fig. 1) were established in *E. coli*. Clones carrying pTLGR11 were easily identified by the maroon color of the colonies whereas clones carrying pTLGR12 appeared brownish-red (results not shown). After transfer of pTLGR11 and pTLGR12 to *L. lactis* and *E. faecalis*, expression of GFP and mCherry proteins was detected in all bacterial strains and could be simultaneously quantified by measuring fluorescence during growth in CDM-riboflavin medium. Table 3 shows the levels of fluorescence related to OD_{480} at exponential and stationary phases of growth. In the three strains, GFP and mCherry were detected by their autofluorescence. The fluorescence of mCherry in *L. lactis* MG1363[pTLGR11] were 4 times higher than that of GFP. In contrast, the opposite situation was observed in *L. lactis*

MG1363[pTLGR12] where levels of GFP were on average 40-fold higher than mCherry. In *E. faecalis*, however, GFP levels were always higher than that of mCherry, independently of the P_{kivD} and $P_{rmaF\text{-}rlrC}$ orientation. In *E. coli* the levels of both active proteins reached similar fluorescence values (Table 3).

Regulatory analyses of divergent promoters

Previous studies had shown that *kivD* transcription in *L. lactis* is specifically affected by isoleucine and peptide contents in the growth medium (De la Plaza et al., 2009). Thus, to further evaluate the application of pTLGR for the study of regulated divergent promoters, the fluorescence of *L. lactis* MG1363[pTLGR11] and MG1363[pTLGR12] were monitored during growth in CDM-riboflavin without isoleucine (CDM-Ile) and in CDM-riboflavin with casitone instead of free amino acids (CDMK) (Table 4). During growth of MG1363[pTLGR11] in CDM-Ile and CDMK, higher levels of mCherry were detected than those of GFP, independently of the growth phase of the cultures. This result indicated that P_{kivD} is a stronger promoter than $P_{rmaF\text{-}rlrC}$ in its natural host. This hypothesis was confirmed by the fact that in MG1363[pTLGR12] levels of GFP were higher than those of mCherry. In addition, the absence of isoleucine in the growth medium (CDM-Ile) caused an average increase of about 5-fold for mCherry fluorescence in MG1363[pTLGR11] and of 3-fold for GFP in MG1363[pTLGR12], when compared with their levels of growth in CDMK.

Discussion

In this study we have described the construction and application of two promoter-probe shuttle vectors and derivatives carrying Gram-positive promoters. The

constructs were based on pAK80 (Israelsen et al. 1995), which carries the pCT1138 replicon functional in *L. lactis* and *E. faecalis* and the replicon of the *E. coli* p15A plasmid.

Firstly, we developed a promoter-probe (pTLR) carrying the *mrfp* gene, which had been previously codon optimized to increase its expression in LAB (García-Cayuela et al. 2011). Then, the strong P_X promoter of the *malXCD* operon (Nieto et al. 2001) was introduced into pTLR to generate the expression pTLR1 shuttle plasmid, in order to evaluate the functional expression of mCherry in LAB (Fig. 2). The expression of mCherry in *L. lactis* and *E. faecalis* as well as in *E. coli* harboring pTLR1 was detected by the colony color produced on plates, and real-time fluorescence evolved in parallel to the growth of the strains. Therefore, pTLR is a suitable promoter-probe vector to validate transcriptional signals in *E. coli*, *L. lactis* and *E. faecalis* and can be used to evaluate promoter strength in LAB. In addition, bacteria carrying pTLR1 and expressing mCherry were easily differentiated from bacteria expressing GFP in mixed cultures with Caco-2 cells (Fig. S1). These results demonstrate the utility of the plasmid to study *L. lactis* and *E. faecalis* interactions with eukaryotic cells and to carry out competition assays with GFP tagged bacteria. The advantage of combining GFP and mCherry as fluorescent markers for imaging mixed bacterial populations is that they can be co-visualized in the same microscopy image, whereas GFP fluorescence overlaps with the channels used to detect other proteins such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (Pereira et al. 2010). Combination of GFP and mCherry to perform live-bacteria imaging has been applied to the study of microbial interactions such as formation of mixed-bacteria biofilms (Lagendijk et al. 2010; Tolker-Nielsen et al. 2000). However, as far as we know, this is the first study reporting the expression of mCherry protein in LAB.

