

Recombinant *Lactobacillus johnsonii* as a mucosal vaccine delivery vehicle

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Abstract

Lactobacilli are considered to be safe organisms making them attractive as vehicles for oral vaccination. We report that *Lactobacillus johnsonii* (Lj) partially survived simulated gastric conditions in vitro, suggesting that it could be used as an oral vaccine delivery vehicle. In order to test this approach, we used the cell wall anchored proteinase PrtB, isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus* as a model antigen. Using a new vector system, we demonstrated expression of both proteinase PrtB alone and a mimotope peptide derived from tetanus toxin integrated in the sequence of proteinase PrtB (TTmim–PrtB fusion protein) on the surface of Lj. Oral immunisation of mice with recombinant Lj, expressing the TTmim–PrtB fusion protein induced a systemic IgG response against Lj and recombinantly expressed proteinase PrtB but no antibody response against the tetanus toxin mimotope suggesting that the mimotope was not sufficiently immunogenic to induce an immune response. Interestingly, a proteinase PrtB specific fecal IgA response was also induced, indicating that the proteinase PrtB fusion protein expressed as a cell surface protein on Lj can induce both systemic and local mucosal immune responses. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Lactobacillus johnsonii*; Proteinase B; Tetanus mimotope

1. Introduction

In recent years, new vaccination strategies have been developed in order to avoid disadvantages associated with parenterally administered vaccines [1]. As many viral, bacterial and parasitic pathogens enter the body via mucosal surfaces, oral administration of antigens might simulate the natural route of infection and be a more effective method of immunisation [2]. In addition, oral vaccination applied on a large scale, is relatively inexpensive and frequently induces both local and systemic immune responses, resulting in an effective elimination of pathogens [3].

Lactobacillus strains have a number of properties making them attractive candidates for oral vaccination. Since centuries they have been used for food processing and food preservation and are considered to be safe organisms for hu-

man consumption. In addition, some strains of *Lactobacillus* have the ability to colonise certain parts of the intestinal mucosa [3,4]. It has also been observed that particular strains of *Lactobacillus* have an adjuvant effect by enhancing antigen specific immune responses when administered in combination with antigen [5]. Several systems have been described recently using *Lactobacillus* as a carrier for expressing foreign antigens in a form that can be presented to and processed by the immune system of the mammalian host. In a murine model, it has been shown that after oral administration a mucosal as well as a systemic immune response can be achieved against tetanus toxin fragment C produced intracellularly in lactobacilli [6]. These observations prompted us to use one strain of *Lactobacillus*, *Lactobacillus johnsonii* (Lj) for the development of an oral vaccine.

In this study, we analysed the ability of Lj to express heterologous antigens and to act as an antigen delivery vehicle for oral immunisation. As it is probable that antigen associated with the bacterial surface may be more immunogenic [7], a vector termed pMD112 was used to express antigens on the bacterial cell surface. This vector allows the incorporation of the antigen into the cell wall by fusing it to the cell

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wall anchored proteinase PrtB isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus* [8]. As model antigens, we used mimotopes, i.e. small, constrained peptides that mimic a portion of a conformational epitope [9,10], and have been shown to monitor an immune response in mice. One of the mimotopes, termed tetanus toxin mimotope (TTmim), is 11 amino acids in length and shows sequence homology to tetanus toxin fragment A (amino acids 141–150). Our data indicate that the TTmim was expressed as a fusion protein with proteinase PrtB on the surface of Lj (TTmim–PrtB) and after oral administration significant anti-PrtB antibodies but no anti-TTmim antibody response were induced.

2. Materials and methods

2.1. Plasmids and oligonucleotides

Plasmid pMD112 was constructed by inserting into pNZ124 [11], the cell wall anchored proteinase *prtB* gene of *L. delbrueckii* subsp. *bulgaricus* (Germond and Delley, unpublished data). TTmim, an 11 mer mimotope recognised by a preparation of affinity purified human anti-TT serum antibodies, was isolated by using random peptide libraries and phage display technology [12]. This mimotope displays homology to amino acids 141–150 of tetanus toxin fragment A. A nonamer mimotope, ϵ 4mim, recognised by the monoclonal mouse anti-human IgE antibody BSW17 [13] has been described elsewhere [14]. Oligonucleotides encoding for TTmim and ϵ 4mim were synthesised by Microsynth GmbH (Balgach, Switzerland) as follows:

TTfwd 5'-CTAGCTGCACAGATCCTTCTGGAGCATCTGCACCTTGCAT-3';

TTrev 5'-GCAAGGTGCAGATGCTCCAGAAGGATCTGTGCAG-3';

ϵ 4fwd 5'-CTAGCTGCATTAATCATAGAGGATATTGGGTTTGCAT-3';

ϵ 4rev 5'-GCAAACCCAATATCCTCTATGATTAATGCAG-3'.

