Expression of the *Giardia lamblia* cyst wall protein 2 in *Lactococcus lactis*

Peter Lee and Gaétan M. Faubert

Correspondence
Gaétan M. Faubert
gaetan.faubert@mcgill.ca

Institute of Parasitology, McGill University, Macdonald Campus, 21 111 Lakeshore Rd,
Ste-Anne de Bellevue, Québec H9X 3V9, Canada

In this study, *Lactococcus lactis* was engineered to express *Giardia lamblia* cyst wall protein 2 (CWP2) at three different subcellular locations, intracellular, secreted or cell-surface-anchored, using nisin as an inducing agent. CWP2 expression did not appear to be detrimental to *L. lactis* viability. No particular subcellular location of CWP2 expression offered any advantages over the others with respect to decreased toxicity towards the bacteria. All recombinant lactococci experienced a similar reduction in growth rate when induced. It was determined whether recombinant lactococcal cells engineered for cell surface expression of CWP2 were capable of inducing a CWP2-specific mucosal IgA antibody response. Recombinant lactococci were successful at inducing CWP2-specific IgA antibodies. Moreover, in a pilot challenge experiment, mice immunized with these recombinant lactococci demonstrated a significant (63%) reduction in cyst output. Thus, it has been demonstrated that *G. lamblia* CWP2 may be expressed in *L. lactis* and that recombinant lactococcal cells elicit *Giardia*-specific antibodies which reduce cyst shedding in a murine model.

Received 29 January 2006

Revised 8 March 2006

Accepted 15 March 2006

INTRODUCTION

Lactic acid bacteria are a group of Gram-positive bacteria widely used in the food industry and include members of the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Carr *et al.*, 2002). *Lactococcus lactis* is amongst the best characterized of the lactic acid bacteria and has been proposed to serve as an oral mucosal vaccine delivery system (Norton *et al.*, 1994; Robinson *et al.*, 1997; Wells *et al.*, 1996). *L. lactis* is being intensively investigated as a delivery vehicle for bacterial/viral antigens and cytokines at mucosal sites. For example, *Brucella abortus* L7/L12 protein (Pontes *et al.*, 2003; Ribeiro *et al.*, 2002), tetanus toxin fragment C of *Clostridium tetani* (Norton *et al.*, 1996, 1997; Robinson *et al.*, 1997, 2004; Wells *et al.*, 1993), human papillomavirus-16 E7 oncoprotein (Bermudez-Humaran *et al.*, 2004; Cortes-Perez *et al.*, 2005) and human IL-10 (Steidler *et al.*, 2000) have all been successfully expressed in *L. lactis*.

Giardia lamblia is an intestinal protozoan parasite disseminated worldwide and a major pollutant of surface water. The infection is spread by the cyst stage and as little as 10 cysts are required for infection. Giardiasis is usually acquired through drinking cyst-contaminated water or eating cyst-contaminated food. The clinical illness is characterized by diarrhoea, abdominal cramps, bloating, weight loss and malabsorption; however, asymptomatic

infections also frequently occur (Faubert *et al.*, 2002). Although giardiasis cases occur sporadically, water-borne outbreaks are well documented. During 1991–2000 in the United States, *Giardia* was identified as a causal agent of 9.4% (10 of 106) of recreational-water-associated outbreaks and 16.2% (21 of 130) of drinking-water-associated gastroenteritis of known or suspected infectious aetiology (Hlavsa *et al.*, 2005).

G. lamblia has a direct life cycle and possesses two stages: the trophozoite, which is the vegetative stage colonizing the small intestine, and the cyst, which is released into faecal material constituting the infective stage. The cyst is composed of a rigid wall structure which protects the two trophozoites inside from the harsh external environment. Cyst wall proteins (CWPs) with a molecular mass ranging from 29 to 102 kDa have been detected by immunostaining (Erlandsen *et al.*, 1990). One of these, CWP2, appears to be a major component of the cyst wall structure. The intestinal mucosal immune system of mice infected with *Giardia muris* recognizes CWP2 as a foreign antigen since specific antibodies are produced locally (Larocque *et al.*, 2003). In addition, we have reported that immunization of BALB/c mice with the 39 kDa form of CWP2 reduces cyst shedding in the murine model of giardiasis when it is delivered orally with cholera toxin (Larocque *et al.*, 2003). CWP2 has two forms. Newly expressed CWP2 has a molecular mass of 39 kDa and possesses a hydrophobic N-terminal signal peptide followed by five leucine-rich repeat regions and a cysteine-rich region with a 13 kDa carboxy end tail region

Abbreviation: CWP, cyst wall protein.

(Lujan *et al.*, 1995). However, mature CWP2, detected within the cyst wall, migrates at 26 kDa since it lacks its 13 kDa tail portion due to post-translational processing. Through epitope mapping, we have determined that the mature 26 kDa form of CWP2 contains the relevant B cell epitopes (P. Lee & G. Faubert, unpublished data). Therefore, we have chosen to focus on this form of CWP2 in our study.

Since lactic acid bacteria have been proposed to serve as oral mucosal vaccine delivery systems, we engineered *L. lactis* for cell surface expression of CWP2. The aims of this study were threefold. First, we sought to determine if *L. lactis* can express a parasite protein derived from *G. lamblia*. Second, using the nisin-inducible expression system, we would then examine the efficiency of subcellular localization of the 26 kDa mature form of CWP2 (intracellular, secreted or cell-surface-anchored) by *L. lactis*. Third, we would determine whether recombinant lactococci expressing CWP2 on the cell surface can successfully deliver CWP2 to the intestinal mucosal sites of mice, generating CWP2-specific IgA antibodies. Secretory antibodies against *Giardia* spp. play a central role in the clearance of this parasite from the intestinal tract of the host (Eckmann, 2003; Langford *et al.*, 2002). Although IgA has been implicated in giardial clearance, there are currently no studies in the literature documenting IgA and its role with *Giardia* cysts. We report that *L. lactis* can successfully express CWP2, a protein of parasite origin, at three different subcellular locations. Moreover, lactococcal cells expressing CWP2 on the cell surface were able to stimulate CWP2-specific IgA antibodies which lead to a significant reduction in cyst shedding. In a pilot challenge experiment, mice immunized with recombinant lactococcal cells demonstrated a 63 % reduction in cyst output. This result is promising since it validates a

strategy to target and control the infective stage of the parasite which warrants further investigation.

