

Comparison of the immune responses induced by local immunizations with recombinant *Lactobacillus plantarum* producing tetanus toxin fragment C in different cellular locations

Nathalie Reveneau^{a,b}, Marie-Claude Geoffroy^c, Camille Locht^b,
Patrice Chagnaud^a, Annick Mercenier^{a,*}

^a Laboratoire de Bactériologie des Ecosystèmes, INSERM IFR17, Institut Pasteur de Lille, 1 rue du Professeur Calmette, F-59019 Lille Cedex, France

^b Laboratoire de Microbiologie Génétique et Moléculaire, INSERM U447 and IFR17, IBL, Institut Pasteur de Lille, 1, rue du Professeur Calmette, F-59019 Lille Cedex, France

^c INSERM 411, Laboratoire de Microbiologie, Faculté de Médecine Necker-Enfants Malades, F- 75015, Paris, France

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Abstract

Lactobacillus plantarum NCIMB8826 was selected as a bacterial carrier for the development of live mucosal vaccines. This strain was reported to display interesting pharmaco-kinetic properties when fed to human volunteers and is also able to persist in the mouse intestine. The non-toxic C fragment of tetanus toxin (TTFC) was used as a model antigen. Recombinant strains producing TTFC in three cellular locations, intracellular, secreted or cell-surface exposed were compared to each other by immunizing mice through the subcutaneous, intranasal and intragastric routes. The three types of constructs were able to induce strong specific immune responses against TTFC by all routes tested. While cell-surface presentation required lower antigen doses to be immunogenic, the highest IgG serum antibody titers were obtained with the strain producing large amounts of TTFC in the cytoplasm. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Most pathogenic microorganisms initiate their infectious cycle at the mucosal surfaces. The respiratory, intestinal and urogenital tracts correspond, in total, to a huge epithelial surface, which needs to be protected against the permanent challenge by invading bacteria, viruses or parasites. Efficient protection against mucosal infections requires the development of new vaccines, which are able to induce protective local immune responses in order to neutralize the pathogen at its infection point [1,2].

In many instances, this necessitates the elicitation of the local production of so-called secretory immunoglobulins A (sIgA) [3]. This can best be achieved by mucosal vaccination, a route which offers several advantages over the traditional parenteral vaccines [4,5]. However, as the local administration of pure antigens or subunit vaccines is usually rather inefficient, several strategies have been developed

to deliver the antigen in an immunogenic and protected form at the targeted mucosa. One promising approach relies on the use of live bacterial vehicles [6]. While most of them were initially derived from attenuated pathogens [7–10], alternative systems based on non-pathogenic Gram-positive bacteria have recently been designed [11,12]. The development of lactic acid bacteria as live bacterial carriers has been achieved in different laboratories during the last decade [13–17]. The “generally recognized as safe” (GRAS) status of dietary lactic acid bacteria, such as *Lactococcus lactis* and *Lactobacillus* spp. render them particularly attractive as mucosal vaccine vectors. While these two types of bacteria offer common advantages [12] they are presently perceived as leading to potentially different delivery systems. *L. lactis* is a commonly used dairy starter which does not persist in the gastro-intestinal tract of humans or mice [16,18,19] but nevertheless has successfully been used as a “live micro-particle” pre-loaded with antigen [13]. In contrast, the lactobacilli include a wide variety of species some of which being able to persist in the gastro-intestinal or urogenital tract for several days or weeks, respectively [20]. Specific *Lactobacillus* strains are also reputed to exert

* Corresponding author. Tel.: +33-320-87-11-87;
fax: +33-320-87-11-92.
E-mail address: annick.mercenier@ibl.fr (A. Mercenier).

beneficial health properties and are thus intensively studied for probiotic applications [21]. We therefore choose to develop *Lactobacillus plantarum* as a candidate vaccine vehicle. Our model strain, *L. plantarum* NCIMB8826 is an isolate of human origin, which has been demonstrated to be particularly resistant to the conditions of the upper digestive tract in humans. When fed to healthy volunteers it has been shown to survive at much higher rates in the terminal ileum than *L. lactis* [19]. We also observed that it is able to persist in the mouse intestine for more than 4–6 days, while *L. lactis* could not be detected in faeces 24 h after cessation of the feeding [18]. Even though the capacity of lactic acid bacteria to act as efficient antigen carriers has been undoubtedly established [22–25] a number of key questions remain to be answered. Among others, it is not yet known whether the cellular location of the foreign antigen is equally important when using persisting, i.e. lactobacilli, rather than non-persisting recombinant strains.

Therefore, we constructed recombinant *L. plantarum* NCIMB8826 strains producing the model antigen tetanus toxin fragment C (TTFC) in three cellular locations, i.e. the cytoplasm, the external milieu or anchored to the cell surface using plasmid constructions that differed only by the targeting/expression signals. The immunogenicity of the three recombinant strains was analyzed after subcutaneous, intranasal or intragastric administration of live bacteria to mice. The highest TTFC-specific immune response was obtained with the recombinant strain synthesizing very high amounts of cytoplasmic antigen. However, the cell-surface associated form of TTFC was found to be intrinsically the most immunogenic.