2.2. Bacteria, reagents

Bacterial strains Lj strain NCC2754 and *Lactococcus lactis* strain MG1363 (Lcl) were grown respectively in MRS broth (Difco Laboratories, Detroit, USA), and in M17 broth (Difco) containing 1% glucose. Chloramphenicol (Fluka Chemie AG, Buchs, Switzerland) was used at a concentration of 10 μ g/ml. All restriction enzymes were purchased from Axon Lab AG (Baden, Switzerland), Promega (Catalys, Wallisellen, Switzerland) and Boehringer Mannheim (Mannheim, Germany).

2.3. Antibodies and antigens

A polyoxime TTmim construct (polyTTmim) was produced by Keith Rose (University Medical Centre, Geneva,

Switzerland) as previously described [15]. Additionally in our construct, a universal T helper epitope (aa 830–843 of tetanus toxin) was C-terminally attached to TTmim. Polyclonal rabbit anti-TTmim serum was produced in our laboratories by immunising rabbits with the polyTTmim construct. A polyclonal rabbit anti-PrtB serum was prepared by immunising a rabbit with *L. delbrueckii* subsp. *bulgaricus* strain ATCC11842 expressing PrtB and was negatively pre-absorbed on Lj. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA and HRP-conjugated sheep anti-mouse IgG was purchased from Cappel (ICN, Switzerland). HRP-conjugated goat anti-rabbit antibodies were obtained from Nordic Immunology Laboratories (Tilberg, The Netherlands). Tetanus toxoid (TTd) was obtained from Dr. A.B. Lang (Swiss Serum and Vaccine Institute, Bern, Switzerland) and used at a concentration of 10 μ g/ml.

2.4. Generation of Lj and Lcl transformants expressing TTmim- or ϵ 4mim–PrtB fusion protein

Wild type plasmid pMD112 was isolated from transformed LclPrtB using a slightly modified QIAfilter Plasmid Midi Kit (Qiagen AG, Basel, Switzerland). For more efficient lysis, 1 mg/ml lysozyme was added to the lysis buffer and the sample was incubated for 1 h at 37 °C before DNA isolation according to the manufacturer's manual. Plasmid pMD112 was digested with *PvuI* and *NheI*, gel purified and ligated to annealed synthetic oligonucleotides encoding either TTmim or ϵ 4mim, respectively. Ligated DNA was then used to transform Lj and Lcl and plated on either MRS or M-17 plates containing chloramphenicol (10 μ g/ml). Clones were then tested for the presence of TTmim or ϵ 4mim DNA by PCR and sequencing analysis (data not shown).

2.5. Gel electrophoresis and Western immunoblots

Transformed bacteria (LjTT, Lj ϵ 4) and wild type Lj were grown overnight in 25 ml medium containing 10 μ g/ml chloramphenicol. Bacterial cells were harvested by centrifugation at 3000 $\times$ g for 15 min at 4 °C and washed with 5 ml TBS. Finally, the bacterial pellet was resuspended in 450 μ l TBS and 150 μ l 4 $\times$ non-reducing sample buffer (80 mM Tris–HCl pH 6.8, 2.5% SDS, 0.15% glycerol, 0.005% bromophenol blue). A volume of 20 μ l aliquots were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (6% acrylamide, 0.5 M Tris–HCl pH 8.8) and run in a 25 mM Tris, 192 mM glycine buffer (pH 8.3) at 100 V for 60 min. The gels were stained with BM Fast stain (Boehringer Mannheim, Germany) or transferred electrophoretically onto Protran BA 83 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After transfer, the membranes were blocked with PBS/5% BSA for 2 h at RT and incubated with either rabbit anti-TTmim serum (1:1000) or anti-PrtB serum (1:2000) overnight at RT followed by incubation for 3 h at RT with a 1:1000 dilution

of HRP-conjugated goat anti-rabbit IgG. Immunoblots were developed with 4-chloro-1-naphthol for 2 min.