Furthermore, the promoterless GFP S65T F64L coding gene was inserted into the pTLR vector in a divergent orientation to the mCherry coding gene for the development of a promoter-probe (pTLGR) shuttle vector carrying two fluorescent reporters, GFP and mCherry (Fig. 1). The pTLGR vector was tested, after generation of two expression (pTLGR11 and pTLGR12) plasmids carrying the divergent non-overlapping P_{kivD} and $P_{rmaF\text{-}rlrC}$ promoters (which are located between the *kivD* gene and the upstream divergent *rmaF* and *rlrC* gene cluster in *L. lactis* IFPL730 (De la Plaza et al. 2009). All clones carrying pTLGR11 and pTLGR12 were easily identified by the different coloration of the colonies. In all bacterial strains, GFP and mCherry were detected by their autofluorescence and could be simultaneously quantified during growth showing the functionality of both promoters, not only in *L. lactis*, but also in *E. coli* and *E. faecalis* (Table 3). The incorporation of MOPS buffer into the medium maintained the cultures at neutral pH, thereby preventing the decreasing to 50% of maximal GFP fluorescence intensity at pH 6.0 (Kneen et al. 1998), as previously reported for *L. lactis* (Fernández de Palencia et al. 2000). On the other hand, the protein mCherry is considerably more acid-tolerant (Lagendijk et al. 2010). Hence, the results showed that pTLGR is a suitable vector to test functionality of divergent promoters in all three species. For the characterization of this type of promoter, the expression seems to depend on the specific regulatory factors of the promoter natural host.

In order to further develop the application of pTLGR, the fluorescence of *L. lactis* MG1363[pTLGR11] and MG1363[pTLGR12] carrying, respectively, $P_{rmaF\text{-}rlrC}$–*gfp*, P_{kivD}–*mrfp* and $P_{rmaF\text{-}rlrC}$–*mrfp*, P_{kivD}-*gfp* transcriptional fusions were monitored during growth in media with differences in amino acid content (Table 4). The results confirmed that transcriptional regulation of *kivD* is specifically affected by isoleucine and peptide contents in the growth medium, as previously demonstrated by Northern

hybridization analyses and determination of ketoisovalerate decarboxylase activity in *L. lactis* IFPL730 (De la Plaza et al., 2009). These results also revealed a previously unknown regulation of gene expression from $P_{rmaF\text{-}rlrC}$. Furthermore, the results indicated that regulatory analyses of divergent promoters with pTLGR would function regardless of the orientation of the promoter.

In conclusion, pTLR and pTLGR are suitable promoter-probe vectors for characterization of single and two divergent promoters, respectively, and may be used in *L. lactis*, *E. faecalis* and *E. coli*. Detection of transformants carrying vectors containing functional promoters is greatly facilitated by the appearance of colored colonies. Moreover, their use could allow real-time detection of the regulation of gene expression by different effectors or environmental conditions. To our knowledge this is the first published study reporting the expression of the mCherry protein as well as construction and testing of a divergent-promoter probe vector in LAB. Although these vectors are useful cloning tools, since they can be propagated in *E. coli*, they have been principally designed for studying divergently-arranged gene regions in *L. lactis* and *E. faecalis*.

Acknowledgements

This work was supported by the Spanish Ministry of Science and Innovation (grants AGL2009-12998-C03-01, AGL2009-13361-C02-02 and CSD2007-00063 Consolider Ingenio 2010 FUN-C-FOOD), the Comunidad de Madrid (grant ALIBIRD P2009/AGR-1469), and the European Union VII framework program (Initial Training Network, grant N° 238490).

We thank Dr. Stephen Elson for the critical reading of the manuscript. We are grateful to M. Angeles Corrales for her technical assistance.