2. Materials and methods

2.1. Bacterial strain and growth conditions

L. plantarum NCIMB8826, a lactic acid bacterium isolated from the human saliva [19], was grown at 37 °C,

without shaking in MRS medium (Difco laboratories, Detroit, MI, USA).

2.2. Plasmids, DNA manipulation and transformation

The plasmids used in this study are listed in Table 1. pMEC127, pMEC160 and pMEC139 were derived from pMEC4. In all cases, expression of the recombinant genes was controlled by the strong constitutive *ldh* promoter, and the transcriptional termination signals were those of the *ldh* gene. pMEC4 was obtained by inserting a fragment coding for TTFC into pGIT032 [26]. This fragment was obtained by polymerase chain reaction (PCR) using pLET2-TTFC [27] as described by Chagnaud et al. (in preparation). pMEC127 (Fig. 1A) was obtained by deleting the 73bp sequence coding for the N-terminus of LDH in pMEC4 in order to place the initiation codon of TTFC at an optimal distance from the *ldh* ribosome binding site (RBS) (Fig. 1B). This was achieved by PCR amplification of a 1460 bp fragment of pMEC4 using the oligonucleotides 5'-GGCATGCTTGCCATGGAATAAG-3' and 5'-GAGGTGAATTCCTGGGAGAGC-3' as primers including a *Nco*I site and a *Sma*I site (underlined), respectively. This fragment was inserted into the *Sma*I–*Nco*I restriction sites to replace the corresponding fragment of pMEC4. The construction was verified by nucleotide sequencing. pMEC160 and pMEC139 were obtained by replacing the gene encoding TTFC in pMEC127 with chimeric genes coding the hybrid proteins M6::TTFC and M6::TTFC::M6', respectively, where M6 designates the N terminal (165 residues) and M6' the C terminal (145 residues) parts of the M6 protein of *Streptococcus pyogenes*. These fragments were obtained by PCR using pSMB158 [17] as a template and the forward primer 5'-CGCCATGGCTAAAAATAACACGAATAG-3' including a *Nco*I site (underlined) in both cases, with the reverse primers 5'-CCCAAGCTTTTAGCTGTTTGC-3' and 5'-CCCAAGCTTTTAGTTTTCTTC-3' (including a *Hind*III

Table 1
Plasmids used in this study

Plasmids	Relevant features	Expected location of recombinant protein	Reference
pGIT032	pLAB1000 replicon; Ap ^r Em ^r ; <i>E. coli</i> Ori _{pBR322} ; <i>Lactobacillus hilgardii</i> Ori _{pLH1} ; <i>Kpn</i> I– <i>Kpn</i> I fragment coding for LDH of <i>L. plantarum</i> DG301	Cytoplasmic	[26]
pMEC4	pGIT032 with the <i>Nco</i> I– <i>Hind</i> III fragment of <i>ldh</i> replaced by a 1368bp fragment coding for TTFC, leading to a <i>ldh</i> :: <i>ttfc</i> fusion gene	Cytoplasmic	This work
pMEC127	pMEC4 with a 73bp deletion of the 5' <i>ldh</i> sequence where translation of TTFC starts at its own initiation codon	Cytoplasmic	This work
pMEC160	pMEC127 where the <i>Nco</i> I– <i>Hind</i> III TTFC encoding fragment was replaced by the genetic fusion coding the chimera M6::TTFC. M6::TTFC comprises the 165 N-terminal residues of M6 including the signal peptide and the full-length gene coding for TTFC including its translation termination codon	Secreted	This work
pMEC139	pMEC127 where the <i>Nco</i> I– <i>Hind</i> III TTFC encoding fragment was replaced by the genetic fusion coding the chimera M6::TTFC::M6'. M6::TTFC::M6' comprises the 165 N-terminal residues of M6 including the signal peptide, the gene coding for TTFC without stop codon and the 145 C-terminal residues of M6 including the anchor sequence (M6')	Cell-surface exposed	This work