METHODS

Bacterial strains and growth conditions. *E. coli* TG1 [*supE hsd* $\Delta 5$ *thi* Δ (*lac-proAB*) F'(*traD36 proAB-lacZ* Δ M15)] (Gibson, 1984) and *L. lactis* *htrA*-NZ9000 (MG1363 derivative carrying *nisK* and *nisR* genes on the chromosome, Em^r, *htrA* disrupted by single-cross-over recombination) (Le Loir *et al.*, 2001) were used as bacterial hosts. *htrA* encodes an *L. lactis* extracellular housekeeping protease responsible for the degradation of exported and fusion proteins (Poquet *et al.*, 2000). *L. lactis* *htrA*-NZ9000 was kindly provided by P. Langella and I. Poquet (INRA, Jouy-en-Josas, France). *E. coli* was grown in Luria–Bertani (LB) medium at 37 °C in an orbital shaker. *L. lactis* was grown without agitation at 30 °C in M17 medium (Difco) (Terzaghi & Sandine, 1975) supplemented with 1 % glucose (GM17). When solid medium was required, agar (1.5 %) was added to GM17 to make GM17 agar plates. When required, antibiotics were added as follows: for *L. lactis*, chloramphenicol was added at a concentration of 10 μ g ml⁻¹ and erythromycin at 5 μ g ml⁻¹; for *E. coli*, chloramphenicol was added at 10 μ g ml⁻¹ and erythromycin at 150 μ g ml⁻¹. Unless indicated, plasmid constructions were first established in *E. coli* (Sambrook *et al.*, 1989) and then transformed into *L. lactis* by electroporation (Langella *et al.*, 1993).

Plasmid engineering. The different plasmids used in this experiment were kindly provided by P. Langella (INRA, Jouy-en-Josas, France); their characteristics are described in Table 1. All plasmids (pCYT:Nuc, pSEC:LEISS:Nuc and pVE5547:L7/L12) were extracted from *E. coli* TG1 by alkali lysis and purified using a CsCl gradient. pCYT and pSEC backbones are derivatives of pNZ8010 (de Ruyter *et al.*, 1996). pCYT:CWP2 and pSEC:LEISS:CWP2 were constructed by replacing the *nuc* gene with the CWP2 gene in pCYT:Nuc and pSEC:LEISS:Nuc. The constructed plasmids together with protein localization are illustrated in Fig. 1.

The *G. lamblia* CWP2 gene sequence was amplified by PCR from plasmid pMM109, a genomic clone kindly provided by Dr T. Nash

Table 1. Plasmids used in this study

Plasmid	Replicon	Relevant characteristics*	Reference
pCYT:Nuc	pWV01	Cm ^r , expression vector encoding mature <i>nuc</i> and expressed under the control of P _{nisA}	Enouf <i>et al.</i> (2001)
pSEC:LEISS:Nuc	pWV01	Cm ^r , pSEC backbone with Usp45 peptide leader expression vector containing mature <i>nuc</i> fused at 5' end with sequence of propeptide LEISSTCDA expressed under the control of P _{nisA}	Le Loir <i>et al.</i> (2001)
pVE5547:L7/L12	pIL252	Em ^r , expression vector containing <i>Brucella abortus</i> L7/L12 fused at 3' end with sequence of cell wall protein M6 under the control of P _{nisA}	Ribeiro <i>et al.</i> (2002)
pCYT:CWP2	pWV01	pCYT:Nuc in which mature <i>nuc</i> was replaced by DNA encoding mature <i>G. lamblia</i> CWP2	This study
pSEC:LEISS:CWP2	pWV01	pSEC:LEISS:Nuc in which mature <i>nuc</i> was replaced by DNA encoding mature <i>G. lamblia</i> CWP2	This study
pVE5547:CWP2	pIL252	Em ^r , expression vector containing <i>G. lamblia</i> CWP2 fused at 3' end with sequence of cell wall protein M6 under the control of P _{nisA}	This study
pVE5547CORE:CWP2	pWV01	pSEC:Nuc backbone with Usp45 signal peptide and <i>nuc</i> replaced with DNA sequence encoding Usp45, CWP2 and M6 from pVE5547:CWP2	This study

**nuc* encodes the staphylococcal nuclease (also called heat-stable nuclease, thermonuclear nuclease and micrococcal nuclease) (Le Loir *et al.*, 1994). Cm, Chloramphenicol; Em, erythromycin.

Fig. 1. Expression constructs for CWP2 expression by *L. lactis*. P_{nisA} , nisin-inducible promoter; RBS, ribosome-binding site of the *usp45* gene; SP_{Usp} , signal peptide of Usp45; LEISS, sequence encoding the LEISSTCDA synthetic propeptide; M6, sequence encoding the cell wall M6 protein; T1T2, termination signal of *L. lactis* tryptophan operon.

(NIH, MD, USA), containing the *cwp2* gene (GenBank accession no. U28965) encoding *G. lamblia* CWP2 (Lujan *et al.*, 1995). *Giardia* lacks introns and in encysting *G. lamblia* trophozoites, cyst wall proteins are transcribed as intron-free mRNAs (Lujan *et al.*, 1995). Using different sets of primers listed in Table 2, CWP2 DNA fragments were amplified to be incorporated into the different vectors. All PCR products were resolved by agarose gel electrophoresis (0.8%) and visualized by ethidium bromide. DNA bands of interest were purified from the gel, using a GFX PCR DNA and Gel Band Purification Kit (Pharmacia Biotech), according to the manufacturer's recommendations.

Primers 1 and 2 were used with the pCYT and pSEC:LEISS vectors. Amplified CWP2 fragments were digested with *NsiI* and *EcoRI*, and ligated to *NsiI*/*EcoRI*-digested pCYT:Nuc or pSEC:LEISS:Nuc to obtain pCYT:CWP2 and pSEC:LEISS:CWP2, respectively. To obtain a cell-wall-anchored form of CWP2, *cwp2* was cloned into pVE5547 using primers 3 and 4. pVE5547 is an expression plasmid previously described by Piard *et al.* (1997a, b). The CWP2 PCR fragment was digested with *Sall* and *EcoRV* enzymes and cloned directly into *Sall*/

EcoRV-digested pVE5547:L7/L12, resulting in pVE5547:CWP2, encoding an in-frame fusion of CWP2 with the M6 cyst wall anchor sequence (CWA_{M6}). However, pVE5547 is a large theta-replicating plasmid that is difficult to manipulate (Dieye *et al.*, 2001). Therefore, an SP_{Usp} -CWP2- CWA_{M6} cassette was transferred from pVE5547:CWP2 into a pGK derivative, a smaller *E. coli* shuttle vector that is easier to manipulate (Bermudez-Humaran *et al.*, 2002, 2003; Kok *et al.*, 1984). Briefly, the SP_{Usp} -CWP2- CWA_{M6} cassette was amplified by PCR from pVE5547:CWP2 using primers 5 and 6 and digested with *BamHI* and *EcoRI*. The digested cassette fragment was then cloned into the pGK backbone purified from pCYT:Nuc digested with *BglII* and *EcoRI*, resulting in plasmid pVE5547CORE:CWP2. *BglII* was used to create a compatible end for the *BamHI* site of the SP_{Usp} -CWP2- CWA_{M6} cassette. A pre-existing *BamHI* site was present elsewhere within pCYT:Nuc.