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Legends to the figures

Figure 1. Schematic diagram showing the construction of pTLR and pTLGR and its derivatives. For details, see Materials and methods. Relevant restriction sites are shown. Specific genes are: *mrfp*, *gfp* and *erm* that encode mCherry, GFP and the protein responsible for the resistance to erythromycin, respectively. Promoters: $P_{X,}$ promoter of the pneumococcal *malXCD* operon; $P_{rmaF\text{-}rlrC}$-P_{kivD}, intergenic region between *rmaF* and *kivD* genes of *Lactococcus lactis* IFPL730.

Figure 2. Detection of expression of mCherry encoded by pTLR1 in *E. coli*, *L. lactis* and *E. faecalis* strains. (A) On Luria-Bertani agar for *E. coli* and on M17-agar containing 0.5% glucose for LAB strains. (B) In CDM medium lacking riboflavin. The growth of cultures was monitored at a wavelength of 480 nm (□). Fluorescence emission of mCherry was recorded at 612 nm (◊) after excitation at a wave length of 587 nm during exponential and stationary phases. (C) Cells were directly analyzed, without fixing, by confocal laser scanning microscopy. Confocal illumination was provided with a ×100 objective and numerical aperture of 1.6 and by fluorescent light mercury lamp with long passes filters for red emissions.

Table 1. Strains and plasmids used in this study

Strains and plasmids	Relevant characteristic(s)	Source or reference
Bacterial strains		
Enterococcus faecalis JH2-2	Host strain for cloning	(Jacob and Hobbs 1974)
E. faecalis JH2-2 [pTLR1]	Contains pTLR1plasmid; Em^R	This study
E. faecalis JH2-2 [pTLGR11]	Contains pTLGR11 plasmid; Em^R	This study
E. faecalis JH2-2 [pTLGR12]	Contains pTLGR12 plasmid; Em^R	This study
Escherichia coli DH5α	Host strain for cloning	(Sambrook and Russell 2001)
E. coli DH5α [pGreenTIRGFP]	Contains pGreenTIRGFP plasmid; Amp^R	(Miller and Lindow 1997)
E. coli DH5α [pTLR]	Contains pTLR plasmid; Em^R	This study
E. coli DH5α [pTLR1]	Contains pTLR1plasmid; Em^R	This study
E. coli DH5α [pTLGR]	Contains pTLGR plasmid; Em^R	This study
E. coli DH5α [pTLGR11]	Contains pTLGR11plasmid; Em^R	This study
E. coli DH5α [pTLGR12]	Contains pTLGR12plasmid; Em^R	This study
Lactobacillus plantarum NCIMB8826	Contains pTV-mCherry plasmid; Amp^R	NCIMB[a]
Lactococcus lactis IFPL730	Contains two divergent non-overlapping promoter consensus sequences (P_{kivD} and $P_{rmaF\text{-}rlrC}$)	(De la Plaza et al. 2009)
L. lactis MG1363	Host strain for cloning	(Gasson 1983)
L. lactis MG1363[pTLR1]	Contains pTLR1plasmid; Em^R	This study
L. lactis MG1363[pTLGR11]	Contains pTLGR11plasmid; Em^R	This study
L. lactis MG1363[pTLGR12]	Contains pTLGR12plasmid; Em^R	
Streptococcus pneumoniae JNR7/87	Contains the P_X promoter from *malXCD* operon	(Bricker and Camilli 1999)
Plasmids		
pTV-mCherry	Plasmid containing *mrfp* gene; Amp^R	NCIMB[a]
pGreenTIRGFP	Plasmid containing *gfp* gene; Amp^R	(Miller and Lindow 1997)
pAK80	Cloning vector; Em^R	(Israelsen et al. 1995)
pTLR	pAK80 derivative containing *mrfp* from pTV-mCherry; Em^R	This study
pTLR1	pTLR derivative containing the promoter P_X; Em^R	This study
pTLGR	pTLR derivative containing *gfp* gene from pGreenTir; Em^R	This study
pTLGR11	pTLGR derivative containing $P_{rmaF\text{-}rlrC}$–*gfp* and P_{kivD}–*mrfp* transcriptional fusions; Em^R	This study
pTLGR12	pTLGR derivative containing $P_{rmaF\text{-}rlrC}$–*mrfp* and P_{kivD}-*gfp* transcriptional fusions; Em^R	This study

[a] NCIMB: National Collections of Industrial and Marine Bacteria, Aberdeen, UK.

