Comparison of expression vectors in *Lactobacillus reuteri* strains

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Abstract

The synthesis of heterologous proteins in lactobacilli is strongly influenced by the promoter selected for the expression. In addition, the activity of the promoters themselves may vary among different bacterial hosts. Three different promoters were investigated for their capability to drive enhanced green fluorescent protein (EGFP) expression in *Lactococcus lactis* spp. *cremoris* MG1363, in *Lactobacillus reuteri* DSM 20016^T and in five *L. reuteri* strains isolated from chicken crops. The promoters of the *Lactobacillus acidophilus* surface layer protein gene (*slp*), *L. acidophilus* lactate dehydrogenase gene (*ldhL*) and enterococcal rRNA adenine *N*-6-methyltransferase gene (*ermB*) were fused to the coding sequence of EGFP and inserted into the backbone of the pTRKH3 shuttle vector (pTRKH3-*slp*GFP, pTRKH3-*ldh*GFP, pTRKH3-*erm*GFP). Besides conventional analytical methods, a new quick fluorimetric approach was set up to quantify the EGFP fluorescence in transformed clones using the Qubit^{TM} fluorometer. *ermB* proved to be the most effective promoter in *L. reuteri* isolates, producing 3.90×10^{-7} g of fluorescent EGFP $(mL\,OD_{stationary\ culture})^{-1}$. Under the same conditions, the *ldhL* promoter produced 2.66×10^{-7} g of fluorescent EGFP $(mL\,OD_{stationary\ culture})^{-1}$. Even though the *slp* promoter was efficient in *L. lactis* spp. *cremoris* MG1363, it was nearly inactive both in *L. reuteri* DSM 20016^T and in *L. reuteri* isolates.

Introduction

During the last decade, the use of lactic acid bacteria (LAB) as vehicles to deliver heterologous antigens has been intensively studied and its possible application to induce immunity to specific antigens, i.e. to 'vaccinate' the host, has raised increasing interest (Cortes-Perez *et al.*, 2005; Ho *et al.*, 2005; Mota *et al.*, 2006; Hou *et al.*, 2007; Ferreira *et al.*, 2008; Mohamadzadeh *et al.*, 2009). Many commensal LABs are considered 'generally recognized as safe' (Adams & Marteau, 1995) and this represents an important advantage for their potential use as live therapeutic vehicles (Mercenier, 1999). Such approaches would be convenient in the mass treatment of farm animals and in particular in chicken breeding, a field facing huge infective emergencies (such as avian flu, with potential zoonotic risks) and where the cost of classic vaccinal procedures heavily influences the earnings of the farm.

Our study focuses on the possibility to obtain engineered bacterial strains able to express high levels of heterologous proteins, starting from *Lactobacillus* strains normally inhabiting the chicken crop.

Young animals are the target for a forced colonization of the crop to cause an immunostimulation by LABs expressing heterologous proteins. In our study, we have chosen to perform our transformation experiments in *Lactobacillus reuteri* strains isolated from the crop because it is the dominant lactobacilli population in young chickens; the presence of *L. reuteri* gradually decreases and is replaced by *Lactobacillus salivarius* during the chicken growth (Guan *et al.*, 2003; Abbas Hilmi *et al.*, 2007). *Lactobacillus reuteri* is a common heterofermentative and fast-growing inhabitant of the digestive tract of vertebrates.

One of the key factors for the successful expression of heterologous proteins in bacteria is the choice of an effective promoter. Studies on constitutive promoters to express the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* or other antigens in *L. reuteri* strains have not yet been described. In previous reports, only nisin-inducible expression vectors were used to express GFP (Wu & Chung, 2006) or GFP:STLT$_B$ (a fusion protein between GFP and the heat-stable enterotoxin ST and heat-labile enterotoxin B LT$_B$ of the enterotoxigenic *Escherichia coli*, ETEC) (Wu &

Chung, 2007) in *L. reuteri* strains. To induce a successful mucosal immune response in the host, both the amount and the persistence of the antigen are critical factors. In the study described by Wu & Chung, the GFP:STLT$_B$ protein secreted by their *L. reuteri* was expressed at a high level during 3 h after the *L. reuteri* had been induced by nisin and orally inoculated in mice, but after that, only a basal amount of protein was predicted to be produced, from the *in vitro* estimation. For this reason, the expression of antigens using constitutive promoters could be an effective alternative.