2.6. Gastric simulation tests

To test the stability of recombinant LjTT gastric simulation tests were performed using a protocol adapted from Astwood et al. [16]. Approximately 10^{10} LjTT were suspended in 1 ml TBS and 1 ml of $2\times$ SGF solution (126 mM HCl containing 0.6% (w/v)) pepsin purchased from Fluka (Buchs, Switzerland). Incubations were performed at 37°C in a water bath and samples of $200\mu\text{l}$ were quenched by neutralisation with $75\mu\text{l}$ $0.16\text{ M Na}_2\text{CO}_3$ at the following time points: 0, 1, 2, 5, 10, 20, 40, 60 and 120 min. Aliquots were plated on agar plates and incubated overnight to determine the titer of surviving bacteria. To test the stability of TTmim–PrtB on the surface of Lj, aliquots of SGF-treated LjTT were coated to wells of an ELISA plate overnight at 37°C . PrtB was detected using a rabbit anti-PrtB serum (1:1000) followed by an incubation with HRP-conjugated goat-anti-rabbit IgG (Fc) at a dilution of 1:1000.

2.7. Immunisation of mice

A group of three female BALB/c mice (8 weeks old) was fed intragastrically with 2×10^9 live LjTT at days 0, 1, 2 using intubation needles. A booster immunisation was given at days 14, 15, 16. A control group of three female BALB/c mice (8 weeks old) was immunised subcutaneously with $25\mu\text{g}$ polyTTmim at days 0 and 14. Sera were collected by retro-orbital bleeding at days 0 and 28, and sera from each of these groups were pooled.

2.8. Evaluation of antibody responses

The antibody response was measured by ELISA. Costar EIA/RIA half-well plates (Costar, Cambridge, MA) were coated overnight at 37°C with approximately 10^8 wild type Lcl, recombinant LclTT and Lcl ϵ 4 or with tetanus toxoid (TTd) at a concentration of $10\mu\text{g/ml}$. Sera were tested at dilutions of 1:100 and 1:1000 by incubation on the coated bacteria and TTd at 37°C for 4 h. Bound antibodies were detected using HRP-conjugated sheep anti-mouse IgG (Fc) incubated for 1.5 h at 37°C . The 3,3',5,5'-tetramethylbenzidine (Fluka, Buchs, Switzerland) was used as substrate. The absorbance was read after 10 min at 450 nm on a molecular devices ELISA reader (Paul Bucher, Basel, Switzerland). Serum of non-immunised mice as well as the pre-bleed of the immunised mice were used as negative controls (background) and subtracted from serum signals. Absorbance in these samples was never higher than OD 0.1.

2.9. Measurement of fecal IgA responses

The mucosal IgA immune response was measured by ELISA. Costar EIA/RIA half-well plates (Costar) were

coated overnight at 37°C with approximately 10^8 wild type Lj, with LjTT or Lj ϵ 4. Fresh fecal pellets (0.1 g) from immunised mice were collected at days 0 and 28 and treated as described elsewhere [17]. Samples were then tested at a dilution of 1:2 by incubation on the coated bacteria at 37°C for 4 h. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgA incubated for 1.5 h at 37°C . Tetramethylbenzidine (Fluka) was used as substrate. The absorbance was read after 10 min at 450 nm on a molecular devices ELISA reader (Paul Bucher). Fecal pellets of non-immunised mice as well as the pellets from day 0 of the immunised mice were used as negative controls (background) and subtracted from signals. Absorbance in these samples was never higher than OD 0.1.

3. Results

3.1. Transformation of lactic acid bacteria with pMD112TT and pMD112 ϵ 4

In order to develop an oral vaccine based on the use of Lj as an antigen delivery vehicle, the plasmid pMD112 was chosen as expression vector. This plasmid contains the entire structural gene of the cell wall anchored PrtB from *L. delbrueckii* subsp. *bulgaricus*. Annealed complementary oligonucleotides coding for the TTmim, a peptide of 11 amino acids derived from tetanus toxin (unpublished results) and for control purpose the ϵ 4 mimotope (ϵ 4mim), an IgE decapeptide [14] were inserted into the *NheI* and *PvuI* restriction sites which are located in the active site of PrtB. The resulting plasmids pMD112TT and pMD112 ϵ 4 thus code for peptides inserted in the sequence of prtB that are expressed as fusion proteins on the bacterial cell surface.