Electroporation. Electroporation was performed as described by Holo & Nes (1995) with modifications as suggested by Geller *et al.* (2001). The electroporation was carried out using a Bio-Rad Gene Pulsar II Electroporator apparatus at 25 mF, 2.00 kV and a Gene Controller set at 200 Ω using 0.2 cm gap electroporation cuvettes. Immediately after electroporation, the cells were resuspended in M17 containing 15% sucrose, 1% glycine and supplemented with 20 mM $MgCl_2$ and 2 mM $CaCl_2$, and incubated at 30 °C for 2 h without any agitation to allow the cells to recover from the electroporation. After recovery, the cells were plated on GM17 agar plates containing antibiotics and incubated for 1–2 days at 30 °C.

Nisin induction. *L. lactis* strains were grown without agitation at 30 °C overnight in GM17 medium containing antibiotics. Selected clones were inoculated in fresh GM17 at a 1:50 dilution and grown until early/mid-exponential phase, which corresponds to an OD_{600} of 0.3–0.5. Induction was carried out using different concentrations (0.1, 1 or 10 ng ml^{-1}) of nisin (Sigma). After induction, cells were grown without agitation at 30 °C for 1–3 h before performing protein extraction from the induced cells.

Growth of recombinant lactococcal cells in the presence of nisin. Overnight cultures of *L. lactis* strains were diluted 1:50 in fresh GM17 medium containing antibiotics. The bacteria were grown to an OD_{600} of 0.4 and induced with 10 ng nisin ml^{-1} , which was previously determined to be the optimal amount of nisin for CWP2 expression. Subsequently, the OD_{600} of all cultures was measured prior to induction and subsequently every half hour for up to 7 h after induction using a Biochrom Ultraspec 2000 Pro UV/Visible Light spectrophotometer. Samples (1 ml) were collected from each bacterial strain under sterile conditions to produce a 7 h growth curve.

Protein extraction. Protein extracts were prepared from exponentially growing cultures as described by Le Loir *et al.* (1998). For cell

Table 2. Primers used in this study

Restriction enzyme sites incorporated in the primers are shown underlined. CA nucleotides (in bold) were added after the restriction site in Primer 1 to ensure the correct reading frame for integration into the vector.

Primer	Direction	Sequence [5'–3'] (restriction enzyme)
1	Sense	GCTATGCATCAGCTTGGCCCTGCCACCGAGGAG (<i>NsiI</i>)
2	Antisense	GGTGTA <u>GAATTC</u> TCAGTGGTCGTGGTCGTGGTCCCGCGGCACTGGACATGAATCTC (<i>EcoRI</i>)
3	Sense	GCTGTCGACATGGCTTGCCCTGCCACCGAGGAG (<i>Sall</i>)
4	Antisense	AGCTAGCGATATCTCGAGATCGATGGTGTGCACTGGACATGAATCTC (<i>EcoRV</i>)
5	Sense	ACAGCTCCAGGATTCAGTCTTATAACTATACTG (<i>BamHI</i>)
6	Antisense	AGAACTAGTGAATTCCTCCCGTTGTTAGTTTGTG (<i>EcoRI</i>)

fractionation, 10 ml *L. lactis* culture was centrifuged for 5 min at 6000 g at 4 °C and both the supernatant and cell pellet fractions were collected for immunoblot analysis.

Immunoblotting. To determine if CWP2 was produced after nisin induction of the vector promoter, immunoblotting was performed. *L. lactis* lysate samples were boiled for 10 min in an equal volume of reducing sample buffer and applied in volumes of 25 µl. Proteins were separated on a 4 % stacking gel and 12 % separating gel using a Bio-Rad Protean II electrophoresis unit. Resolved proteins were transferred onto a nitrocellulose membrane using a mini-gel Bio-Rad transfer apparatus set at 100 V for 1 h at 4 °C. Non-specific binding sites on the blotted nitrocellulose membrane were blocked with TBS containing 1 % Tween-20 (TTBS) and 5 % skim milk overnight. The nitrocellulose membrane was then washed with TTBS and incubated for 1·5 h at room temperature with mAb 8C5.C11 diluted 1:1500, or rabbit polyclonal 10F5 antibody diluted 1:3500. The polyclonal antibody 10F5 is specific against M6 (SIGA), while mAb 8C5.C11 is specific against CWP2 (Campbell & Faubert, 1994a). Membranes were then washed with TTBS and subsequently incubated with a goat anti-mouse horseradish-peroxidase-conjugated secondary antibody (1:3000; Amersham Pharmacia) or with a goat anti-rabbit horseradish-peroxidase-conjugated secondary antibody (1:5000; Cedar Lane Labs) for 1 h. Probed proteins were visualized by chemiluminescence using Super Signal West Pico Chemiluminescent Substrate (Pierce), according to the manufacturer's recommendations. They were subsequently exposed to a Kodak Scientific Imaging X-OMAT AR film for 5–10 min and developed in a Kodak M35A Film Processor.

Immunofluorescence. To confirm the presence of the CWP2-M6 fusion protein on the cell surface of recombinant lactococci, cells were examined by immunofluorescence as described by Cortes-Perez *et al.* (2003). Briefly, 5 ml nisin-induced *L. lactis* cells were harvested at an OD₆₀₀ of 0·5–0·6, corresponding to mid-exponential phase. The cells were incubated overnight at room temperature with mAb 8C5.C11 (1:1500), specific against CWP2, diluted in PBS/1 % BSA. After three washes with PBS/Tween 0·05 %, the immune complex was incubated for 6 h at room temperature with a goat anti-mouse IgG conjugated to fluorescein isothiocyanate diluted 1:100. Cells were washed three times. Smears were prepared, air-dried and heat-fixed. A cover slip was added to the slides and mounted using Vectashield mounting medium (Vector Labs). The slides were examined under UV illumination using a Nikon Eclipse 800 compound microscope at ×100 magnification with an FITC filter (502 nm).

Preparation of *L. lactis* for immunization. Selected clones were inoculated in fresh GM17 at 1:50 dilution and grown until early/mid-exponential phase, corresponding to an OD₆₀₀ of 0·3–0·4. Lactococcal cell pellets were harvested by centrifugation at 3000 g for 15 min at 4 °C. Cells were washed three times with sterile PBS and were subsequently resuspended in GM17 (no antibiotics) to a final concentration of $2·5 \times 10^{10}$ c.f.u. ml⁻¹.