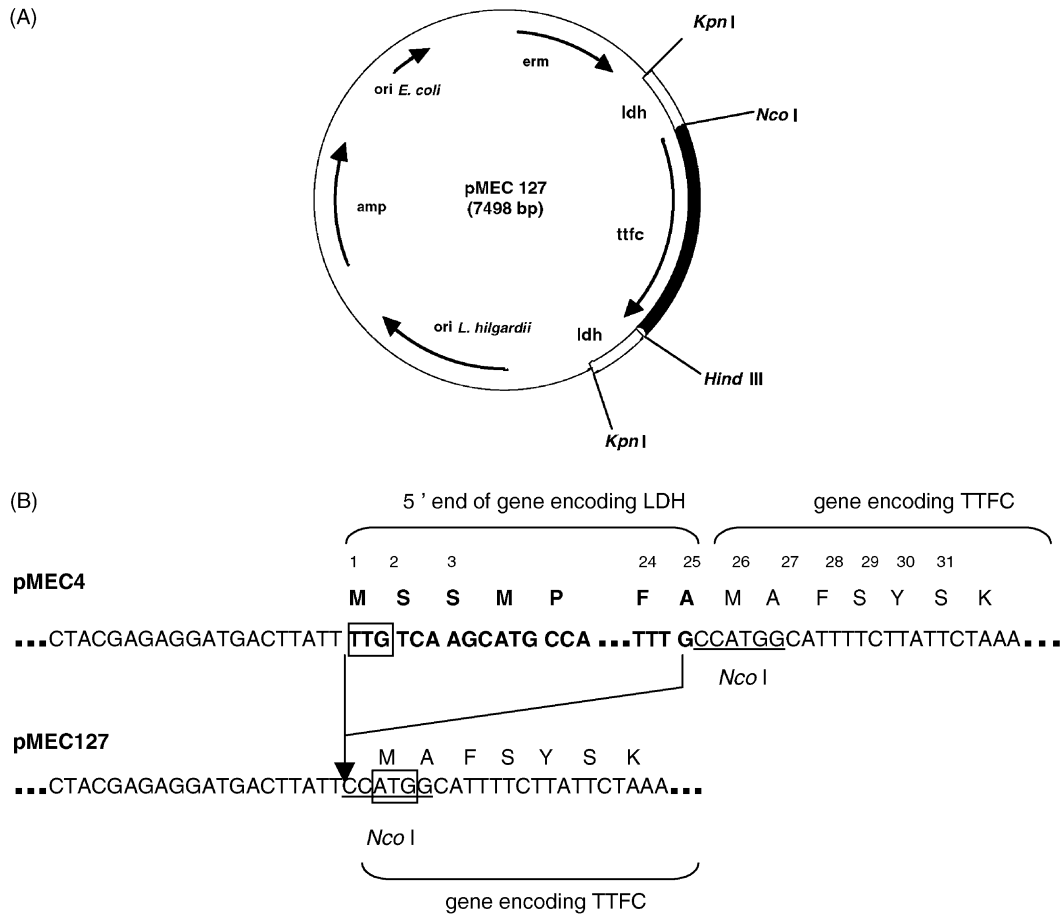


Fig. 1. Structure of pMEC127: (A) partial restriction map of pMEC127 carrying the gene encoding tetanus toxin fragment C (TTFC) under the control of the *L. plantarum* L-lactate dehydrogenase promoter (*PldhL*); (B) deletion of 73 bp of the *ldh* 5' end in pMEC4 to obtain pMEC127. The start (ATG) codon of TTFC in pMEC127 is boxed.

site), for the genes encoding the fusions M6::TTFC and M6::TTFC::M6', respectively (Table 1).

All DNA manipulations were carried out as described by Sambrook et al. [28]. Electroporation of *L. plantarum* was performed according to the method of Josson et al. [29] and plasmid DNA was isolated as described by Posno et al. [30]. Recombinant *L. plantarum* strains were selected on MRS medium containing 5 µg/ml of erythromycin. The presence and the integrity of the constructions carried by the *L. plantarum* transformants were checked by extraction of plasmid DNA, electroporation into *E. coli* XL1-Blue, DNA extraction from this host followed by restriction analyses and sequencing (data not shown).

2.3. Protein extraction and immunoblotting

Total cell protein extracts, cell-wall extracts and culture supernatants were prepared from cells harvested at mid-exponential phase (o.d.₆₀₀ ≈ 1.5). A volume corresponding to 10⁹ colony forming units (CFU) was centrifuged at 4000 rpm for 10 min at 4 °C, 1:40 of 1% sodium

deoxycholate (DOC) was added to the supernatant and the mixture was incubated for 20 min at 4 °C. After addition of 1:16 of 100% trichloroacetic acid (TCA) and incubation for 5 min at room temperature, the suspension was centrifuged at 3500 rpm for 20 min and the pellet was washed once with ice-cold ethanol and resuspended in 1 ml of 1 M Tris-HCl (pH 8).

The bacteria were washed twice in 100 mM Tris-HCl (pH 7.5) and resuspended in the same buffer to obtain a suspension at 10⁹ CFU/ml. Total cell extracts were obtained from the bacteria by disruption with a French pressure cell (Bioritech). In order to release protein that might be attached to the cell wall, the bacteria were treated with lysozyme to degrade the cell surface. The cell wall extracts were obtained by washing the bacteria three times in 20 mM Tris-HCl (pH 7.5) and 10% (w/v) sucrose supplemented with 50 µl 10 mg/ml lysozyme. After 30 min at 37 °C, the suspension was centrifuged for 15 min at 3500 rpm. The supernatant was then treated with 1% DOC and 100% TCA as described above. After a centrifugation for 20 min at 3500 rpm, the pellet was resuspended in 1 M

Tris–HCl (pH 8). The amounts of total proteins present in the extracts were determined using a Bradford assay [31] with bovin gamma globulin as a standard. Ten microliters (corresponding to about 10^7 CFU) of total cell extracts and concentrated supernatant were heated at 100°C for 5 min in Laemmli sample buffer [32] and subjected to 10% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane by electroblotting [33]. Immunoblots were developed with rabbit anti-TTFC polyclonal serum diluted at 1:1000 (Innogenetics, Ghent, Belgium) and with AP-linked secondary antibody (Promega, Madison, WI, USA) diluted at 1:7000.