Table 2. Oligonucleotides and PCR conditions.

Target	Primer	Sequence[a] 5′→3′	Restriction enzymes	PCR cycle conditions		
				Annealing	Extension at 72ºC	Product size (pb)
mrfp	FormRFP	AAAA**CCCGGG**GGATACGCACGAGTTTCAA	*Sma*I	Cycles (1-20): 47°C, 10 s Cycles (21-30): 68°C, 30 s	60 s	780
	RevmRFP	CGGCGCG**GTCGAC**TTATTTATATAATAATTCGTCC	*Sal*I			
gfp	ForGFP	CCGC**CTGCAG**TTCTGATTAACTTTATAAGGAGGA	*Pst*I	Cycles (1-20): 45°C, 10 s Cycles (21-30): 57°C, 60 s Cycles (31-40): 60°C, 60 s	60 s	768
	RevGFP	CCG**CTCGAG**CCTATTTGTATAGTTCATCCATGCC	*Xho*I			
P_X	ForP_X	AT**CTGCAG**CGTGTTAAAATAATGGAACGT	*Pst*I	Cycles (1-20): 50.5 °C, 10 s Cycles (21-30): 60°C, 60 s Cycles (31-40): 63°C, 60 s	60 s	529
	RevP_X	AT**GGATCC**CCCCAAAGAATAGCAAGTTTTATTG	*Bam*HI			
$P_{rmaF\text{-}rlrC}$-P_{kivD}	ForP_{KivD}	AGC**GGATCC**CCGAAGTAAAATAAAGCCAAATC	*Bam*HI	Cycles (1-20): 53°C, 30 s Cycles (21-30): 63°C, 30 s	30 s	281
	RevP_{KivD}	AGC**GGATCC**TTTCTTCAATTCCTAACTCGTGTAA	*Bam*HI			

[a]Restriction enzyme sites are indicated with bold letters

Table 3. Evaluation of simultaneous expression of GFP and mCherry fluorescence during growth in chemically defined medium.

Strain	Incubation Time	Growth[a] OD_{480}	Fluorescence[a]		Ratio Fluorescence/OD	
			GFP	mCherry	GFP	mCherry
E. coli DH5-α[pTLGR11]	T1	0.59 ± 0.13	105.04 ± 1.48	103.08 ± 5.29	179.2	175.9
	T2	1.19 ± 0.05	112.26 ± 7.25	102.85 ± 2.78	94.7	86.8
	T3	1.21 ± 0.00	124.87 ± 8.93	121.80 ± 2.65	103.5	101.0
E. coli DH5-α[pTLGR12]	T1	0.63 ± 0.3	37.69 ± 3.74	110.69 ± 11.48	59.6	174.9
	T2	1.11 ± 0.04	63.47 ± 0.18	107.56 ± 5.93	57.4	97.3
	T3	1.15 ± 0.06	65.41 ± 0.83	112.01 ± 10.59	56.9	97.5
E. faecalis JH2-2[pTLGR11]	T1	0.49 ± 0.08	202.88 ± 4.00	30.17 ± 2.66	418.2	62.2
	T2	0.93 ± 0.07	429.51 ± 9.66	58.44 ± 2.89	460.3	62.6
	T3	0.93 ± 0.01	431.76 ± 22.70	84.98 ± 6.17	463.3	91.2
E. faecalis JH2-2[pTLGR12]	T1	0.51 ± 0.00	117.59 ± 10.15	6.95 ± 0.04	231.2	13.7
	T2	0.92 ± 0.01	231.96 ± 16.89	15.74 ± 0.26	251.0	17.0
	T3	0.89 ± 0.02	235.09 ± 13.81	21.81 ± 0.80	263.8	24.5
L. lactis MG1363[pTLGR11]	T1	0.55 ± 0.06	8.56 ± 3.39	29.52 ± 0.29	15.7	54.1
	T2	1.00 ± 0.07	8.83 ± 3.56	30.63 ± 0.66	8.9	30.8
	T3	0.81 ± 0.01	9.54 ± 3.55	41.20 ± 1.02	11.8	51.1
L. lactis MG1363[pTLGR12]	T1	0.48 ± 0.15	404.8 ± 18.81	10.57 ± 7.44	837.7	21.9
	T2	0.83 ± 0.02	822.84 ± 13.15	13.33 ± 9.97	990.6	16.0
	T3	0.76 ± 0.01	740.89 ± 127.62	25.00 ± 2.33	970.3	32.7