Lj was transformed with the recombinant plasmids pMD112TT and pMD112 ϵ 4 (Table 1). For control purposes Lcl was transformed with the same recombinant plasmids. DNA was prepared from chloramphenicol resistant transformants and tested by PCR and sequencing analysis for the presence of the recombinant plasmids using primers specific for the different mimotopes and the wild type PrtB (data not shown).

3.2. Expression of the recombinant TT–PrtB fusion protein in Lj

The expression of the TTmim–PrtB fusion protein in Lj was analysed by SDS-PAGE and Western blots using wild type Lj and recombinant LjTT. Recombinant Lj ϵ 4 was used as a control and analysed on the same gels. Fig. 1A shows the presence of protein bands with molecular weights between 90 and 200 kDa in both recombinant Lj clones (lanes 2 and 3) but not in wild type Lj (lane 1). On the Western blot, these bands showed reactivity with rabbit-anti PrtB serum (Fig. 1B), indicating that the PrtB protein was expressed by the Lj bacteria and that these bands

Table 1
Bacterial clones and designations

Clone designation	Bacterial strain	Plasmid used for transformation	Mimotope fused to PrtB	Expressed fusion protein ^a
Lj	<i>L. johnsonii</i>	None	None	None
Lcl	<i>L. lactis</i>	None	None	None
LclPrtB	<i>L. lactis</i>	pMD112	None	PrtB
LjTT	<i>L. johnsonii</i>	pMD112TT	TTmim	Ttmim–PrtB
LclTT	<i>L. lactis</i>	pMD112TT	TTmim	Ttmim–PrtB
Ljε4	<i>L. johnsonii</i>	pMD112ε4	Cε4mim	ε4mim–PrtB
Lclε4	<i>L. lactis</i>	pMD112ε4	Cε4mim	ε4mim–PrtB

^a Expression was monitored by Western blot and ELISA (see Section 3 and Fig. 1).

correspond to full length (molecular weight 200 kDa) and to fragments of PrtB. The bacterial clones were then tested for expression of the TTmim–PrtB fusion protein using rabbit anti-TTmim serum. As shown in Fig. 1C (lane 2) only LjTT bacteria express a protein corresponding to full length PrtB that reacts strongly with the rabbit anti-TTmim serum indicating that the TTmim was expressed as fusion protein to PrtB. No reactivity was observed with either wild type Lj or recombinant Ljε4. Expression of the TTmim–PrtB fusion protein on the LjTT cell surface was confirmed by ELISA. Live recombinant LjTT bacteria were coated onto ELISA plates and shown to react with anti-PrtB and anti-TTmim sera (data not shown). The same reactivity was obtained with LclTT and Lclε4 bacteria when incubated with rabbit anti-PrtB serum and with LclTT bacteria when anti-TTmim serum was used (data not shown).

3.3. Stability of Lj and of surface displayed proteinase PrtB in simulated gastric fluid

Bacteria used for oral immunisations should be able to survive the harsh conditions of the stomach. In order to test the stability of recombinant LjTT clones under such conditions, a simulated gastric fluid (SGF) digestion test [16] was performed. Fig. 2A shows the survival curve obtained for LjTT clones and in comparison for LclTT and for *Escherichia coli* expressed as the number of colony forming units (CFU) after various times of digestion. An appreciable proportion of the lactobacilli survived the simulated gastric conditions up to 20 min and even after 120 min more than 10^4 viable cells were found. In contrast, the number of viable *E. coli* bacterial cells dropped to $<10^3$ after 10 min and after 120 min, no viable cells could be found anymore. In the case of LclTT,