2.4. Immunization

Eight weeks old C57 Bl/6 N Crl BR female mice (Charles River Laboratories, St. Aubin-les-Elbeuf, France) were immunized subcutaneously (s.c.), intranasally (i.n.) or intragastrically (i.g.) with a suspension of recombinant *L. plantarum* prepared as follows. Freshly cultured bacteria were harvested at mid-exponential phase ($\text{o.d.}_{600} \approx 1.5\text{--}2$) and washed twice with sterile PBS (pH 7.2). The bacterial cell concentrations were adjusted to 10^{10} CFU/ml (s.c. or i.g. administration) or 10^{11} CFU/ml (i.n. administration). Plate counts were performed with all inoculum samples to confirm the number of CFU administered to the mice. The antigen production was verified by immunoblotting for each inoculum at each administration.

For subcutaneous immunizations, groups of five mice were injected with $100\ \mu\text{l}$ of a bacterial suspension containing 10^9 CFU at days 1, 15 and 30. Control mice received identical volumes of PBS.

Intranasal immunizations were performed on groups of eight mice anesthetized as described by Grangette et al. [25]. 10^9 CFU suspended in $10\ \mu\text{l}$ of PBS were administered into one nostril of anesthetized mice on two consecutive days. Three administrations (one priming and two boosts) were performed at 3-week-intervals.

Groups of 8 mice were immunized by the intragastric route with 10^9 CFU in $100\ \mu\text{l}$ of a buffer containing 250 mM NaHCO_3 , 0.5% casein and 1% glucose [22]. Three consecutive daily doses of 10^9 CFU each were given to the mice 3 times at 3-week-intervals.

2.5. Collection of biological fluids

Serum samples were taken 7 days after each administration and bronchoalveolar lavage (BAL) fluids were obtained 9 days after the final boost as described by Grangette et al. [25]. The intestinal lavages were performed 9 days after the last boost by collecting the small intestine, and washing once in PBS to eliminate traces of blood. The intestine was cut in its length and washed with 1 ml of protease inhibitors in PBS. The lavage was centrifuged for 10 min at 3500 rpm at 4°C and the supernatant was stored at -20°C until analysis.

2.6. Antibody determinations

The levels of anti-TTFC antibodies in sera and BAL fluids were determined by enzyme-linked immunosorbent assays (ELISA) as described by Grangette et al. [25]. End point titers were defined as the highest dilution that gave an absorbance 3 times higher than background for serum samples or twice for BALF samples, using the Kc4 program (Kinetic for windows-Bio-tek instruments Inc., Virooski, VT).

2.7. Statistical analysis

The results are expressed as the mean $\pm$ S.E.M. Statistical significance was evaluated by Student's *t*-test. Differences were considered significant at $P < 0.05$ level.

2.8. Bacterial enumeration in faeces

Freshly passed faeces were collected 3 h after oral feeding of live lactobacilli and then every day during 10 days. Samples of each group of mice were pooled. Faecal pellets were weighed and crushed in MRS medium to obtain a suspension at 100 mg/ml. Appropriate dilutions of $100\ \mu\text{l}$ were plated onto selective medium and bacterial colonies were counted after 48 h incubation at 37°C . The results were expressed as CFU/10 mg of faeces.

3. Results

3.1. TTFC production by recombinant *L. plantarum*

Three recombinant *L. plantarum* strains were engineered to produce TTFC in the cytoplasm (*L. plantarum* (pMEC127)), in the supernatant (*L. plantarum* (pMEC160)) or on the surface of the bacterium (*L. plantarum* (pMEC139)). Cell surface and extracellular production of TTFC were achieved by using the N- and C-terminal ends of M6, a surface protein of *S. pyogenes* [17]. The synthesis of TTFC was examined in the cytoplasm for the three strains, in the supernatant for *L. plantarum* (pMEC160) and in cell-wall preparations for *L. plantarum* (pMEC139). As shown in Fig. 2A, specific immunoreactive proteins were detected in the cell extracts for the three recombinant strains at the expected sizes, i.e. 51, 65 and 80 kDa for *L. plantarum* harboring pMEC127, pMEC160 and pMEC139, respectively. An immunoreactive protein band of the expected size was also detected in the culture supernatant of *L. plantarum* (pMEC160) (Fig. 2B, lane 1) and in the cell-wall extract of *L. plantarum* (pMEC139) (Fig. 2B, lane 2). TTFC was produced at high levels in the cytoplasm of *L. plantarum* (pMEC127) (Fig. 2A, lane 2), while the levels of TTFC in the supernatant of *L. plantarum* (pMEC160) or cell wall extracts of *L. plantarum* (pMEC139) were significantly lower (Fig. 2B, lanes 1 and 2). No signal was detected in extracts or culture supernatants of the control strain harbor-