[a]Growth (OD_{480}) and fluorescence were assayed at the middle of the exponential (T1) as well as early (T2) and late (T3) stationary phases of growth. Fluorescence of GFP was determined by excitation at 488 nm and detection at 511 nm, and fluorescence of mCherry was detected at 612 nm after excitation at 587 nm. Values are mean ± SD from at least two independent experiments and analyzed in triplicate.

Table 4. Evaluation of simultaneous expression of GFP and mCherry fluorescence during growth in chemically defined medium without isoleucine (CDM-Ile) and CDM with casitone instead of free amino acids (CDMK).

Strain	Medium	Incubation Time	Growth[a] OD_{480}	Fluorescence[a]		Ratio Fluorescence/OD	
				GFP	mCherry	GFP	mCherry
L. lactis MG1363[pTLGR11]	CDM-Ile	T1	0.23 ± 0.01	4.59 ± 0.29	28.36 ± 0.70	19.7	121.5
		T2	0.61 ± 0.02	46.21 ± 1.04	83.43 ± 4.88	76.1	137.4
		T3	0.54 ± 0.01	65.65 ± 1.47	208.07 ± 8.95	119.8	379.5
L. lactis MG1363[pTLGR12]	CDM-Ile	T1	0.29 ± 0.01	341.91 ± 16.37	10.48 ± 0.31	1189.9	36.5
		T2	0.67 ± 0.01	1614.63 ± 21.64	27.49 ± 1.55	2418.5	41.2
		T3	0.60 ± 0.01	1865.00 ± 58.99	100.36 ± 2.99	3104.3	167.1
L. lactis MG1363[pTLGR11]	CDMK	T1	0.67 ± 0.02	4.32 ± 0.16	25.14 ± 0.77	6.5	23.9
		T2	1.15 ± 0.02	10.07 ± 0.23	40.40 ± 1.13	8.8	35.2
		T3	1.03 ± 0.01	11.42 ± 0.37	52.29 ± 1.14	11.4	52.3
L. lactis MG1363[pTLGR12]	CDMK	T1	0.56 ± 0.02	347.11 ± 2.04	7.98 ± 0.41	621.1	14.3
		T2	1.06 ± 0.03	936.32 ± 8.11	14.00 ± 0.70	886.8	13.3
		T3	0.97 ± 0.01	902.75 ± 13.45	21.29 ± 0.90	933.7	22.0

[a]Growth (OD_{480}) and fluorescence were assayed during exponential (T1) as well as early (T2) and late (T3) stationary phases of cultures growth. Fluorescence of GFP was determined by excitation at 488 nm and detection at 511 nm, and fluorescence of mCherry was detected at 612 nm after excitation at 587 nm. Values are mean ± SD from at least two independent experiments and analyzed in triplicate.

