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 20016T 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-slpEGFP, pTRKH3-ldhL-EGFP, pTRKH3-ermEGFP). Besides conventional analytical methods, a new quick fluorimetric approach was set up to quantify the EGFP fluorescence in transformed clones using the QubitTM 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 20016T 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.
Constitutive promoters in \textit{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-STLTB protein secreted by their \textit{L. reuteri} was expressed at a high level during 3 h after the \textit{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 \textit{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 \textit{L. reuteri} strains, we compared the expression of the \textit{gfp} gene under the control of three constitutive promoters: the lactate dehydrogenase (\textit{ldhL}) promoter from \textit{Lactobacillus acidophilus} (Kim \textit{et al.}, 1991), which is reported to be a highly efficient promoter, the surface (S)-layer protein (\textit{slp}) promoter from \textit{L. acidophilus}, responsible for the high level of transcription of stable mRNAs coding the S-protein monomers (Boot \\textit{et al.}, 1996; Boot \textit{et al.}, 1996) and the erythromycin ribosomal methylase (\textit{ermB}) promoter from the broad-host range plasmid pAM\textit{B}I isolated from \textit{Enterococcus faecalis} (Swinfield \textit{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 \textit{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 \textit{et al.}, 2002; Mota \textit{et al.}, 2006). Besides these important features of the S-layer proteins, we considered it essential to evaluate the activity of their promoter in \textit{L. reuteri} by comparison with those of \textit{ldhL} and \textit{ermB}.

The activity of the vectors bearing these different promoters was tested in reference strains of \textit{Lactococcus lactis}, \textit{L. reuteri} and in five selected strains of \textit{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.

\textbf{Materials and methods}

\textbf{Bacterial strains, isolation and identification of indigenous lactobacilli from chicken crop}

\textit{Lactococcus lactis} spp. \textit{crenoris} 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 \textit{L. reuteri} DSM 20016T 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 seven 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. \textit{Lactobacillli} 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 \\textit{et al.}, 1997; Moreira \textit{et al.}, 2005). Uncertain identifications were confirmed by sequencing of 16S rRNA gene. Lactobacilli and \textit{L. lactis} transformants were selected with 10 and 5 µg mL\textsuperscript{-1} of erythromycin, respectively.
sequence. pQE-slpGFP3 was restricted by EcoRI and PstI and cloned into pBlueScript, yielding pBS-slpGFP. Finally pBS-slpGFP was digested by BamHI and SalI and inserted into pTRKH3 resulting in pTRKH3-slpGFP.

**pTRKH3 ermGFP**

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 ermGFP. Finally, pBS ermGFP was digested by BamHI and Sall and inserted into pTRKH3, yielding pTRKH3 ermGFP.

**Electroporation**

To screen the activity of these vectors in a standard Gram-positive host, pTRKH3-ldhGFP, pTRKH3 slpGFP and pTRKH3 ermGFP were initially introduced by electroporation into *L. lactis* spp. cremoris MG1363 following the protocol described by Holo (Hol & Nes, 1989). After showing that the plasmids could replicate in a Gram-positive host, they were electroporated into *L. reuteri* DSM 20016T 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 μg mL^-1_ erythromycin) or in a buffered MRS broth (containing 0.2 M potassium phosphate, pH 7.0, and

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

| Strains, plasmids or primers | Relevant features* | Sources or references |
|-------------------------------|-------------------|----------------------|
| Strains | Lactococcal reference strain | Gasson (1983) |
| *L. reuteri* | DSM 20016T | Type culture for *L. reuteri* species | Kandler et al. (1980) |
| | H09 | Chicken crop isolate | This work |
| | I09 | Chicken crop isolate | This work |
| | N09 | Chicken crop isolate | This work |
| | N10 | Chicken crop isolate | This work |
| Plasmids | pTRKH3 Emr, Te, Enteroococcus faecalis plasmid pAMβ1 origin, Escherichia coli plasmid p15A origin | O'Sullivan & Klaenhammer (1993) |
| | pQE-GFP Ap, pQE 30 (Qiagen, Milan, Italy) derivative containing egfp | Our previous work |
| | pBSGFP3 Ap, pBlueScript derivative containing GFP3fw + rev PCR product | This work |
| | pBS-ldhGFP Ap, pBSGFP3 derivative containing P<sub>ldhL</sub> PCR product | This work |
| | pTRKH3-ldhGFP Emr, pTRKH3 derivative containing GFP3fw + rev PCR product downstream of P<sub>ldhL</sub> | This work |
| | pQE-slpGFP3 Ap, pQE-GFP derivative containing P<sub>slp</sub> PCR product | This work |
| | pBS-slpGFP Ap, pBlueScript derivative containing GFP3fw + rev PCR product downstream of P<sub>slp</sub> | This work |
| | pTRKH3-slpGFP Emr, pTRKH3 derivative containing GFP3fw + rev PCR product downstream of P<sub>slp</sub> | This work |
| | pBS ermGFP Ap, pBSGFP3 derivative containing P<sub>ermB</sub> PCR product | This work |
| | pTRKH3 ermGFP Emr, pTRKH3 derivative containing GFP3fw + rev PCR product downstream of P<sub>ermB</sub> | This work |
| Primers | GFP3fw | 5'-TCGGAAATCTAGTAAAGGAGAAGAA-3' | This work |
| | GFP3rev | 5'-TCAGGATCTCCATTGTATAGTCATCC-3' | This work |
| | LA<sub>ldh</sub>4 | 5'-CTCAAGCTTTTATGCAATGTCCTTC-3' | This work |
| | LA<sub>ldh</sub>3 | 5'-TCAGAATTCAATTTCCTTTTATTAGTG-3' | This work |
| | slpLfw | 5'-CTGGAAATCTGTGAATAGAACGTCG-3' | This work |
| | slpLrev | 5'-CTCAAGCTCTGGTCCAGTAGATACACG-3' | This work |
| | erm6 | 5'-CTCAAGCTTTAATATTGCAATGCA-3' | This work |
| | erm4 | 5'-CTCAAGCTTCTTCCAATGTCCTTC-3' | This work |