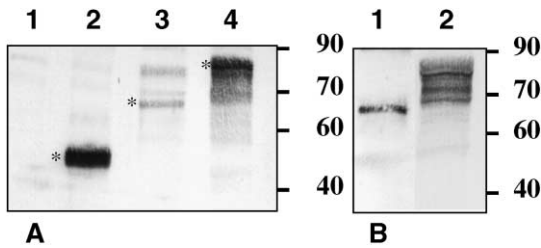


Fig. 2. Production of tetanus toxin fragment C (TTFC) by *L. plantarum* NCIMB 8826 transformants. (A) Immunoblotting of total cell extracts. Lane 1: *L. plantarum* (pGIT032), lane 2: *L. plantarum* (pMEC127), lane 3: *L. plantarum* (pMEC160), lane 4: *L. plantarum* (pMEC139). Protein extracts corresponding to approximately 10^7 CFU of bacteria were immunoblotted and revealed with rabbit polyclonal anti-TTFC serum (*: expected band for TTFC or TTFC fusions); (B) immunoblotting of cell-wall extract of *L. plantarum* (pMEC139) (lane 1) and culture supernatant of *L. plantarum* (pMEC160) (lane 2). The sizes (in kDa) of the molecular mass markers are shown in the right margin.

ing pGIT032 (Fig. 2A, lane 1). As seen in Fig. 2A, export or anchoring was not total as part of the antigen remained inside of the cells of *L. plantarum* (pMEC160) and *L. plantarum* (pMEC139) which led to a number of additional immunoreactive bands.

The respective amounts of TTFC synthesized by the three recombinant *L. plantarum* strains were evaluated by scanning immunoblots of relevant cellular fractions (cytoplasm, supernatant or cell wall) corresponding to 10^7 , 3×10^6 and 10^6 CFU of each strain (data not shown). The results indicated that the production of TTFC is approximately 5 times higher in *L. plantarum* (pMEC127) (cytoplasmic) as compared to *L. plantarum* (pMEC139) (cell-surface presentation) which in turn synthesizes about four times more TTFC than *L. plantarum* (pMEC160) (secretion). In addition, the surface association of the chimeric protein in *L. plantarum* (pMEC139) was assessed by flow cytometry using polyclonal TTFC-specific antiserum. It was observed that TTFC was exposed at the cell surface in only a small portion of the bacterial population ($\geq 15\%$) (data not shown).

3.2. Immune responses induced by systemic immunization

The immunogenicity of the different recombinant strains was evaluated by s.c. administration of 10^9 CFU (at days 1, 5 and 30) to mice. As shown in Fig. 3, high levels of anti-TTFC IgG (end point titers of 10^4 to 10^5) were obtained in all groups of mice that had received live TTFC-producing strains. No anti-TTFC antibody response was observed in the groups of mice that had received PBS or the strain not producing TTFC (*L. plantarum* (pGIT02)). After two administrations of *L. plantarum* producing TTFC in the cytoplasm (*L. plantarum* (pMEC127)) the anti-TTFC serum IgG titers were somewhat higher ($P < 0.05$) than after administration of the strains producing TTFC in the extracellular medium (pMEC160) or on the cell surface (pMEC139) (i.e.

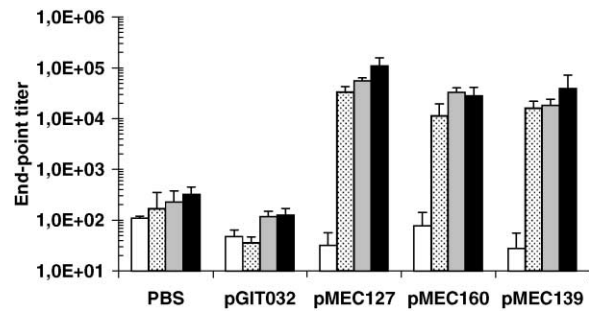


Fig. 3. Anti-TTFC antibody response induced after subcutaneous immunization with recombinant *L. plantarum* NCIMB8826 strains. Groups of five mice were immunized with 10^9 CFU of *L. plantarum* (pGIT032) (negative control), *L. plantarum* (pMEC127) (intracellular), *L. plantarum* (pMEC160) (secretion) or *L. plantarum* (pMEC139) (cell surface anchoring). Individual serum were collected from preimmune mice (open bar) and 7 days after each administration: priming (dotted bar), boost 1 (grey bar) and boost 2 (solid bar) and tested by ELISA for TTFC specific IgG. An additional IgG control group of mice received only PBS. Bars represent the mean IgG titer $\pm$ S.E.M. in each group.

end point titers of 6×10^4 versus 3×10^4 and 2×10^4). The IgG end point titers increased only slightly after the boosts, reaching 1×10^5 with *L. plantarum* harboring pMEC127, 3×10^4 with *L. plantarum* harboring pMEC160 and 4×10^4 with *L. plantarum* harboring pMEC139.