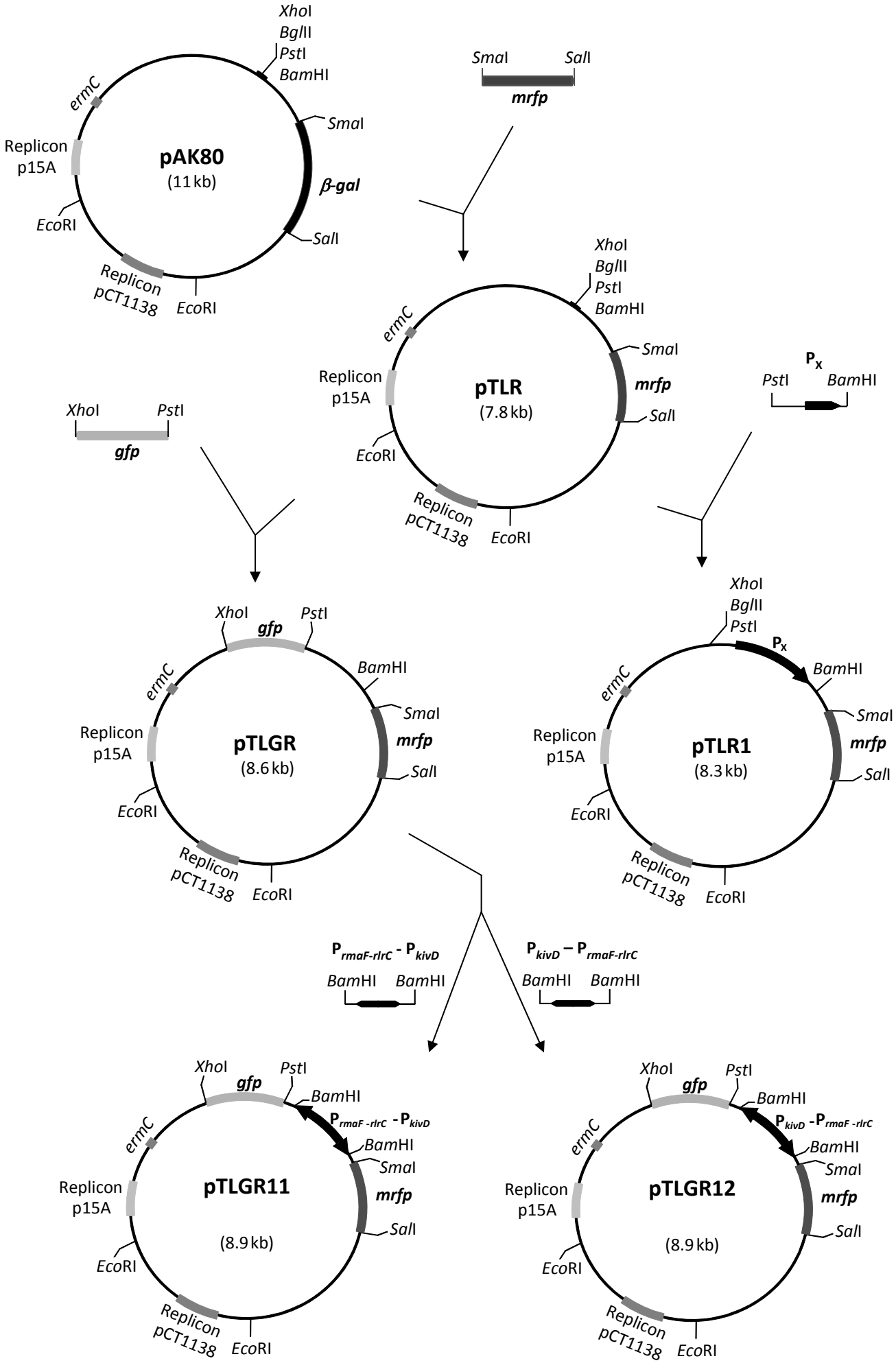
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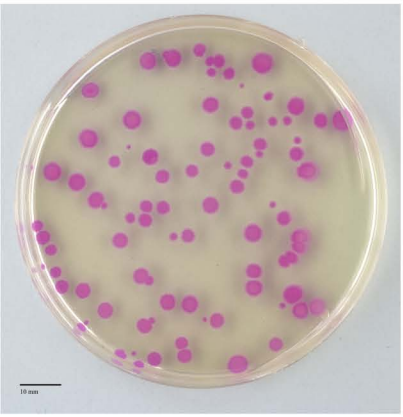
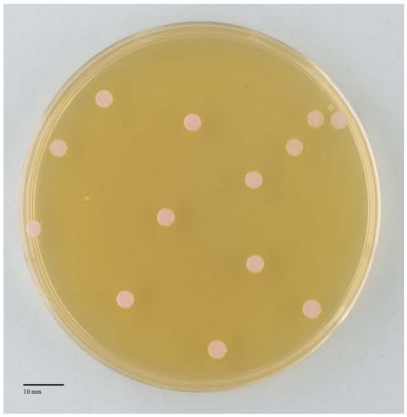
Figure 2