*Underlined base pairs in primers indicate introduced restriction sites; bold base pairs shown in primers indicate the RBS. Ap<sup>+</sup>, Ampicillin resistant; Emr<sup>+</sup>, erythromycin resistant; Te<sup>+</sup>, tetracycline resistant.*
10 μg 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 μg mL⁻¹ erythromycin. The pellets of the GFP-expressing cells were resuspended in phosphate-buffered saline (PBS), from which 10 μL 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™ 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 μg) 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 OD₆₀₀ nm) were centrifuged at 1800 g for 5 min, washed with PBS, resuspended in 200 μL 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™ 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 ermA 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 20016ᵀ. Following the testing in the L. reuteri DSM 20016ᵀ 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-ermGFP, while L. lactis transformed with pTRKH3-ldhGFP and pTRKH3-slpGFP 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 20016ᵀ, 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 37 °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
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 exposure 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. 1. Three different strains of Lactobacillus reuteri isolated from chicken crop transformed with pTRKH3-ermGFP. Upper panels show fluorescence following DNA-intercalating bisbenzimide staining. Lower panels show GFP fluorescence. (a) Strain H909, 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/pTRKH3-ermGFP grown in different culture conditions. Cells were grown in several combinations of the following parameters: MRS broth (–) or MRS broth buffered with KHPO₄ (B), 30 °C (30°) or 37 °C (37°), anaerobiosis (–) or aeration (O₂) (see Materials and methods). (b) GFP content in lag-phase cell lysates of L. reuteri I09/pTRKH3-ermGFP 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 (O₂), MRS broth (–) or MRS broth buffered with KHPO₄ (Buff.). The GFP content is referred to the same amount of cells (measured as OD₆₀₀nm). w.t., Wild type.
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 20016T (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 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 200163 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.

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References
Abbas Hilmi HT, Surakka A, Apajalahi J & Saris PE (2007) Identification of the most abundant Lactobacillus species in the crop of 1- and 5-week-old broiler chickens. Appl Environ Microb 73: 7867–7873.

Adams MR & Marteau P (1995) On the safety of lactic acid bacteria from food. Int J Food Microb 27: 263–264.

Avall-Jaaskelainen S, Kila-Nikkila K, Kahala M, Miikkulainen-Lahti T & Palva A (2002) Surface display of foreign epitopes on the Lactobacillus brevis S-layer. Appl Environ Microb 68: 5943–5951.

Boot HJ & Pouwels PH (1996) Expression, secretion and antigenic variation of bacterial S-layer proteins. Mol Microbiol 21: 1117–1123.

Boots HJ, Kolen CP, Andreazaki FJ, Leer RJ & Pouwels PH (1996) The Lactobacillus acidophilus S-layer protein gene expression site comprises two consensus promoter sequences, one of which directs transcription of stable mRNA. J Bacteriol 178: 5388–5394.

Cortes-Perez NG, Azevedo V, Alcocer-Gonzalez JM, Rodriguez-Padilla C, Tamez-Guerra RS, Corthier G, Gruss A, Langella P & Bermudez-Humaran LG (2005) Cell-surface display of E7 antigen from human papillomavirus type-16 in Lactococcus lactis and in Lactobacillus plantarum using a new cell-wall anchor from lactobacilli. J Drug Target 13: 89–98.

Ferreira PC, Campos IB, Abe CM, Trabulsi LR, Elias WP, Ho PL & Oliveira ML (2008) Immunization of mice with Lactobacillus casei expressing intimin fragments produces antibodies able to inhibit the adhesion of enteropathogenic Escherichia coli to cultivated epithelial cells. FEMS Immunol Med Mic 54: 245–254.

Gasson MJ (1983) Plasmid complements of Streptococcus lactis NCDO 712 and other lactic streptococci after protoplast-induced curing. J Bacteriol 154: 1–9.