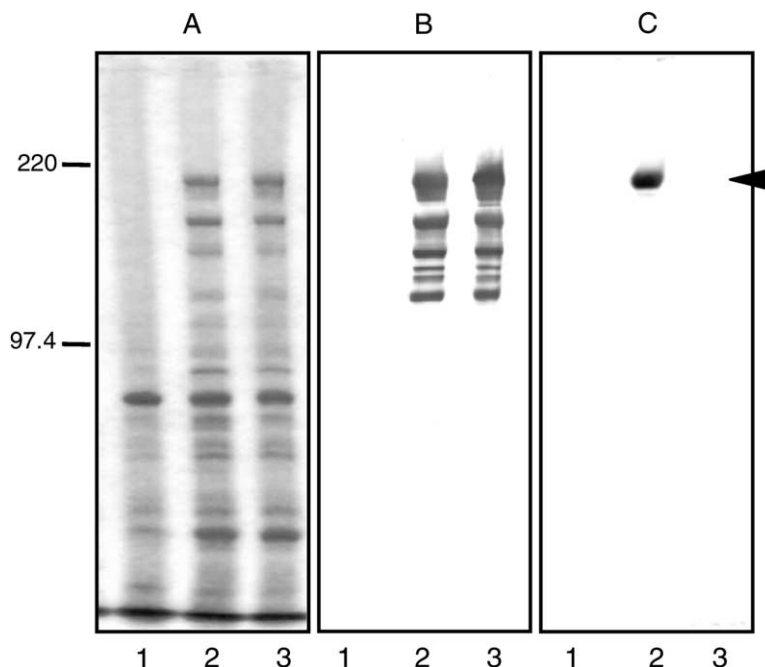


Fig. 1. Recombinant expression of PrtB fusion proteins in Lj. Whole bacterial cell lysates from overnight cultures of Lj (lane 1), LjTT (lane 2) or Ljε4 (lane 3) were separated by SDS-PAGE 6% and stained with BM Fast stain (A) or transferred to nitrocellulose filters and incubated with either rabbit anti-PrtB serum (B) or rabbit anti-TTmim serum (C). Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG (Fc) antibodies. The arrow indicates the height of full-length TTmim–PrtB and ε4mim–PrtB.