Immunization of BALB/c mice. To assess the immunogenicity of recombinant lactococcal cells, 26 BALB/c mice were divided into four groups and immunized by oral gavage. The first group (*n*=7) was not immunized and served as a negative control. The second group (*n*=6) was immunized with *htrA*-NZ9000 which served as a bacterial control group. The third group (*n*=6) was immunized with lactococcal cells expressing M6 protein (C-terminal half of the molecule) fused to *B. abortus* L7/L12 antigen. This group served as a control for M6 protein being expressed on the cell surface. The last group of mice (*n*=7) received lactococcal cells carrying pVE5547CORE:CWP2 and expressing CWP2-M6 fusion protein on the cell surface. Every mouse received 10^{10} c.f.u. bacteria per dose in a volume of 0·4 ml GM17 medium. Mice received six doses within a 30 day period (days 1, 2, 3, 15, 16 and 17).

Collection of gut lavage fluids. Gut lavage fluids were obtained by flushing the excised small intestine with 5 ml PBS containing 50 mM EDTA and 1 % BSA (Boehringer Mannheim) as described by Wu & Russell (1993). Subsequently, lavage samples were vortexed and centrifuged at 1000 g for 15 min at 4 °C. Supernatants were removed and 50 µl 100 mM PMSF (Sigma) was added to the supernatants before they were vortexed and spun at 5000 g for 20 min at 4 °C. Supernatants were dispensed into aliquots and frozen at -20 °C. until further use.

CWP2-specific antibody responses. Anti-CWP2 IgA antibodies in intestinal lavage fluids were measured by ELISA as described by Medaglini *et al.* (2001). Flat-bottom microtitre ELISA plates (Falcon) were coated with 100 µl *G. lamblia* encysting cell antigen at a concentration of 1 µg ml⁻¹ in PBS and blocked with PBS/1 % albumin. Encysting cell antigen was prepared as described by Larocque *et al.* (2003) and contains native CWP2 expressed by *G. lamblia*. Intestinal lavage samples were diluted 1:2 followed by two-fold dilutions. Plates were incubated at room temperature for 1 h. After washing, 100 µl anti-mouse IgA diluted 1:10 000 (Caltag Laboratories) conjugated to horseradish peroxidase was added to the wells. After the plates were incubated for 1 h at room temperature, the substrate 3,3',5,5'-tetramethylbenzidine (Sigma) was added to the wells. Plates were read at 450 nm after 20 min using an EL309 Microplate Reader (Bio-Tek Instruments). Results are expressed as the ratio of the amount of CWP2 specific antibodies (µg) to the amount of total antibody (mg) in the sample for IgA. The amount of CWP2-specific antibodies cannot be compared alone between groups since the amount of IgA in intestinal lavage fluids may vary from mouse to mouse which may influence the amount of specific antibodies. As such, the amount of CWP2-specific antibodies was normalized to the total amount of antibodies detected within the sample in the form of a ratio to compare groups.

Challenge of BALB/c mice with *G. muris* cysts. Twelve BALB/c mice were divided into four groups of three mice each. Mice were immunized as described above. Thirteen days after the last dose (day 30), all mice were challenged by gavage without anaesthesia with 6×10^5 live *G. muris* cysts. The *Giardia* mouse model of infection was selected for two reasons: first, mice can be challenged with *G. muris* cysts and second, cross-reactivity exists between *G. muris* and *G. lamblia* cyst antigens. Campbell & Faubert (1994a) were able to stain both *G. lamblia* and *G. muris* cysts using mAb 8C5.C11, thus indicating the existence of a *G. muris* CWP2 murine homologue containing conserved epitopes. Cyst output was followed for all mice for a period of 15 days post-challenge. No adverse signs were noted in any mice and no mice died during challenge with *G. muris*.

Isolation of cysts from faecal specimens. For a period of 15 days (days 6–20 post-challenge), individual mice were placed in separate cages and the faecal pellets excreted over a 1 h period were collected in 12 × 75 mm glass borosilicate tubes. Cysts were isolated by a sucrose gradient centrifugation technique as described previously (Campbell & Faubert, 1994b). Briefly, faeces were collected, weighed, emulsified in PBS, layered on sucrose (specific gravity 1·12) and centrifuged at 400 g for 15 min. Cysts were counted with a Spencer Bright Line haemocytometer (Fisher Scientific).

Statistics. Statistical significance was determined by Student's unpaired *t*-test performed using SigmaStat 3.11 statistical analysis software from SYSTAT Software Inc. The significance level was set at *P* ≤ 0·001.

RESULTS

CWP2 Expression in *L. lactis*

To determine whether CWP2 can be expressed by *L. lactis*, lactococcal cells were engineered with three different expression vectors. These vectors were induced with varying nisin concentrations and bacterial lysates were analysed. As expected, in the absence of nisin, CWP2 was not expressed, demonstrating tight regulatory control of the expression vector by nisin (Fig. 2a–c). However, with the addition of nisin at a concentration of 1 or 10 ng ml⁻¹, CWP2 was expressed (Fig. 2a–c). With the Western blot assay, a band at ~26 kDa was detected using CWP2-specific mAb 8C5.C11 for lactococcal cells harbouring vector pCYT expressing CWP2 (Fig. 2a) or vector pSEC:LEISS expressing CWP2 (Fig. 2b), regardless of the amount of nisin used for induction. This band at 26 kDa corresponds to the molecular mass of CWP2. In addition, when lactococcal cells were transformed with vector pVE5547CORE and induced with 1 or 10 ng nisin ml⁻¹, a band at the 52 kDa mark was detected (Fig. 2c). The increase in molecular mass of CWP2 is due to pVE5547CORE expressing CWP2 as a fusion protein with M6 (~26 kDa), therefore doubling its molecular mass. Using rabbit polyclonal antibodies specific

Fig. 2. Western blot analysis of CWP2 expression with anti-CWP2 mAb 8C5.C11. (a) pCYT expressing CWP2 in cytosol; (b) pSEC:LEISS secreting CWP2 into culture medium; (c) pVE5547CORE expressing CWP2 anchored to the cell surface. Protein extracts were prepared 1 h after induction with 0, 0.1, 1 or 10 ng nisin ml⁻¹. Data presented are representative of three independent experiments.

Fig. 3. Localization of CWP2 expressed by *L. lactis* by Western blotting. (a) pCYT; (b) pSEC:LEISS; (c) pVE5547CORE. The expression of CWP2 was confirmed using mAb 8C5.C11 to be restricted within the cell pellet fraction (C) or the culture medium supernatant fraction (S). Protein extracts in induced exponential-phase cultures of lactococci were prepared 2 h after they had been induced with 10 ng nisin ml⁻¹. Data presented are representative of three independent experiments.

against the M6 anchoring protein, a similar band of ~52 kDa was detected, confirming that CWP2 is expressed as a CWP2-M6 fusion protein when vector pVE5547CORE is used (data not shown).