Grangette C, Müller-Allof H, Guerdcourt D, Geoffroy MC, Turneer M & Mercenier A (2001) Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant Lactobacillus plantarum. Infect Immun 69: 1547–1553.

Hansen MC, Palmer RJ Jr, Udsen C, White DC & Molin S (2001) Assessment of GFP fluorescence in cells of Streptococcus gordonii under conditions of low pH and low oxygen concentration. Microbiology 147: 1383–1391.

Ho PS, Kwang J & Lee YK (2005) Intragastric administration of Lactobacillus casei expressing transmissible gastroenteritis coronavirus spike glycoprotein induced specific antibody production. Vaccine 23: 1335–1342.

Holo H & Nes IF (1989) High-frequency transformation, by electroporation, of Lactococcus lactis subsp. cremoris grown with glycine in osmotically stabilized media. Appl Environ Microb 55: 3119–3123.

Hou XL, Yu LY, Liu J & Wang GH (2007) Surface-displayed porcine epidemic diarrhea viral (PEDV) antigens on lactic acid bacteria. Vaccine 26: 24–31.

Kandler O, Stetter KO & Kohl R (1980) Lactobacillus reuteri sp. nov. a new species of heterofermentative lactobacilli. Zbl Bakt Mik Hig I C 1: 264–269.

Kim SF, Baek SJ & Pack MY (1991) Cloning and nucleotide sequence of the Lactobacillus casei lactate dehydrogenase gene. Appl Environ Microb 57: 2413–2417.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Mercenier A (1999) Lactic acid bacteria as live vaccines. Probiotics: A Critical Review (Tannock GM, ed), pp. 113–127. Horizon Scientific Press, Wymondham, Norfolk, UK.

Mercenier A, Müller-Allof H & Grangette C (2000) Lactic acid bacteria as live vaccines. Curr Issues Mol Biol 2: 17–25.

Mohamadzadeh M, Duong T, Sandwick SJ, Hoover T & Klænhammer TR (2009) Dendritic cell targeting of Bacillus anthracis protective antigen expressed by Lactobacillus acidophilus protects mice from lethal challenge. P Natl Acad Sci USA 106: 4331–4336.

Moreira JL, Mota RM, Horta MF, Teixeira SM, Neumann E, Nicolli JR & Nunes AC (2005) Identification to the species level of Lactobacillus isolated in probiotic prospecting studies of human, animal or food origin by 16S–23S rRNA restriction profiling. BMC Microbiol 5: 15.

Mota RM, Moreira JL, Souza MR, Horta MF, Teixeira SM, Neumann E, Nicolli JR & Nunes AC (2006) Genetic
transformation of novel isolates of chicken \textit{Lactobacillus} bearing probiotic features for expression of heterologous proteins: a tool to develop live oral vaccines. \textit{BMC Biotechnol} \textbf{6}: 2.

O’Sullivan DJ & Klaenhammer TR (1993) High- and low-copy-number \textit{Lactococcus} shuttle cloning vectors with features for clone screening. \textit{Gene} \textbf{137}: 227–231.

Pérez-Arellano I & Pérez-Martínez G (2003) Optimization of the green fluorescent protein (GFP) expression from a lactose-inducible promoter in \textit{Lactobacillus casei}. \textit{FEMS Microbiol Lett} \textbf{222}: 123–127.

Reveneau N, Geoffroy MC, Locht C, Chagnaud P & Mercenier A (2002) Comparison of the immune responses induced by local immunizations with recombinant \textit{Lactobacillus plantarum} producing tetanus toxin fragment C in different cellular locations. \textit{Vaccine} \textbf{20}: 1769–1777.

Sambrook J, Fritsch EF & Maniatis T (1989) \textit{Molecular Cloning: A Laboratory Manual}, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Swinfield TJ, Oultram JD, Thompson DE, Breehm JK & Minton NP (1990) Physical characterisation of the replication region of the \textit{Enterococcus faecalis} plasmid pAMβ1. \textit{Gene} \textbf{87}: 79–90.

Thompson K & Collins MA (1996) Improvement in electroporation efficiency for \textit{Lactobacillus plantarum} by the inclusion of high concentrations of glycine in the growth medium. \textit{J Microbiol Meth} \textbf{26}: 73–79.

Tilsala-Timisjarvi A & Alatossava T (1997) Development of oligonucleotide primers from the 16S–23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. \textit{Int J Food Microbiol} \textbf{35}: 49–56.

Wu CM & Chung TC (2006) Green fluorescent protein is a reliable reporter for screening signal peptides functional in \textit{Lactobacillus reuteri}. \textit{J Microbiol Meth} \textbf{67}: 181–186.

Wu CM & Chung TC (2007) Mice protected by oral immunization with \textit{Lactobacillus reuteri} secreting fusion protein of \textit{Escherichia coli} enterotoxin subunit protein. \textit{FEMS Immunol Med Mic} \textbf{50}: 354–365.