3.3. Immune responses induced by i.n. immunization

As the three recombinant *L. plantarum* strains were shown to induce strong immune responses by the s.c. route, their immunogenicity was also tested by i.n. immunization. A priming and two boosts were performed at 3-week-interval and the mice received two consecutive doses (days 0 and 1) of 10^9 live bacteria at each administration. After the second boost, high levels of serum anti-TTFC IgG were obtained by intranasal administration of *L. plantarum* (pMEC127), *L. plantarum* (pMEC160) or *L. plantarum* (pMEC139), whereas no anti-TTFC antibodies were observed in the control groups of mice that received PBS or *L. plantarum* (pGIT032) (Fig. 4A). Maximum levels of anti-TTFC antibodies (IgG end point titer of 4×10^5) were reached after the first boost when the mice were immunized with *L. plantarum* (pMEC127) and a significant systemic response was already observed after priming (serum IgG end point titer of 3×10^2). In contrast, significant anti-TTFC serum antibody responses were detected only after the first boost when the mice were immunized with *L. plantarum* (pMEC160) or *L. plantarum* (pMEC139). End-point titers reached levels of 2×10^4 and 6×10^4 , respectively, after the second boost. After the last boost, the level of anti-TTFC IgG was significantly higher ($P < 0.05$) in mice that had received *L. plantarum* (pMEC139) than in those that were given *L. plantarum* (pMEC160).

The local response was also studied by measuring the anti-TTFC IgA response in BAL fluids (Fig. 5A).

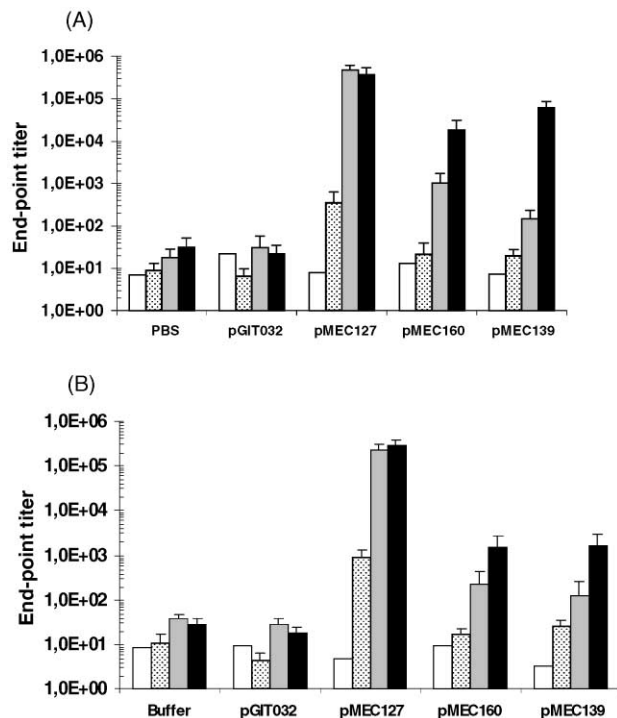


Fig. 4. Anti-TTFC serum IgG titers induced after mucosal immunizations with recombinant *L. plantarum* NCIMB8826 strains. Inocula of 10^9 CFU were prepared from cultures collected in mid-exponential phase and administered by intranasal (A) or intragastric (B) route. Groups of eight mice were immunized with the control strain not producing TTFC (pGIT032), or each of the three TTFC-producing *L. plantarum* strains (pMEC127, pMEC160 or pMEC139). Individual sera were collected from pre-immune mice (open bar) and 7 days after each administration: priming (dotted bar), boost 1 (grey bar) and boost 2 (solid bar). Bars represent the mean IgG titer $\pm$ S.E.M. in each group.

Immunization with *L. plantarum* (pMEC127) led to a significantly higher response (end-point titer of 160) than administration of *L. plantarum* (pMEC160) (end-point titer of 44) or *L. plantarum* (pMEC139) (end-point titer of 85). No response was observed after administration of *L. plantarum* (pGIT032) or PBS.

3.4. Immune responses induced by i.g. immunization

We next examined whether specific responses against TTFC could be induced in the serum of mice after i.g. administration of each of the TTFC-producing strain described in this paper. As shown in Fig. 4B, this was clearly found to be the case, while no response was elicited in the control group receiving PBS or the control strain (*L. plantarum* (pGIT032)). Notably, anti-TTFC IgG levels were already high (end point titer of 9×10^2) after the first administration of *L. plantarum* (pMEC127) and reached maximum levels as soon as after the first boost (titer of 2.5×10^5). In contrast, anti-TTFC antibodies were detected only after the second administration of *L. plantarum* (pMEC160) (titer of 2×10^2) or *L. plantarum* (pMEC139) (titer of 1×10^2) and