Specificity of subcellular localization of recombinant CWP2 in *L. lactis*

Since we wished to develop *L. lactis* as an antigen delivery system for CWP2, lactococcal cells were engineered for expression of CWP2 within the cytosol, secreted in culture medium or expressed on the cell surface. All vectors were

Fig. 4. Immunofluorescence assay confirming expression of CWP2 on the cell membrane. Lactococcal cells with vector pVE5547:Nuc or pVE5547CORE:CWP2 were incubated with mAb 8C5.C11 specific to CWP2 (a, b) or mAb 10F5 specific to M6 (c, d). The secondary antibody was an anti-mouse antiserum conjugated to fluorescein isothiocyanate (FITC). Slides were observed under UV light using a Nikon Eclipse 800 compound microscope (Nikon) at $\times 100$ magnification with an FITC filter (502 nm). Lactococcal cells engineered with pVE5547:Nuc served as a negative control.

induced with $10 \text{ ng nisin ml}^{-1}$. As expected, CWP2 was expressed in all three locations, respecting the selectivity of the vector used. With vector pCYT, CWP2 was expressed in the cytosol only (Fig. 3a). Vectors pSEC:LEISS (Fig. 3b) and pVE5547CORE (Fig. 3c) secreted CWP2 into the supernatant and expressed it on the cell surface, respectively. A band at 26 kDa appeared when CWP2 was expressed in the cytosol or secreted into the culture medium (Fig. 3b). When CWP2 was anchored to the cell surface, a band appeared at 52 kDa due to the fusion of CWP2 with M6 (Fig. 3c).

Cell surface expression of CWP2

To confirm the results obtained by Western blotting on the expression of CWP2 on the cell surface, we performed an IFA using lactococcal cells engineered with pVE5547CORE vector. For this purpose, mAb 8C5.C11, which is specific to CWP2, or mAb 10F5, which is specific to M6 anchoring protein, was used. In this assay, *L. lactis* transformed with vector pVE5547:Nuc served as a negative control for mAb 8C5.C11 and as a positive control for mAb 10F5. This vector expresses *Staphylococcus aureus* nuclease A as a fusion protein with M6. Lactococcal cells carrying vector pVE5547:Nuc did not stain with mAb 8C5 (Fig. 4a), but did stain positive with mAb 10F5 (Fig. 4c). On the other hand, cells engineered with vector pVE5547: CORE, expressing CWP2 as a fusion protein with M6, stained positive with both mAbs (Fig. 4b and d).

Mucosal immune response to recombinant CWP2

To determine whether recombinant lactococcal cells can adequately deliver CWP2 to intestinal mucosal sites, we infected mice with engineered lactococcal cells and tested

intestinal lavages for the presence of anti-CWP2 antibodies. Using ELISA, we measured the level of CWP2-specific IgA antibodies. Significant levels of CWP2-specific IgA ($P < 0.001$) were observed with intestinal lavage samples from mice infected with lactococcal cells expressing CWP2 (CWP2 group) compared to negative control animals which were not infected (Fig. 5). The mean ratio of the CWP2 group was fivefold higher than the non-infected control mice [$203.93 \pm 24.31 \mu\text{g (mg total IgA)}^{-1}$ versus $41.07 \pm 8.80 \mu\text{g (mg total IgA)}^{-1}$]. All mice (7 out of 7) within the CWP2 group were positive for the presence of

Fig. 5. CWP2-specific IgA antibody response. Results are expressed as the ratio of the amount of CWP2-specific antibodies (μg) to the amount of total IgA antibody (mg) in the sample. Intestinal lavage samples were tested by ELISA. The dotted line represents the cutoff point and is calculated as the mean ± 2 SEM of naive animals. The asterisk indicates significance at $P < 0.001$. Data presented are representative of two independent experiments.

CWP2-specific intestinal IgA using the mean of the values of the non-infected control mice plus 2 SEM as a cut-off (dotted line). No significant levels of CWP2-specific IgA were observed with the other groups, thus indicating that lactococcal cells by themselves (NZ9000), the presence of the *Streptococcus pyogenes* M6 anchor protein or the *B. abortus* L7/L12 protein (L7/L12) do not induce antibodies which cross-react with the CWP2 antigen. The NZ9000 group had a mean ratio of $95.13 \pm 22.66 \mu\text{g (mg total IgA)}^{-1}$ whereas the L7/L12 group had a mean ratio of $69.40 \pm 17.60 \mu\text{g (mg total IgA)}^{-1}$.

Challenge infection with live *G. muris* cysts

We tested if the CWP2-specific IgA elicited by the recombinant *L. lactis* cells could interfere with cyst shedding. Recombinant lactococci was first administered to mice, and then 14 days after the last administration mice were challenged with live *G. muris* cysts. Faeces were collected for a period of 20 days. A significant reduction ($P < 0.001$) in cyst output was observed for the group of mice infected with lactococcal cells expressing CWP2 as compared to the naive group of mice. The CWP2 group shed $1.16 \times 10^5 \pm 0.12 \times 10^5$ cysts (g faeces) $^{-1}$ as opposed to $3.11 \times 10^5 \pm 0.31 \times 10^5$ cysts (g faeces) $^{-1}$ for the non-immunized group. This difference in cyst output represents a 63 % reduction. With regard to the other two groups of lactococcal-treated mice, neither group shed significantly less cysts when compared to the non-immunized group. NZ9000 mice, which were used to examine whether bacteria alone can affect cyst shedding, shed $3.01 \times 10^5 \pm 0.30 \times 10^5$ cysts (g faeces) $^{-1}$. Meanwhile, L7/L12 immunized mice, which were used to examine whether M6 protein alone may affect cyst shedding, shed $3.56 \times 10^5 \pm 0.36 \times 10^5$ cysts (g faeces) $^{-1}$.

DISCUSSION

L. lactis has served as an efficient expression vehicle for several heterologous proteins (Enouf *et al.*, 2001; Gilbert *et al.*, 2000; Miyoshi *et al.*, 2002). We report, for the first time, on the expression of an intestinal parasite protein by *L. lactis*. This protein originates from the protozoan parasite *G. lamblia* which is a major pollutant of surface water (Faubert *et al.*, 2002). CWP2 is an important building block of the cyst wall which protects the parasite against the harsh external environment (Lujan *et al.*, 1995). Using the appropriate vector and the optimal concentration of nisin, *L. lactis* can have CWP2 expressed intracellularly, secreted into culture medium or anchored to the cell surface. In addition, we report that intragastric immunization of BALB/c mice with recombinant lactococcal cells was able to deliver CWP2 to the mucosal site. In a pilot experiment, mice immunized with recombinant lactococci expressing CWP2 on the cell surface and challenged with live *Giardia* cysts shed a significantly lower number of cysts.