To test the effectiveness of different expression vectors in crop-derived *L. reuteri* strains, we compared the expression of the *gfp* gene under the control of three constitutive promoters: the lactate dehydrogenase (*ldlL*) promoter from *Lactobacillus acidophilus* (Kim *et al.*, 1991), which is reported to be a highly efficient promoter, the surface (S)-layer protein (*slp*) promoter from *L. acidophilus*, responsible for the high level of transcription of stable mRNAs coding the S-protein monomers (Boot & Pouwels, 1996; Boot *et al.*, 1996) and the erythromycin ribosomal methylase (*ermB*) promoter from the broad-host range plasmid pAMβ1 isolated from *Enterococcus faecalis* (Swinfield *et al.*, 1990).

Because the S-layer proteins represent up to 10–15% of the total protein content of an S-layer-carrying bacterial cell (Boot *et al.*, 1996), the expression and secretion signals of S-layer protein genes have a potential for the construction of efficient vectors to display antigens on the cell surface of LABs (Avall-Jaaskelainen *et al.*, 2002; Mota *et al.*, 2006). Besides these important features of the S-layer proteins, we considered it essential to evaluate the activity of their promoter in *L. reuteri* by comparison with those of *ldhL* and *ermB*.

The activity of the vectors bearing these different promoters was tested in reference strains of *Lactococcus lactis*, *L. reuteri* and in five selected strains of *L. reuteri*, isolated from chicken crop, using a rapid method to detect the GFP fluorescence using the Qubit™ fluorometer (Invitrogen, Milan, Italy), besides the classical direct observation by epifluorescence microscopy and Western blot analysis.

Materials and methods

Bacterial strains, isolation and identification of indigenous lactobacilli from chicken crop

Lactococcus lactis spp. *cremoris* MG1363 (Gasson, 1983) was cultured in GM17 medium (M17 medium supplemented with 0.5% glucose) (Merck KGaA, Darmstadt, Germany) at 30 °C in aerobiosis and *L. reuteri* DSM 20016^T was cultured in MRS medium (Oxoid, Cambridge, UK) in anaerobiosis at 37 °C. Lactobacilli were isolated by plating on Rogosa agar (Merck KGaA) from 12 chicken crops obtained from two different chicken farms (seven and five chickens, respectively). The first sampling was performed by collecting crops

from a commercial plant where a stock of fowls from a commercial breeder was under slaughtering. The second set of samples was obtained from an experimental facility where a stock of commercial pullet had been grown under standard conditions. All the chickens were sacrificed at the age of 8 weeks. *Lactobacillus* isolates were cultured in MRS broth and identified to the species level by PCR-ARDRA on the 16S–23S rRNA gene spacer region (Tilsala-Timisjarvi & Alatossava, 1997; Moreira *et al.*, 2005). Uncertain identifications were confirmed by sequencing of 16S rRNA gene. Lactobacilli and *L. lactis* transformants were selected with 10 and 5 µg mL^{-1} of erythromycin, respectively.

Construction of expression vectors

DNA cloning was performed using standard protocols in *E. coli* DH5α according to Sambrook *et al.* (1989). All the final DNA constructs were checked by sequencing (BMR Genomics s.r.l., Padova, Italy). pTRKH3 (O'Sullivan & Klaenhammer, 1993), a shuttle cloning vector for Gram-positive and Gram-negative bacteria, was used as the backbone for the construction of our expression vectors.