A





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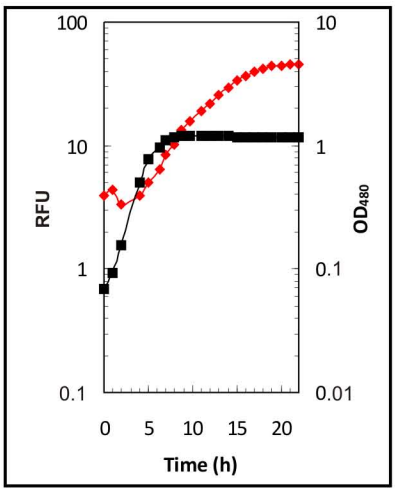

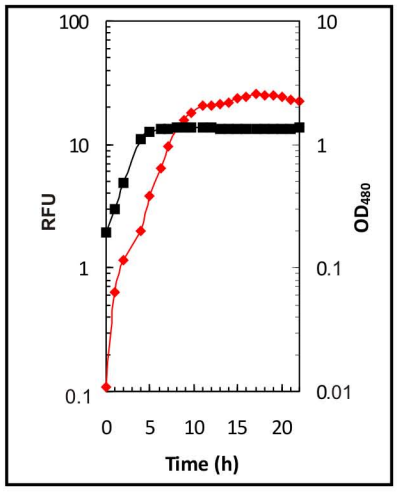

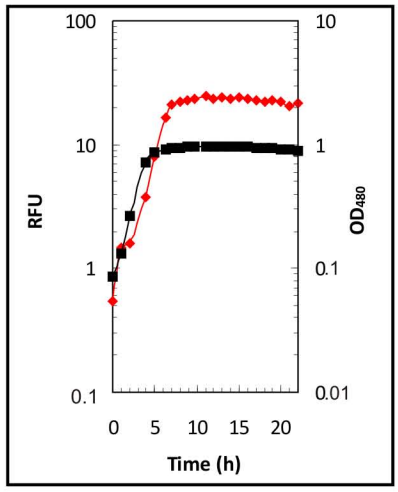

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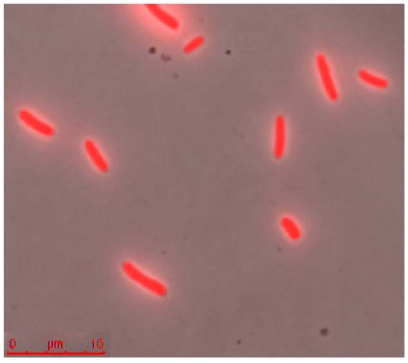

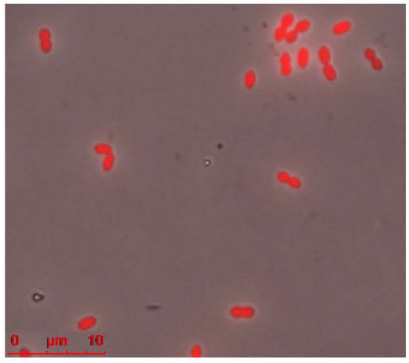

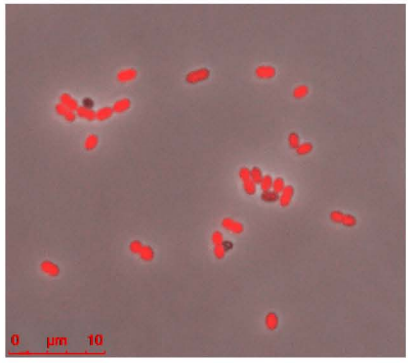

E. coli **DH5α[pTLR1]** ***E. faecalis*****[pTLR1]** ***L. lactis*** **MG1363[pTLR1]**

Supplementary Material

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