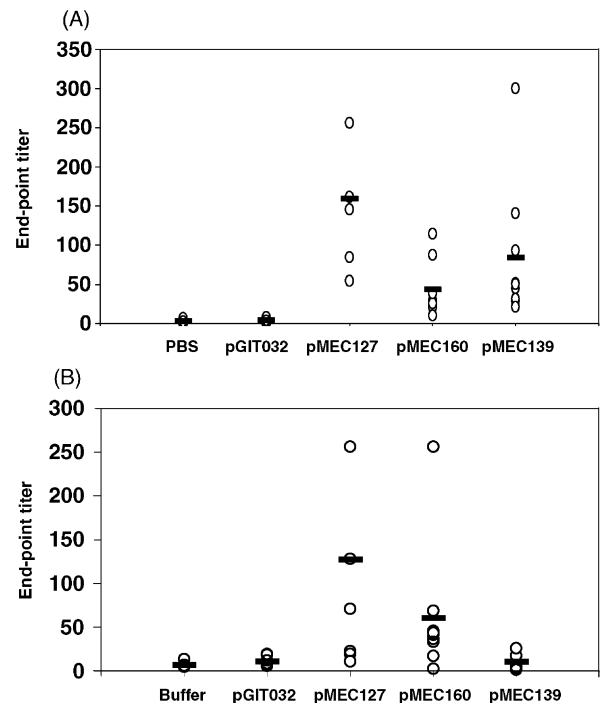


Fig. 5. TTFC-specific local IgA responses in (A) bronchoalveolar lavage fluids after intranasal immunization or (B) in intestinal lavages after intragastric immunization. Groups of eight mice received 2 (intranasal administration) or 3 (intragastric administration) consecutive doses of 10^9 CFU, 3 times at 3-week intervals. Mice received 10^9 CFU of *L. plantarum* containing pGIT032 (negative control), pMEC127, pMEC160 or pMEC139. A naïve group received buffer alone. BAL fluids and intestinal lavages were collected 9 days after the final boost. Bars represent the mean IgG titer $\pm$ S.E.M. in each group.

were substantially increased by a second boost leading to IgG titers of 2×10^3 and 1.5×10^3 , respectively.

Fig. 5B shows that anti-TTFC IgA was present in intestinal lavages after administration of *L. plantarum* (pMEC127) or *L. plantarum* (pMEC160). However, no local response was observed after administration of *L. plantarum* (pMEC139).

3.5. Intestinal transit time of the recombinant *L. plantarum* strains

Since it is difficult to foresee whether the metabolic burden imposed by the synthesis of heterologous molecules in various cellular locations would impact differently on the individual capacity of the strains to maintain themselves in the intestine, the persistence of each of the recombinant strains was studied by bacterial enumeration in the faeces of mice after intragastric administration (Fig. 6). Bacteria were recovered during 8 days in the faeces after administration of the TTFC-producing *L. plantarum* strains, and the persistence curves were not statistically different ($P > 0.05$) from that obtained with the strain not producing TTFC.

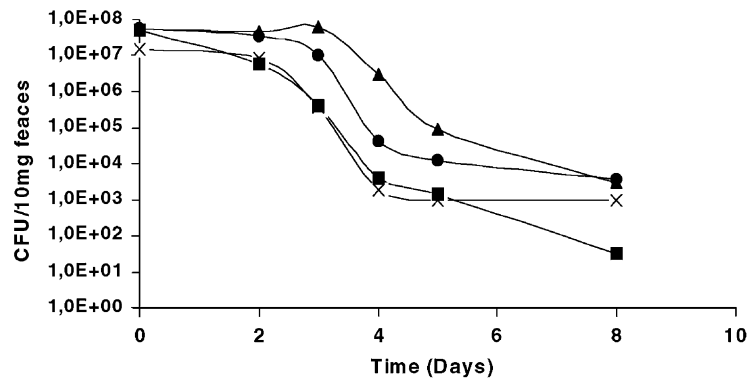


Fig. 6. Persistence of *L. plantarum* recombinant strains in the mouse digestive tract. Groups of eight mice were fed 10^9 CFU of the non-producing strain, *L. plantarum* pGIT032 (cross) or each of the three TTFC producing strains, *L. plantarum* pMEC127 (closed square), *L. plantarum* pMEC160 (closed circle) or *L. plantarum* pMEC139 (closed triangle). Bacteria were enumerated by plating faecal suspensions on selective medium.

4. Discussion

In this study we conducted a controlled comparison of the immunogenicity of three recombinant *L. plantarum* strains producing TTFC in the cytoplasm, in the external milieu or on the cell surface, after administration to mice by different mucosal routes. In comparison with the previously described expression vector pMEC4 [25] the improved construction described in this paper led to much higher cytoplasmic synthesis (5 to 10 times higher) of the antigen by the recombinant strain *L. plantarum* (pMEC127). In contrast, secretion in the culture medium by *L. plantarum* (pMEC160) or anchoring to the cell wall in *L. plantarum* (pMEC139) remained notably lower. In our hands, the three recombinant *L. plantarum* strains led to essentially similar TTFC-specific IgG serum responses by subcutaneous administration.