The EGFP-coding sequence (735 bp) was PCR amplified from pQE-GFP with the primers GFP3fw and GFP3rev (Table 1). The *egfp* CDS was derived from the vector pCSGFP3, a kind gift from Enrique Amaya, Wellcome/CRC Institute, Cambridge, UK. The primers introduced, respectively, an EcoRI site and a BamHI site (underlined) at the two sides of the amplified fragment. The PCR product was then cloned into pBlueScript, yielding pBSGFP3.

pTRKH3-*ldh*GFP

The *ldhL* promoter was amplified from genomic DNA of *L. acidophilus* ATCC4356^T by PCR with the oligonucleotides LA*ldh*4 and LA*ldh*3 (Table 1). The first one included a HindIII site (underlined), and the second one contained an EcoRI site (underlined) and the *ldhL* ribosome-binding site (RBS) (bold). The 290-bp PCR product was cloned into pBSGFP3, yielding pBS-*ldh*GFP. The *ldh*GFP was then excised from pBS-*ldh*GFP by SalI and BamHI digestion and inserted into pTRKH3, yielding pTRKH3-*ldh*GFP.

pTRKH3-*slp*GFP

The *slp* promoter/leader sequence (the CDS corresponding to the signal peptide of the *slp*) was amplified from *L. acidophilus* ATCC4356^T by PCR with the primers *slp*PLfw and *slp*PLrev (Table 1): the former introduced an EcoRI and the latter a BglII site (both underlined). The 317-bp PCR product, including the RBS, was inserted into pQE30-GFP, yielding pQE-*slp*GFP3. In this configuration, the CDS of EGFP is fused *in frame* downstream the *slp* signal peptide

Table 1. Bacterial strains, plasmids and primers used in this study

Strains, plasmids or primers	Relevant features*	Sources or references
Strains		
L. lactis spp. cremoris MG1363	Lactococcal reference strain	Gasson (1983)
L. reuteri		
DSM 20016^T	Type culture for *L. reuteri* species	Kandler *et al.* (1980)
H09	Chicken crop isolate	This work
I09	Chicken crop isolate	This work
N07	Chicken crop isolate	This work
N09	Chicken crop isolate	This work
N10	Chicken crop isolate	This work
Plasmids		
pTRKH3	Em^r, Tet^r, *Enterococcus faecalis* plasmid pAMβ1 origin, *Escherichia coli* plasmid p15A origin	O'Sullivan & Klaenhammer (1993)
pQE-GFP	Ap^r, pQE 30 (Qiagen, Milan, Italy) derivative containing *egfp*	Our previous work
pBSGFP3	Ap^r, pBlueScript derivative containing GFP3fw+rev PCR product	This work
pBS-*ldh*GFP	Ap^r, pBSGFP3 derivative containing P$_{ldhL}$ PCR product	This work
pTRKH3-*ldh*GFP	Em^r, pTRKH3 derivative containing GFP3fw+rev PCR product downstream of P$_{ldhL}$	This work
pQE-*slp*GFP3	Ap^r, pQE-GFP derivative containing P$_{slp}$ PCR product	This work
pBS-*slp*GFP	Ap^r, pBlueScript derivative containing GFP3fw+rev PCR product downstream of P$_{slp}$	This work
pTRKH3-*slp*GFP	Em^r, pTRKH3 derivative containing GFP3fw+rev PCR product downstream of P$_{slp}$	This work
pBS-*erm*GFP	Ap^r, pBSGFP3 derivative containing P$_{ermB}$ PCR product	This work
PTRKH3-*erm*GFP	Em^r, pTRKH3 derivative containing GFP3fw+rev PCR product downstream of P$_{ermB}$	This work
Primers		
GFP3fw	5′-TCG<u>GAATTC</u>ATGAGTAAAGGAGAAGAA-3′	This work
GFP3rev	5′-TCA<u>GGATCC</u>TTATTTGTATAGTTCATCC-3′	This work
LA*ldh*4	5′-TCT<u>AAGCTT</u>TTTAGTCCAATGCCCTTC-3′	This work
LA*ldh*3	5′-TCA<u>GAATTC</u>AAG**TCTCCT**TTTTTATTAGTG-3′	This work
*slp*PLfw	5′-CTG<u>GAATTC</u>GTGGTAAGTAATAGGACGTGC-3′	This work
*slp*PLrev	5′-CTC<u>AGATCT</u>GCTAACAGTAGATACAGC-3′	This work
*erm*6	5′-TCT<u>AAGCTT</u>AGTCTAGAATCGATACGA-3′	This work
*erm*4	5′-TCA<u>GAATTC</u>ACT**CCTTCT**TAATTAC-3′	This work