L. lactis has been used to express successfully proteins from infectious agents and a variety of cytokines (Nouaille *et al.*, 2003). This study with CWP2 is only the second report in

the literature documenting the expression of a protein of parasitic origin. The other study described a hybrid recombinant protein derived from the N-terminal end of the glutamate-rich protein and the C-terminal portion of the merozoite surface protein 3 of *Plasmodium falciparum* which was successfully expressed and secreted by *L. lactis* (Theisen *et al.*, 2004). However, in that study, *L. lactis* was used as an expression system, not as a live vaccine delivery system.

CWP2 is considered to be one of the major proteins involved in the composition of the cyst wall, a structure allowing *G. lamblia* to survive outside of its host in the environment. CWP2 is generally not secreted into the medium when expressed by the parasite, but it can be found at the cell surface within the cyst wall. Using vector pVE5547CORE together with nisin as an inducing agent, CWP2 was expressed by *L. lactis* and anchored to the cell surface, mimicking how CWP2 is normally expressed by the parasite. This form of expression may have important implications for vaccine development since the immune system normally sees CWP2 in this context (i.e. displayed on the cell surface). CWP2 was also expressed in the cytosol of *L. lactis* or secreted into the culture medium. We did not observe any advantage in having CWP2 expressed in any one particular form with respect to growth rates. No significant differences in growth rates were seen between lactococcal cells harbouring different vectors when CWP2 expression was induced. These findings reinforce the versatility of *L. lactis* as an expression vehicle for foreign proteins, since *L. lactis* can be targeted to multiple subcellular locations using nisin-inducible expression system vectors (de Vos, 1999; Miyoshi *et al.*, 2002; Nouaille *et al.*, 2003).

Although we demonstrated that CWP2 can be expressed at different subcellular locations, we were predominantly interested in having CWP2 anchored to the cell surface for the purpose of developing an oral vaccine. To minimize proteolysis of CWP2 when expressed on the cell surface, we utilized *L. lactis* *htrA*-NZ9000, a mutant strain where the *htrA* gene has been inactivated. *htrA* encodes an extracellular housekeeping protease responsible for the degradation of exported and fusion proteins (Frees *et al.*, 2001; Nilsson *et al.*, 1994; Poquet *et al.*, 2000). To confirm this, Western blotting revealed one major predominant CWP2 band when *L. lactis* *htrA*-NZ9000 was employed, whereas multiple CWP2 bands were detected with *L. lactis* NZ9000 (data not shown). The presence of multiple bands were indicative of CWP2 being degraded, supporting the idea that an *htrA*-deficient *L. lactis* strain provides higher protein stability at the cell surface (Miyoshi *et al.*, 2002).

Previous studies comparing different forms of antigen expression and subsequent immune responses have indicated that cell-surface expression results in a better immune response than intracellular or secreted forms of antigen presentation (Bermudez-Humaran *et al.*, 2004; Cortes-Perez *et al.*, 2003). It is believed that the bacterial cell wall may provide an adjuvant activity, enhancing the host

immunological response (Vitini *et al.*, 2000). In addition, cell-surface-displayed antigens are less soluble than their secretable counterparts in most cases (Bernasconi *et al.*, 2002; Lindholm *et al.*, 2004) and may be less exposed to degrading or denaturing agents, such as proteinases, or acid-rich environments such as the stomach (Nouaille *et al.*, 2003; Piard *et al.*, 1997a, b). Lactococcal cells expressing CWP2 on the cell surface were immunogenic, generating a significant amount of CWP2-specific secretory IgA antibodies detected from intestinal lavage samples of mice immunized with recombinant lactococci. Giardiasis in humans and in mice results in the production of anti-giardial antibodies of the IgA and IgM isotypes in mucosal secretions and IgG in serum (Faubert *et al.*, 2002). Moreover, specific antibody production in mucosal sites correlates with giardial clearance (Daniels & Belosevic, 1994; Heyworth, 1992, 1986; Heyworth *et al.*, 1987; Nash *et al.*, 1987; Snider & Underdown, 1986). However, the physiological role of IgA and IgM in clearing *Giardia* infection from the intestine is unknown.

Mice immunized with recombinant lactococcal cells expressing CWP2 on the cell surface released 63 % less cysts per gram faeces than non-immunized control mice. Our observed reduction level compares quite well with the 75 % reduction level demonstrated from a previous study where mice were orally immunized with the full-length 39 kDa form of recombinant CWP2 produced by *E. coli* (Larocque *et al.*, 2003). Since both stages of the *Giardia* life cycle occur simultaneously in the intestine (Campbell & Faubert, 1994b), the immune clearance of the cysts from the intestinal lumen may be due to antibodies directed at the trophozoite stage. However, the delivery of CWP2 to the intestinal mucosa of mice by engineered lactococcal cells gave rise to cyst-specific antibodies only. No trophozoite-specific antibodies were detected at the intestinal mucosal site. Moreover, the number of trophozoites was compared between the different groups of mice (data not shown). No differences were observed between the CWP2-immunized and the non-immunized group, indicating that the trophozoite population was not targeted by the immune response. Thus, reduction of cyst shedding is not due to the immune elimination of the trophozoite stage, but due to the antibodies directed against CWP2, which is a major component of the cyst wall structure, thereby inhibiting the formation of the cyst structure.

Experiments are in progress to elucidate the immune mechanisms involved.

ACKNOWLEDGEMENTS

The authors would like to thank Philippe Langella for helpful discussions during the course of this work and for reading this manuscript. This work was supported by an operating grant of the National Sciences and Engineering Research Council of Canada (NSERC) to G. M. Faubert. Research at the Institute of Parasitology is supported by Le Fonds québécois de la recherche sur la nature et les technologies (FQRNT), Québec, Canada.