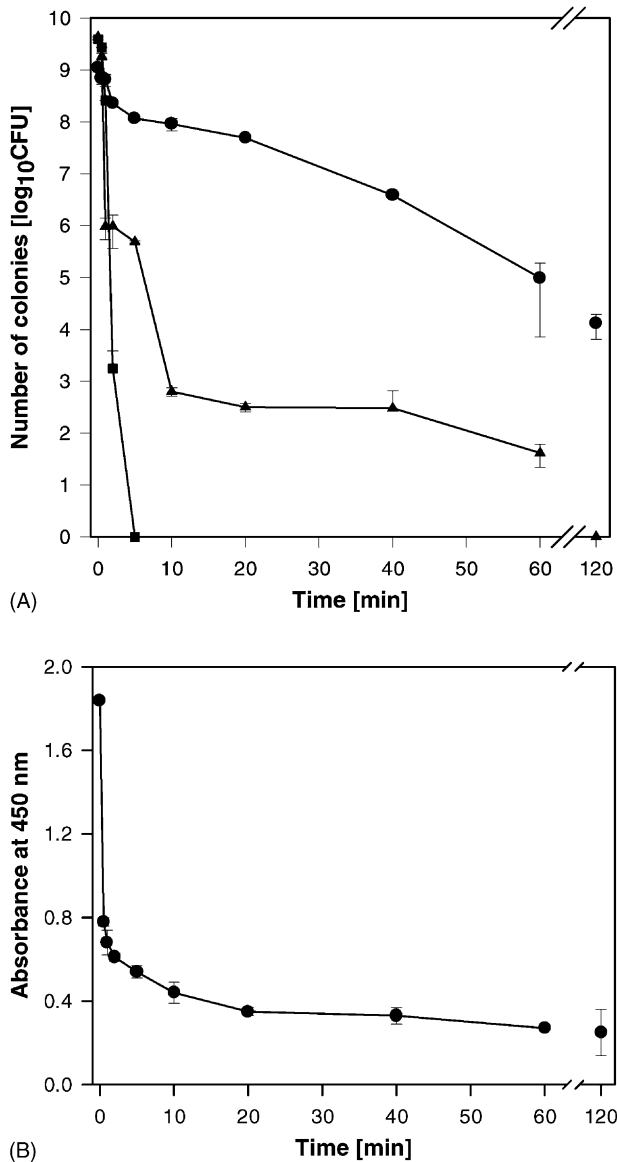


Fig. 2. Survival of LjTT and stability of TTmim–PrtB in SGF. (A) The 10^9 LjTT bacteria (circle) were incubated in tubes containing SGF and digested at 37 °C. At various time points, aliquots were neutralised and streaked out on agar plates. Survival of bacterial cells was measured by counting the CFU. For control purposes 10^9 *E. coli* XL-1 blue (triangle) and 10^9 LclTT (square) were treated the same way. (B) Stability of TTmim–PrtB in SGF was tested by digestion of 10^9 LjTT in tubes containing SGF at 37 °C. At various time points, aliquots were neutralised and coated on ELISA plates. After washing and blocking wells were incubated with rabbit anti-PrtB serum. Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG (Fc) antibodies.

the proportion of viable cells diminished even more drastically and already after 5 min, no viable cells were detected.

To analyse the stability of the cell surface PrtB, an ELISA assay was performed using, as the solid phase, the LjTT bacteria clone after various times of digestion (Fig. 2B). The results, expressed as ELISA OD values, show that almost immediately after exposure (10 s) recombinant Lj bacteria

lost reactivity with the anti-PrtB serum. These data indicate that PrtB was digested in the SGF and suggests that in vivo the surface expressed PrtB might not resist gastric digestion.

3.4. Immunisation of mice with LjTT

To investigate whether the expressed TTmim–PrtB fusion protein could induce an antibody response specific for the TTmim and TTd in vivo, groups of mice were fed intragastrically with 10^9 live LjTT at days 0, 1 and 2 and boosted intragastrically at days 14, 15, 16. Sera were tested by ELISA 28 days after the first immunisation using TTd, wild type Lj and recombinant LjTT and Ljε4 clones as coating antigens (Fig. 3). Sera of mice immunised orally with LjTT showed reactivity not only against Lj expressing the TTmim and an unrelated mimotope (ε4mim) but also against wild type Lj bacteria indicating that an antibody response was induced against cell constituents of Lj. In contrast, no reactivity was observed with TTd indicating that no antibody response was induced against TTd after immunisation.

To specifically distinguish between anti-Lj and anti-TTmim–PrtB fusion protein reactivity, sera of mice immunised with LjTT were incubated on ELISA plates coated with wild type Lcl bacteria and recombinant Lcl clones, which also expressed either the TTmim–PrtB or the ε4mim–PrtB fusion protein (LclTT and Lclε4). As shown in Fig. 4, no cross reactive antibodies were observed against wild type Lcl bacteria which do not express a PrtB fusion protein. In contrast, both LclTT and Lclε4 were strongly recognised by sera of mice orally immunised with LjTT. However, according to the Student's *t*-test, no significant difference ($P = 0.08$) between LclTT and Lclε4 was

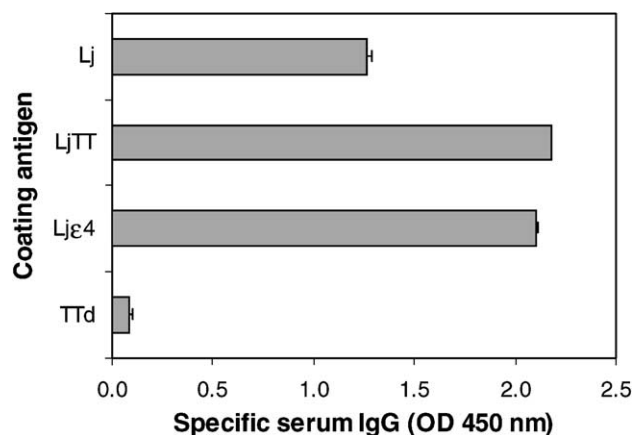


Fig. 3. Binding specificities of serum IgG from mice orally immunised with LjTT. Wells were coated with Lj, LjTT, Ljε4 (approximately 10^8 bacteria per well) or with TTd (10 μg/ml) and incubated with pooled sera collected 28 days after the first immunisation (diluted 1:100). Bound antibodies were detected using HRP-conjugated sheep anti-mouse IgG (Fc) antibodies. Bars represent mean values ($\pm$ S.D.) for three mice. Lj value is significantly smaller than LjTT ($P < 0.01$) and Ljε4 ($P < 0.01$) values, whereas, there is no difference between LjTT and Ljε4.