*Underlined base pairs in primers indicate introduced restriction sites; bold base pairs shown in primers indicate the RBS.
Ap^r, Ampicillin resistant; Em^r, erythromycin resistant; Tet^r, tetracycline resistant.

sequence. pQE-*slp*GFP3 was restricted by EcoRI and PstI and cloned into pBlueScript, yielding pBS-*slp*GFP. Finally pBS-*slp*GFP was digested by BamHI and SalI and inserted into pTRKH3 resulting in pTRKH3-*slp*GFP.

pTRKH3-*erm*GFP

The *ermB* promoter was PCR amplified from pTRKH3 with the primers *erm6* and *erm4* (Table 1). Again, the first sequence included a HindIII site, and the second one contained an EcoRI site (underlined) and the *ermB* RBS (bold). The 556-bp PCR product was cloned into pBSGFP3, yielding pBS-*erm*GFP. Finally, pBS-*erm*GFP was digested by BamHI and SalI and inserted into pTRKH3, yielding pTRKH3-*erm*GFP.

Electroporation

To screen the activity of these vectors in a standard Gram-positive host, pTRKH3-*ldh*GFP, pTRKH3-*slp*GFP and pTRKH3-*erm*GFP were initially introduced by electroporation into *L. lactis* spp. cremoris MG1363 following the protocol described by Holo (Holo & Nes, 1989). After showing that the plasmids could replicate in a Gram-positive host, they were electroporated into *L. reuteri* DSM 20016^T as an electroporation control and into five different strains of *L. reuteri* isolated from chicken crops (Thompson & Collins, 1996). Plasmids were isolated from transformed lactobacilli by a lysozyme-alkaline lysis procedure and checked by restriction analysis.

Detection of GFP fluorescence

Measurement of GFP activity in prokaryotes is reversibly affected by protein oxidation, the pH value of the medium and temperature (Hansen *et al.*, 2001). *Lactobacillus reuteri* transformants were grown in MRS broth (including $10 \, \mu g \, mL^{-1}$ erythromycin) or in a buffered MRS broth (containing 0.2 M potassium phosphate, pH 7.0, and

$10\,\mu g\,mL^{-1}$ erythromycin) at 30 or 37 °C with or without aeration, in several combinations (Pérez-Arellano & Pérez-Martínez, 2003; Wu & Chung, 2006). *Lactococcus lactis* transformants were grown in GM17 broth containing $5\,\mu g\,mL^{-1}$ erythromycin. The pellets of the GFP-expressing cells were resuspended in phosphate-buffered saline (PBS), from which $10\,\mu L$ of the bacterial suspension was transferred onto slides. These smears were then directly observed by epifluorescence microscopy (TMD, Nikon) under an oil immersion objective. Subsequently, the Qubit^{TM} fluorometer, which is able to measure fluorochromes such as SyBR Green, was used to obtain a direct quantification of GFP fluorescence. Indeed, the excitation wavelength provided by the blue light-emitting diodes (LEDs) of the instruments has a peak around 480 nm and the emission of SyBr Green stains shows a maximum around 521 nm; these values are not far from those of EGFP, having the excitation peak at 488 nm and the emission peak at 510 nm. Even though the exact properties of the instrument and the composition of the kits for this fluorometer are not declared by the manufacturer, we demonstrated its ability to detect small amounts of GFP fluorescence, producing a linear and reliable response. Using the 'Quant-iT Protein Assay' program of the fluorometer, we generated a GFP fluorescence calibration curve including three different concentrations of a recombinant 6xHis-EGFP (0, 1 and $2\,\mu g$) as standards. Recombinant 6xHis tagged-EGFP was produced in *E. coli* DH5α bearing the plasmid pQE-GFP and purified by immobilized metal affinity chromatography. For each sample, similar amounts of GFP-expressing cells (measured as $OD_{600\,nm}$) were centrifuged at 1800 g for 5 min, washed with PBS, resuspended in $200\,\mu L$ of PBS and subjected to fluorimetric reading.