When the three strains were administered i.n. or i.g., *L. plantarum* (pMEC127) performed substantially better than *L. plantarum* (pMEC160) or *L. plantarum* (pMEC139). As estimated by quantitative immunoblot analysis, this difference could be related to varying levels of TTFC production depending on the cellular location of the antigen. The immune responses induced by i.n. or i.g. administration of *L. plantarum* (pMEC127) were very high already after priming and reached maximal levels after a single boost. No statistically significant difference was observed between the i.n. and i.g. routes. This strong immunogenicity most probably results from the high level of cytoplasmic TTFC production in *L. plantarum* (pMEC127), since similar experiments conducted with the original strain *L. plantarum* (pMEC4) producing substantially lower antigen amounts led to significant antibody titers only after i.n. administration [Grangette et al., submitted]. These observations are consistent with the fact that *L. plantarum* NCIMB8826 resists quite well the gastric barrier which may limit TTFC release in vivo when the antigen is produced intracellularly.

Even though *L. plantarum* (pMEC160) and *L. plantarum* (pMEC139) produced significantly less TTFC (i.e. 20 and 5

times, respectively) compared to *L. plantarum* (pMEC127) they induced high immune responses by the i.n. route. For both strains, serum anti-TTFC IgG end-point titers were approximately one order of magnitude lower than those elicited by the *L. plantarum* (pMEC127) strain after two boosts, and the kinetics of antibody induction were slower. The difference in immunogenicity between the two strains was even more pronounced after immunization by the i.g. route (Fig. 4). However, considering the respective amounts of TTFC synthesized in the three cellular locations, the cell surface associated and secreted forms of TTFC were more immunogenic than the cytoplasmic form of the antigen, as illustrated by the fact that only *L. plantarum* (pMEC127) was immunogenic after oral administration, whereas *L. plantarum* (pMEC4) was not [Grangette et al., in preparation].

These results contrast with those published by Shaw et al. [24] who found that cell surface presentation of TTFC in strain *L. plantarum* 256 was not inducing detectable immune responses by the i.g. route. Several factors may explain these differences. Successful mucosal immunization requires the delivery of a minimal threshold load of antigen. This dose dependence has clearly been demonstrated for *L. plantarum* NCIMB8826 administered by the i.n. route [25]. It is therefore possible that the recombinant strain described by Shaw et al. produces insufficient antigen quantities at the cell surface. Alternatively, intrinsic immunostimulatory properties of strain NCIMB8826, yet to be determined, may differ from those of *L. plantarum* 256, used by Shaw et al. [24]. Differences in these properties may perhaps explain why *L. plantarum* 256 producing cytoplasmic TTFC led to only modest antigen-specific IgG levels (i.e. 10^{2-4}) after i.g. administration [24], whereas anti-TTFC serum titers were very high (i.e. 2.5×10^5) with *L. plantarum* NCIMB8826 producing high cytoplasmic levels of TTFC (i.e. *L. plantarum* (pMEC127)). In addition, while no major difference is expected by the i.n. route between uptake and processing of gut colonizing or non-colonizing strains, efficient immunization by the i.g. route could be influenced much

more importantly by the nature of the bacterial vector used. If persisting strains replicate actively in vivo, the induced immune responses might vary as a result of the antigen location and the nature of the bacterial vehicle itself as was also hypothesized by Shaw et al. [24].

We verified whether the persistence time of the three recombinant *L. plantarum* NCIMB8826 strains in the mouse intestine was identical to that of the non-expressor strain. This was found to be the case. Nevertheless, in our hands, it seems that in vivo replication of *L. plantarum* NCIMB8826 is unable to compensate totally for the lower antigen dose produced by strains *L. plantarum* (pMEC160) (secretion) and *L. plantarum* (pMEC139) (cell surface) as both strains turned out to be less immunogenic by mucosal routes than the high-expressor strain *L. plantarum* (pMEC127). It is also possible that in these “exposed” locations TTFC is more susceptible to degradation by the intestinal milieu.

In summary, we have shown that lower antigen doses are sufficient to elicit specific serum IgG responses by systemic and mucosal routes when TTFC is exposed to the external milieu of the bacterial carrier. However several factors, such as persistence, in vivo genetic stability and replication ability of the recombinant strains, intrinsic adjuvanticity of the bacterial carrier, or immunological and biochemical properties of the protective antigen may all affect the immunogenicity of the host-vector combination by a specific route of administration. Therefore, we performed this comparative study keeping as many parameters constant as possible. In the system described here, the best results (i.e. high serum TTFC-specific IgG and local IgA responses) were obtained with the strains producing high intracellular levels of TTFC, by both the i.n. and i.g. routes. However, this required optimization of the corresponding expression vector. Systems aiming to improving cell surface presentation or in vivo antigen delivery are thus presently being developed in the laboratory.

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