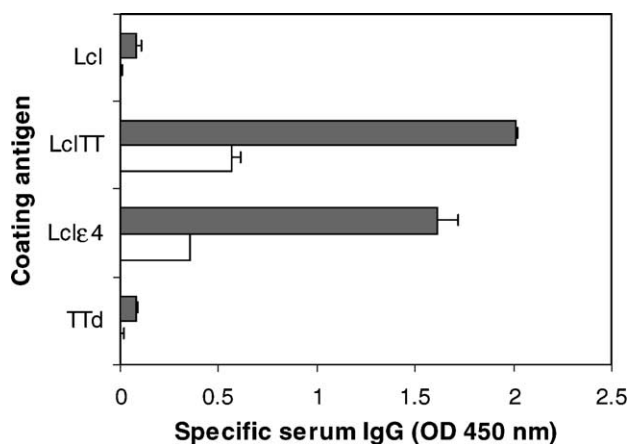


Fig. 4. Detection of serum IgG specific for TTmim–PrtB fusion protein. Pooled sera of mice orally immunised with LjTT react with TTmim–PrtB and ε4mim–PrtB expressed on recombinant Lcl. Wells were coated with wild type Lcl and recombinant LclTT and Lclε4 clones (approximately 10^8 bacteria per well). TTd was coated as a control at a concentration of $10 \mu\text{g/ml}$. Wells were incubated with pooled sera collected 28 days after the first immunisation from mice orally immunised with LjTT diluted 1:100 (hatched bars) or 1:1000 (white bars) in PBS/2% FCS/0.05% Tween. Bound antibodies were detected using HRP-conjugated sheep anti-mouse IgG (Fc) antibodies. Bars represent mean values ($\pm$ S.D.) for three mice. Lcl value is significantly smaller than LclTT ($P < 0.01$) and Lclε4 ($P = 0.01$) values, whereas, there is no difference between LclTT and Lclε4.

observed indicating that the antibody response in the sera was against PrtB and not against the TTmim.

In order to study whether a mucosal immune response had been induced, fresh fecal pellets were collected from mice 28 days after the first oral immunisation with LjTT. Extracts of these fecal pellets were tested for the presence of IgA antibodies against LjTT by ELISA (Fig. 5), using wild

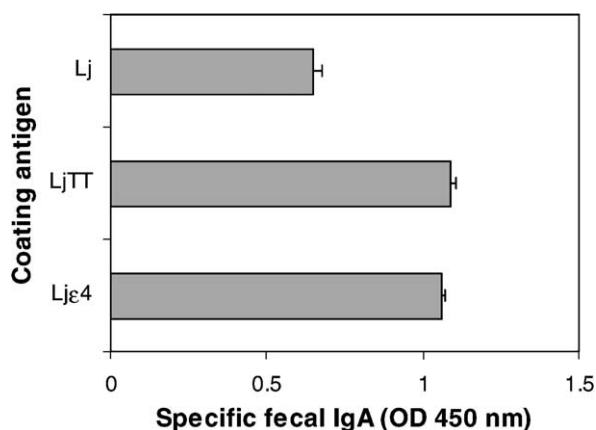


Fig. 5. Binding specificities of fecal IgA from mice orally immunised with LjTT. Wells were coated with different Lj clones and incubated with pooled fecal extracts collected 28 days after the first immunisation diluted 1:2 in PBS. Bound antibodies were detected with HRP-conjugated goat anti-mouse IgA (Fc) antibodies. Bars represent mean values ($\pm$ S.D.) for three mice. Lj value is significantly smaller than LjTT ($P < 0.01$) and Ljε4 ($P = 0.01$) values, whereas, there is no difference between LjTT and Ljε4.

type Lj and recombinant Lj clones (LjTT and Ljε4) as coating antigens. As shown in Fig. 5, there was an IgA response to both wild type Lj and recombinant Lj expressing either the TTmim–PrtB or ε4mim–PrtB fusion proteins. Based on a Student's *t*-test, significant differences were observed between reactivities to Lj and LjTT ($P = 0.001$) as well as between Lj and Ljε4 ($P = 0.01$). However, there was no significant difference between LjTT and Ljε4 ($P = 0.12$) indicating that the antibody response was directed against PrtB. Thus, LjTT bacteria were able to induce both systemic and mucosal antibody responses against PrtB fusion protein.