Western blotting

Total protein extracts were prepared from exponentially growing cultures. Bacteria were disrupted by sonication using an Ultrasonic Processor (W380; Heat Systems, Farmingdale, NY). Cell lysates were centrifuged to remove cell debris. The total protein concentration was determined by fluorimetry using a Qubit^{TM} fluorometer and the Quant-iT Protein Assay Kit (Invitrogen). A recombinant 6xHis-EGFP was used as a control in electrophoresis. The samples were mixed with denaturing buffer, boiled and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli (1970) on a 4–12% gel. Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Bio-Rad Laboratories, Richmond, CA) by electroblotting. GFP was detected using a mouse Anti-GFP antibody (Roche) and the BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) (Roche) according to the protocol of the manufacturer.

Results and discussion

Bacterial transformation and measurement of GFP fluorescence

Three plasmids expressing GFP under the control of, respectively, *ldhL*, *slp* and *ermB* promoters were generated into the backbone of the shuttle vector pTRKH3 and cloned in *E. coli* DH5α. The expression level of the three plasmids was assessed upon electroporation in *L. lactis* and *L. reuteri* DSM 20016^{T}. Following the testing in the *L. reuteri* DSM 20016^{T} reference strain, five different erythromycin-sensitive strains of *L. reuteri* isolated from chicken crops (H09, I09, N07, N09, and N10) were chosen for transformation trials. Transformed colonies were obtained from all the strains. I09 and N09 isolates were cultured in MRS at 37 °C, instead of H09, N07 and N10, which had their optimal growth condition in MRS at 40 °C. The GFP fluorescence was examined in all the recombinant bacteria. The fluorescence was initially observed in pelleted cells upon blue LED light (470 nm) looking through a green polyester filter (Lime#8, Lee filters) and by epifluorescence microscopy on unfixed cells. A high level of fluorescence was detectable in *L. lactis*/pTRKH3-*erm*GFP, while *L. lactis* transformed with pTRKH3-*ldh*GFP and