REFERENCES

- Bermudez-Humaran, L. G., Langella, P., Miyoshi, A., Gruss, A., Guerra, R. T., Montes de Oca-Luna, R. & Le Loir, Y. (2002). Production of human papillomavirus type 16 E7 protein in *Lactococcus lactis*. *Appl Environ Microbiol* **68**, 917–922.
- Bermudez-Humaran, L. G., Cortes-Perez, N. G., Le Loir, Y., Gruss, A., Rodriguez-Padilla, C., Saucedo-Cardenas, O., Langella, P. & Montes de Oca-Luna, R. (2003). Fusion to a carrier protein and a synthetic propeptide enhances E7 HPV-16 production and secretion in *Lactococcus lactis*. *Biotechnol Prog* **19**, 1101–1104.
- Bermudez-Humaran, L. G., Cortes-Perez, N. G., Le Loir, Y., Alcocer-Gonzalez, J. M., Tamez-Guerra, R. S., de Oca-Luna, R. M. & Langella, P. (2004). An inducible surface presentation system improves cellular immunity against human papillomavirus type 16 E7 antigen in mice after nasal administration with recombinant lactococci. *J Med Microbiol* **53**, 427–433.
- Bernasconi, E., Germond, J. E., Delley, M., Fritzsche, R. & Corthesy, B. (2002). *Lactobacillus bulgaricus* proteinase expressed in *Lactococcus lactis* is a powerful carrier for cell wall-associated and secreted bovine beta-lactoglobulin fusion proteins. *Appl Environ Microbiol* **68**, 2917–2923.
- Campbell, J. D. & Faubert, G. M. (1994a). Recognition of *Giardia lamblia* cyst-specific antigens by monoclonal antibodies. *Parasite Immunol* **16**, 211–219.
- Campbell, J. D. & Faubert, G. M. (1994b). Comparative studies on *Giardia lamblia* encystation *in vitro* and *in vivo*. *J Parasitol* **80**, 36–44.
- Carr, F. J., Chill, D. & Maida, N. (2002). The lactic acid bacteria: a literature survey. *Crit Rev Microbiol* **28**, 281–370.
- Cortes-Perez, N. G., Bermudez-Humaran, L. G., Le Loir, Y., Rodriguez-Padilla, C., Gruss, A., Saucedo-Cardenas, O., Langella, P. & Montes de Oca-Luna, R. (2003). Mice immunization with live lactococci displaying a surface anchored HPV-16 E7 oncoprotein. *FEMS Microbiol Lett* **229**, 37–42.
- Cortes-Perez, N. G., Azevedo, V., Alcocer-Gonzalez, J. M., Rodriguez Padilla, C., Tamez-Guerra, R. S., Corthier, G., Gruss, A., Langella, P. & Bermudez-Humaran, L. G. (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.
- Daniels, C. W. & Belosevic, M. (1994). Serum antibody responses by male and female C57Bl/6 mice infected with *Giardia muris*. *Clin Exp Immunol* **97**, 424–429.
- de Ruyter, P. G., Kuipers, O. P. & de Vos, W. M. (1996). Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl Environ Microbiol* **62**, 3662–3667.
- de Vos, W. M. (1999). Gene expression systems for lactic acid bacteria. *Curr Opin Microbiol* **2**, 289–295.
- Dieye, Y., Usai, S., Clier, F., Gruss, A. & Piard, J. C. (2001). Design of a protein-targeting system for lactic acid bacteria. *J Bacteriol* **183**, 4157–4166.
- Eckmann, L. (2003). Mucosal defences against *Giardia*. *Parasite Immunol* **25**, 259–270.
- Enouf, V., Langella, P., Commissaire, J., Cohen, J. & Corthier, G. (2001). Bovine rotavirus nonstructural protein 4 produced by *Lactococcus lactis* is antigenic and immunogenic. *Appl Environ Microbiol* **67**, 1423–1428.
- Erlandsen, S. L., Bemrick, W. J., Schupp, D. E., Shields, J. M., Jarroll, E. L., Sauch, J. F. & Pawley, J. B. (1990). High resolution immunogold localization of *Giardia* cyst wall antigens using field emission SEM with secondary and backscatter electron imaging. *J Histochem Cytochem* **38**, 625–632.