4. Discussion

Previous attempts have described the use of certain *Lactobacillus* species as antigen delivery systems for oral immunisation purposes [18]. In this study, we engineered for the first time Lj to express a cell surface antigen, the PrtB of *L. delbrueckii* subsp. *bulgaricus* linked to a TTmim. This recombinant Lj strain was able to induce both mucosal and systemic humoral responses after oral immunisation in mice. Recently, Shaw et al. have also constructed recombinant *Lactobacillus plantarum* and *casei* strains expressing an exogenous antigen, tetanus toxin fragment C (TTFC) for oral immunisation [6]. The antigen was expressed either as an intracellular antigen or a cell surface exposed antigen but this surface exposed TTFC failed to induce a TTFC specific serum IgG. Their results might be explained by the fact that production of heterologous antigen on the cell surface is less efficient compared to intracellular production [19]. In our study, we used a plasmid vector which allows the antigen to be expressed as a fusion protein with the PrtB [8]. Cell surface PrtBs have been isolated from different *Lactobacillus* strains and therefore represent common *Lactobacillus* structures that are well expressed on the surface of the bacteria [20,21]. Thus these PrtBs are ideal candidates to target heterologous proteins to the outer bacterial surface.

Immunisation of mice with recombinant Lj expressing the TTmim–PrtB fusion protein induced PrtB specific IgG serum titers as well as PrtB specific IgA in the feces but no anti-TTmim and anti-TTd antibody responses. The absence of antibody reactivities against TTd may be explained by the fact that the short TTmim (only 11 amino acids long) was not sufficiently immunogenic to compete with proteins and non proteinaceous molecules of larger size present on the surface of the lactobacillus for inducing an immune response. Recently, studies have demonstrated the presence of a non proteinaceous component identified as lipoteichoic acid (LTA) on the surface of Lj with antigenic activity and a molecular mass of 1000 kD [22]. In the present report, the proteinase PrtB, which is a large protein (200 kD) was immunogenic and able to induce both systemic and mucosal responses. Therefore future experiments will be designed to fuse larger antigenic proteins such as anti-idiotypic single chain Fv (scFv), that mimic the epsilon mimotopes, to PrtB.

Recombinant live Gram positive bacteria expressing cell surface protein antigens have already been reported to induce mucosal and systemic immune responses after oral immunisation [23,24]. However, the two strains used in these studies, *Streptococcus gordonii* and *Staphylococcus xylosus*, are not involved in the food chain and are therefore not considered to be safe organisms even though they are commensal organisms and constituents of the human gastro-intestinal flora [25]. Notably in these and other studies using Gram positive commensals, oral immunisations were performed in the presence of bicarbonate solution to compensate for the gastric acid environment [6,18]. We show the induction of an immune response without the addition of bicarbonate solution indicating that Lj bacteria are as previously shown in the SGF test resistant to gastric conditions and survive the gastro intestinal tract. Ingestion of Lj without the use of buffered solutions represents a major advantage for routine application of Lj which could thus be administered alone or as part of a food diet. Additionally serum analysis of the mice indicated a high antibody titer against the constituents of Lj. This confirms other data showing the adjuvant and immunogenic properties of *Lactobacillus* when given parenterally and orally [3,5,26].

Our data show that the PrtB did not survive the SGF conditions but nevertheless an anti-PrtB response was measured in both serum and fecal pellets. This suggests that recombinant Lj bacteria not only acted as passive carriers for the antigen but were able to express PrtB in the gastro intestinal tract. An anti-PrtB response was induced indicating that PrtB was resistant to intestinal enzymes. Recently Zegers et al. have used recombinant *Lactobacillus casei* expressing the protective antigen of *Bacillus anthracis* (PA) for oral immunisation [27]. Their results showed a low *Lactobacillus* specific antibody response and no specific antibody response against PA. However in their system, the PA antigen was expressed intracellularly and in low concentration. Additionally, previous studies have shown that *Lactobacillus* species differ greatly in their properties such as adjuvanticity and persistence in the gut [28]. The fact that we were able to induce antibody responses against both Lj and the whole fusion protein renders Lj functional as an antigen carrier with the appropriate adjuvant properties.

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