pTRKH3-*slp*GFP showed a very low intensity. The fluorescence of pelleted *L. lactis* cells required a washing step with PBS to be detectable. Very particular conditions were needed to achieve optimal fluorescence in *L. reuteri*, as reported by Pérez-Arellano & Pérez-Martínez (2003) in *Lactobacillus casei*. *Lactobacillus reuteri* DSM 20016^{T}, *L. reuteri* N09 and I09 were grown in MRS medium under several combinations of the following culture conditions: incubation at 30 or 37 °C, unbuffered or buffered MRS medium, with or without aeration. No fluorescence could be detected when *L. reuteri* was grown in an unbuffered medium at 37 °C, either with or without aeration. Growth temperature and pH were the most important factors affecting the synthesis or the stability of the GFP protein, because in buffered medium at 30 °C, fluorescence was clearly visible (Fig. 1). In contrast with the results of Wu & Chung (2006), we found that aeration conditions barely influenced GFP expression, achieving similar results with or without aeration in *L. reuteri* strains (Fig. 2a). Concordant data were obtained by fluorimetry (Fig. 2b). In H09, N07 and N10 strains, fluorescence was clearly detectable when these isolates were grown in buffered MRS at 37 °C without aeration, due to their different optimal growth conditions (Fig. 1). As observed in our *in vitro* experiments, although GFP is considered as a suitable reporter to be used in bacteria, detection of this protein *in vivo* could be problematic due to the high rate of denaturation observed at low pH levels developed and tolerated by LAB during their growth. Actually, satisfactory fluorescence visualization in
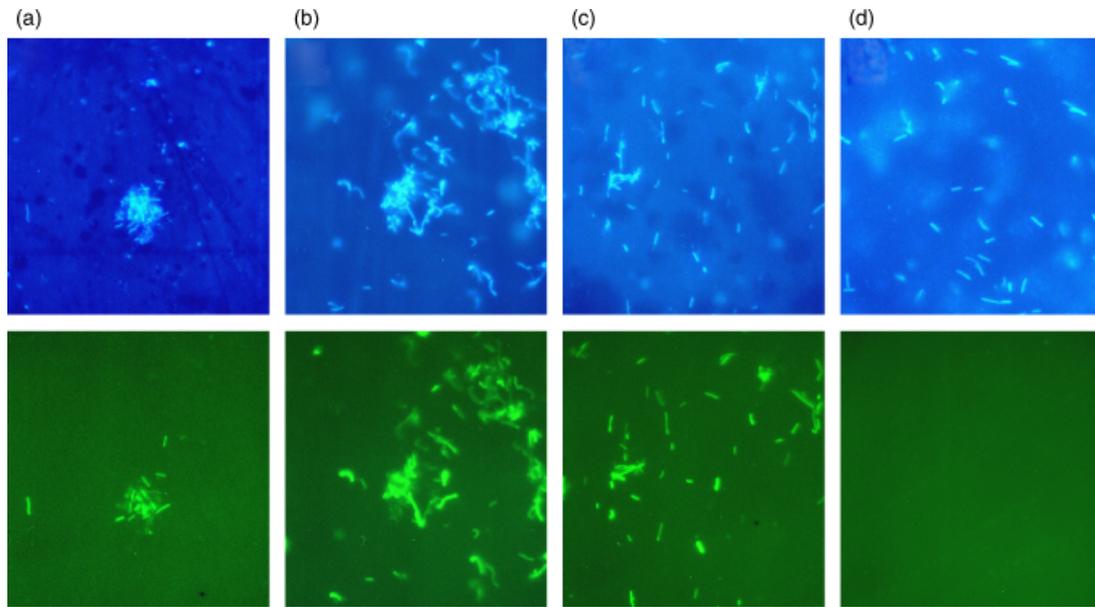
Fig. 1. Three different strains of *Lactobacillus reuteri* isolated from chicken crop transformed with pTRKH3-*erm*GFP. Upper panels show fluorescence following DNA-intercalating bisbenzimide staining. Lower panels show GFP fluorescence. (a) Strain H09, transformed. (b) Strain N10, transformed. (c) Strain N09, transformed. (d) Strain N09, wild type.
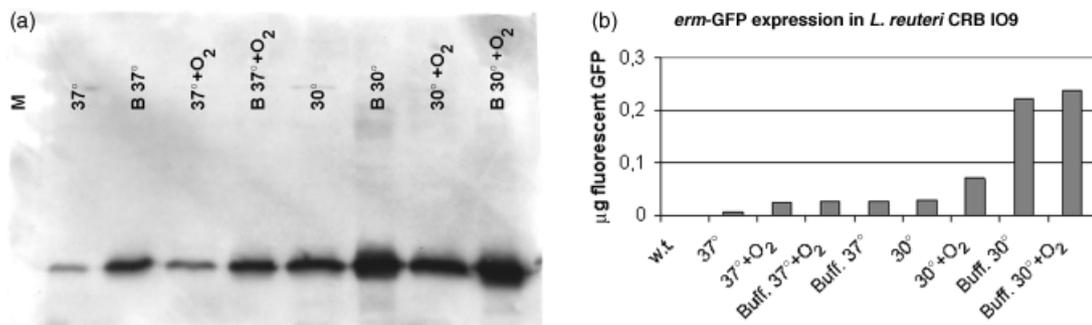