- Faubert, G. M., Lee, P. & Abdul-Wahid, A. (2002). *Giardia duodenalis*. In *Infections of the Gastrointestinal Tract*, pp. 978–1006. Edited by M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg & R. L. Guerrant. New York: LWW Publishing.
- Frees, D., Varmanen, P. & Ingmer, H. (2001). Inactivation of a gene that is highly conserved in Gram-positive bacteria stimulates degradation of non-native proteins and concomitantly increases stress tolerance in *Lactococcus lactis*. *Mol Microbiol* **41**, 93–103.
- Geller, B. L., Wade, N., Gilberts, T. D., Hruby, D. E., Johanson, R. & Topisirovic, L. (2001). Surface expression of the conserved C repeat region of streptococcal M6 protein within the Pip bacteriophage receptor of *Lactococcus lactis*. *Appl Environ Microbiol* **67**, 5370–5376.
- Gibson, T. J. (1984). PhD thesis, University of Cambridge.
- Gilbert, C., Robinson, K., Le Page, R. W. & Wells, J. M. (2000). Heterologous expression of an immunogenic pneumococcal type 3 capsular polysaccharide in *Lactococcus lactis*. *Infect Immun* **68**, 3251–3260.
- Heyworth, M. F. (1986). Antibody response to *Giardia muris* trophozoites in mouse intestine. *Infect Immun* **52**, 568–571.
- Heyworth, M. F. (1992). Immunology of *Giardia* and *Cryptosporidium* infections. *J Infect Dis* **166**, 465–472.
- Heyworth, M. F., Carlson, J. R., Ermak, T. H. (1987). Clearance of *Giardia muris* infection requires helper/inducer T lymphocytes. *J Exp Med* **165**, 1743–1748.
- Hlavsa, M. C., Watson, J. C. & Beach, M. J. (2005). Giardiasis surveillance – United States, 1998–2002. *Morbidity Mortal Wkly Rep Surveill Summ* **54**, 9–16.
- Holo, H. & Nes, I. F. (1995). Transformation of *Lactococcus* by electroporation. *Methods Mol Biol* **47**, 195–199.
- Kok, J., van der Vossen, J. M. & Venema, G. (1984). Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. *Appl Environ Microbiol* **48**, 726–731.
- Langella, P., Le Loir, Y., Ehrlich, S. D. & Gruss, A. (1993). Efficient plasmid mobilization by pIP501 in *Lactococcus lactis* subsp. *lactis*. *J Bacteriol* **175**, 5806–5813.
- Langford, T. D., Housley, M. P., Boes, M., Chen, J., Kagnoff, M. F., Gillin, F. D. & Eckmann, L. (2002). Central importance of immunoglobulin A in host defense against *Giardia* spp. *Infect Immun* **70**, 11–18.
- Larocque, R., Nakagaki, K., Lee, P., Abdul-Wahid, A. & Faubert, G. M. (2003). Oral immunization of BALB/c mice with *Giardia duodenalis* recombinant cyst wall protein inhibits shedding of cysts. *Infect Immun* **71**, 5662–5669.
- Le Loir, Y., Gruss, A., Ehrlich, S. D. & Langella, P. (1994). Direct screening of recombinants in gram-positive bacteria using the secreted staphylococcal nuclease as a reporter. *J Bacteriol* **176**, 5135–5139.
- Le Loir, Y., Gruss, A., Ehrlich, S. D. & Langella, P. (1998). A nine-residue synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*. *J Bacteriol* **180**, 1895–1903.
- Le Loir, Y., Nouaille, S., Commissaire, J., Bretigny, L., Gruss, A. & Langella, P. (2001). Signal peptide and propeptide optimization for heterologous protein secretion in *Lactococcus lactis*. *Appl Environ Microbiol* **67**, 4119–4127.
- Lindholm, A., Smeds, A. & Palva, A. (2004). Receptor binding domain of *Escherichia coli* F18 fimbrial adhesin FedF can be both efficiently secreted and surface displayed in a functional form in *Lactococcus lactis*. *Appl Environ Microbiol* **70**, 2061–2071.
- Lujan, H. D., Mowatt, M. R., Conrad, J. T., Bowers, B. & Nash, T. E. (1995). Identification of a novel *Giardia lamblia* cyst wall protein with leucine-rich repeats. Implications for secretory granule formation and protein assembly into the cyst wall. *J Biol Chem* **270**, 29307–29313.
- Medaglini, D., Ciabattini, A., Spinosa, M. R., Maggi, T., Marcotte, H., Oggioni, M. R. & Pozzi, G. (2001). Immunization with recombinant *Streptococcus gordonii* expressing tetanus toxin fragment C confers protection from lethal challenge in mice. *Vaccine* **19**, 1931–1939.
- Miyoshi, A., Poquet, I., Azevedo, V. & 7 other authors (2002). Controlled production of stable heterologous proteins in *Lactococcus lactis*. *Appl Environ Microbiol* **68**, 3141–3146.
- Nash, T. E., Herrington, D. A., Losonsky, G. A. & Levine, M. M. (1987). Experimental human infections with *Giardia lamblia*. *J Infect Dis* **156**, 974–984.
- Nilsson, D., Lauridsen, A. A., Tomoyasu, T. & Ogura, T. (1994). A *Lactococcus lactis* gene encodes a membrane protein with putative ATPase activity that is homologous to the essential *Escherichia coli* *ftsH* gene product. *Microbiology* **140**, 2601–2610.
- Norton, P. M., Brown, H. W. & Le Page, R. W. (1994). The immune response to *Lactococcus lactis*: implications for its use as a vaccine delivery vehicle. *FEMS Microbiol Lett* **120**, 249–256.
- Norton, P. M., Brown, H. W., Wells, J. M., Macpherson, A. M., Wilson, P. W. & Le Page, R. W. (1996). Factors affecting the immunogenicity of tetanus toxin fragment C expressed in *Lactococcus lactis*. *FEMS Immunol Med Microbiol* **14**, 167–177.
- Norton, P. M., Wells, J. M., Brown, H. W., Macpherson, A. M. & Le Page, R. W. (1997). Protection against tetanus toxin in mice nasally immunized with recombinant *Lactococcus lactis* expressing tetanus toxin fragment C. *Vaccine* **15**, 616–619.
- Nouaille, S., Ribeiro, L. A., Miyoshi, A., Pontes, D., Le Loir, Y., Oliveira, S. C., Langella, P. & Azevedo, V. (2003). Heterologous protein production and delivery systems for *Lactococcus lactis*. *Genet Mol Res* **2**, 102–111.
- Piard, J. C., Hautefort, I., Fischetti, V. A., Ehrlich, S. D., Fons, M. & Gruss, A. (1997a). Cell wall anchoring of the *Streptococcus pyogenes* M6 protein in various lactic acid bacteria. *J Bacteriol* **179**, 3068–3072.
- Piard, J. C., Jimenez-Diaz, R., Fischetti, V. A., Ehrlich, S. D. & Gruss, A. (1997b). The M6 protein of *Streptococcus pyogenes* and its potential as a tool to anchor biologically active molecules at the surface of lactic acid bacteria. *Adv Exp Med Biol* **418**, 545–550.
- Pontes, D. S., Dorella, F. A., Ribeiro, L. A., Miyoshi, A., Le Loir, Y., Gruss, A., Oliveira, S. C., Langella, P. & Azevedo, V. (2003). Induction of partial protection in mice after oral administration of *Lactococcus lactis* producing *Brucella abortus* L7/L12 antigen. *J Drug Target* **11**, 489–493.
- Poquet, I., Saint, V., Seznec, E., Simoes, N., Bolotin, A. & Gruss, A. (2000). HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. *Mol Microbiol* **35**, 1042–1051.
- Ribeiro, L. A., Azevedo, V., Le Loir, Y., Oliveira, S. C., Dieye, Y., Piard, J. C., Gruss, A. & Langella, P. (2002). Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Appl Environ Microbiol* **68**, 910–916.
- Robinson, K., Chamberlain, L. M., Schofield, K. M., Wells, J. M. & Le Page, R. W. (1997). Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nat Biotechnol* **15**, 653–657.
- Robinson, K., Chamberlain, L. M., Lopez, M. C., Rush, C. M., Marcotte, H., Le Page, R. W. & Wells, J. M. (2004). Mucosal and cellular immune responses elicited by recombinant *Lactococcus lactis* strains expressing tetanus toxin fragment C. *Infect Immun* **72**, 2753–2761.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Snider, D. P. & Underdown, B. J. (1986). Quantitative and temporal analyses of murine antibody response in serum and gut secretions to infection with *Giardia muris*. *Infect Immun* **52**, 271–278.

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W. & Remaut, E. (2000). Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **289**, 1352–1355.

Terzaghi, B. E. & Sandine, W. E. (1975). Improved medium for lactic streptococci and their bacteriophages. *Appl Environ Microbiol* **29**, 807–813.

Theisen, M., Soe, S., Brunstedt, K., Follmann, F., Bredmose, L., Israelsen, H., Madsen, S. M. & Druilhe, P. (2004). A *Plasmodium falciparum* GLURPMSP3 chimeric protein; expression in *Lactococcus*

lactis, immunogenicity and induction of biologically active antibodies. *Vaccine* **22**, 1188–1198.

Vitini, E., Alvarez, S., Medina, M., Medici, M., de Budeguer, M. V. & Perdigon, G. (2000). Gut mucosal immunostimulation by lactic acid bacteria. *Biocell* **24**, 223–232.

Wells, J. M., Wilson, P. W., Norton, P. M., Gasson, M. J. & Le Page, R. W. (1993). *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. *Mol Microbiol* **8**, 1155–1162.

Wells, J. M., Robinson, K., Chamberlain, L. M., Schofield, K. M. & Le Page, R. W. (1996). Lactic acid bacteria as vaccine delivery vehicles. *Antonie van Leeuwenhoek* **70**, 317–330.

Wu, H. Y. & Russell, M. W. (1993). Induction of mucosal immunity by intranasal application of a streptococcal surface protein antigen with the cholera toxin B subunit. *Infect Immun* **61**, 314–322.