Fig. 2. Influence of culture conditions on *erm*-GFP expression in *Lactobacillus reuteri*. (a) Western blot analysis of cell lysates and cell-free medium from *L. reuteri* DSM 20016^T/pTRKH3-*erm*GFP grown in different culture conditions. Cells were grown in several combinations of the following parameters: MRS broth (–) or MRS broth buffered with $KHPO_4$ (B), 30 °C (30°) or 37 °C (37°), anaerobiosis (–) or aeration (O_2) (see Materials and methods). (b) GFP content in lag-phase cell lysates of *L. reuteri* I09/pTRKH3-*erm*GFP was assessed after culturing in different conditions. Cells were grown in several combinations of the following parameters: 30 °C (30°) or 37 °C (37°), anaerobiosis (–) or aeration (O_2), MRS broth (–) or MRS broth buffered with $KHPO_4$ (Buff.). The GFP content is referred to the same amount of cells (measured as $OD_{600 nm}$). w.t., Wild type.

Lactococcus and *Lactobacillus* requires a neutralization step performed by washing the cells in a neutral phosphate buffer. Even though visible fluorescence can be recovered by the treatment, it is still unclear whether the totality of the protein can be renatured in this way or whether a part of it remains irreversibly 'switched off' following extended exposition to low pH levels. To overcome such potential problems *in vivo*, some alternative reporter proteins could be tested to replace EGFP *in vivo*, such as the red fluorescent protein from *Discosoma* sp., which is more stable in acidic environments.

The GFP produced in recombinant *L. lactis* and *L. reuteri* strains was analyzed by Western blotting with mouse Anti-GFP antibody (Roche). Analysis confirmed quantitative data collected by fluorimetry, providing additional information concerning the processing and release of the reporter protein in the extracellular environment.

In *L. lactis*, the expression level of GFP driven by the *slp* promoter was clearly detectable, but still lower compared with the *erm*-GFP vector. In Western blot analysis, the *in-frame* fusion of the sequence coding the leader peptide of the SLP with the *GFP* CDS resulted in the presence of a
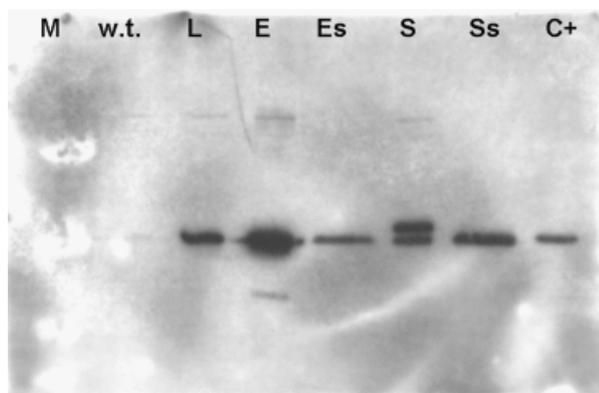
Fig. 3. Western blot analysis of cell lysates and surnatants from *Lactococcus lactis* MG1363 bearing different vectors. M, Molecular weight marker; w.t., lysate, *L. lactis* MG1363, untransformed; L, lysate, *ldhL* promoter; E, lysate, *ermB* promoter; Es, surnatant, *ermB* promoter; S, lysate, *slp* promoter; Ss, surnatant, *slp* promoter; C+, 100 pg of purified 6xHis-EGFP. The lysate from cells transformed with the *slp*-vector shows a double band, corresponding to the presence of the SLP-leader peptide fused to the N-terminal of the reporter (upper band) and to the processed peptide (lower band). In the next lane, the sample containing the spent culture medium (Ss) shows only the secreted processed GFP. The concentration of GFP in such sample is higher than in the corresponding one (Es) obtained with the 'stronger' *ermB* vector.

Fig. 4. Activity of the three promoters in different species. w.t., Wild type; Erm, pTRKH3-*erm*GFP transformants; Ldh, pTRKH3-*ldh*GFP transformants; Slp, pTRKH3-*slp*GFP transformants. Fluorescent GFP content in lag-phase cell lysates was assessed for the three vectors in *Lactococcus lactis* MG1363 cultured in GM17 at 30 °C, *Lactobacillus reuteri* DSM 20016^T and *L. reuteri* N09 both cultured in buffered MRS at 30 °C. The fluorescence of each sample was compared with a purified 6xHis-EGFP standard by means of a Qubit^{TM} fluorometer. The fluorescent GFP content is reported to a similar amount of cells (measured as OD_{600nm}) for each species.

double band in the lane corresponding to the *L. lactis* bearing *slp*-GFP vector (Fig. 3), which was interpreted, respectively, as the propeptide and the leaderless processed form of the protein. To confirm this hypothesis and the possible active secretion of the processed GFP, a sample of bacterial lysate was analyzed together with the concentrated spent culture medium (Fig. 3). In the culture medium, only the processed form of the protein was detected and its amount was higher than in the medium from *erm*-GFP transformed *L. lactis*. Unfortunately, the *slp* promoter proved to be worthless in our isolate *L. reuteri* N09, due to the very low activity observed upon transformation (Fig. 4).

In a comparative analysis, the *ermB* promoter appears to be the most active in all the tested species, even though *ldhL* proved to be similarly effective in *L. reuteri* DSM 20016^T (data not shown) and in our isolate N09 (Fig. 5). The choice of promoters is one of the most important features to consider when expressing specific antigens in LABs to 'vaccinate' the host. Even if a high level of antigen synthesis is not always a prerequisite to elicit the host immunity, i.e. for antigens that are membrane associated or that show some insolubility or toxicity to bacterial cells (Mercenier *et al.*, 2000), the failure in stimulating the production of antibodies in hosts may also be the result of the low level of expression of heterologous proteins in the recombinant LAB. This may be due to the absence of the specific inducer in the gastrointestinal tract of the host. Several studies (Grangette *et al.*, 2001; Reveneau *et al.*, 2002) have shown
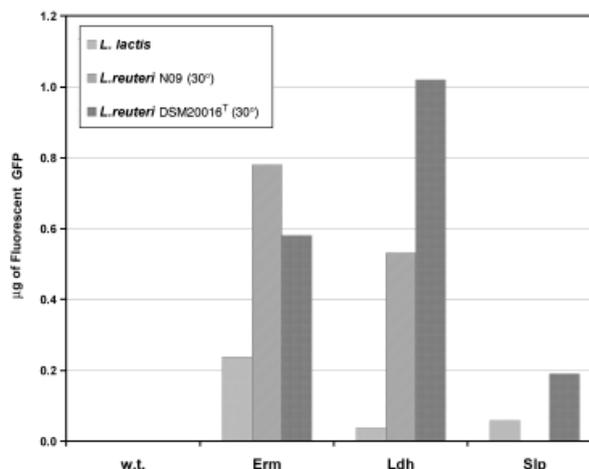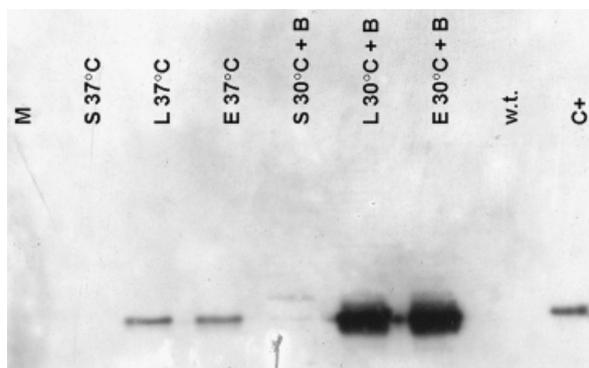

Fig. 5. Western blot analysis of cell lysates from the crop isolate N09 of *Lactobacillus reuteri* transformed with three different vectors and cultured in MRS at 37 °C or in buffered MRS at 30 °C. M, Molecular weight marker; S 37 °C, *slp*-GFP vector, MRS at 37 °C; L 37 °C, *ldh*-GFP vector, MRS at 37 °C; E 37 °C, *erm*-GFP vector, MRS at 37 °C; S 30 °C+B, *slp*-GFP vector, buffered MRS at 30 °C; L 30 °C+B, *ldh*-GFP vector, buffered MRS at 30 °C; E 30 °C+B, *erm*-GFP vector, buffered MRS at 30 °C; w.t., *L. reuteri* N09 untransformed; C+, 300 pg of purified 6xHis-EGFP.

that the absolute level of the antigen produced by *Lactobacillus* vaccine strains is a key factor in determining the level of immune responses obtained, and that the addition of an antigen dose leads to an enhancement of the immune response.

The *slp* promoter responsible for the transcription of stable mRNAs coding the S-layer protein monomers may be

a good candidate to direct mRNA synthesis of chimerical genes for expression of heterologous proteins on the surface of the cells, as reported by Mota *et al.* (2006) in *Lactobacillus crispatus*, but in our study in *L. reuteri*, we demonstrated a low level of GFP expression, comparing the *slp* promoter activity with the ones of *ldhL* and *ermB* promoters in *L. reuteri* DSM 20016$^{\text{T}}$ and in our isolate N09. How this observation may be related to the natural absence of the S-layer protein in *L. reuteri* needs to be investigated.

In conclusion, the constructed vectors were successfully used to express GFP in *L. reuteri* strains, showing the effectiveness of constitutive promoters to produce heterologous proteins in these LABs as an effective alternative to nisin-inducible expression vectors.

Acknowledgements

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