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Surface Display of N-Terminally Anchored Invasin by *Lactobacillus plantarum* Activates NF-κB in Monocytes

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The probiotic lactic acid bacterium *Lactobacillus plantarum* is a potential delivery vehicle for mucosal vaccines because of its generally regarded as safe (GRAS) status and ability to persist at the mucosal surfaces of the human intestine. However, the inherent immunogenicity of vaccine antigens is in many cases insufficient to elicit an efficient immune response, implying that additional adjuvants are needed to enhance the antigen immunogenicity. The goal of the present study was to increase the proinflammatory properties of *L. plantarum* by expressing a long (D1 to D5 [D1-D5]) and a short (D4-D5) version of the extracellular domain of invasin from the human pathogen *Yersinia pseudotuberculosis.* To display these proteins on the bacterial surface, four different N-terminal anchoring motifs from *L. plantarum* were used, comprising two different lipoprotein anchors, a transmembrane signal peptide anchor, and a LysM-type anchor. All these anchors mediated surface display of invasin, and several of the engineered strains were potent activators of NF-κB when interacting with monocytes in cell culture. The most distinct NF-κB responses were obtained with constructs in which the complete invasin extracellular domain was fused to a lipoanchor. The proinflammatory *L. plantarum* strains constructed here represent promising mucosal delivery vehicles for vaccine antigens.

Lactobacilli belong to the lactic acid bacteria (LAB), which are indigenous organisms in a variety of ecological niches, including vegetables, meat, dairy products, and the human gastrointestinal tract (53). These organisms have been ingested for centuries and are regarded as safe for human consumption. In addition, some strains of lactobacilli have documented health benefits and are marketed as probiotics (6). Over the past decade, there has been an increasing interest in developing lactobacilli as mucosal delivery vehicles for a wide range of therapeutic and prophylactic proteins (64). The mucosal surfaces represent the major portal of entry for pathogens, and it has been shown that it is possible to induce both systemic and mucosal immune responses via these surfaces (5). Promising results have been obtained with mucosal delivery of vaccine antigens through the intranasal, oral, or genital mucosal surfaces by both commensal and attenuated pathogenic bacteria (62). There has been much focus on vaccine delivery by attenuated pathogenic bacteria because of their intrinsic immunostimulatory properties, but such strains could potentially regain their virulence and, also, confer a risk to immunocompromised individuals (18). While commensal strains are considered a safer alternative, they may lack the potentially beneficial immunostimulatory properties of pathogens. It has been claimed, however, that certain lactobacilli have intrinsic immunomodulatory properties (63).

The probiotic *Lactobacillus plantarum* WCFS1, the first *Lactobacillus* sequenced, is a human saliva isolate that has been successfully exploited as a vaccine delivery vehicle (13, 25). Still, the use of nonpathogenic bacteria such as *L. plantarum* in immunization strategies usually does not result in complete protection against the disease, suggesting that vaccine adjuvants are needed to boost the immune response generated by the antigen-expressing bacteria (62). Early studies with LAB have shown that both systemic and mucosal immune responses improve substantially upon coexpression of adjuvants, such as interleukin-12 (IL-12) and IL-6, with an antigen (4, 60). Recently, novel strategies have been employed to increase the immunogenicity of mucosal vaccine antigens—in particular, coexpression of proteins from pathogenic bacteria that target immune cells in the gut (14, 47). Antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs), are promising target cells in this context. These cells are able to detect microbes and present antigenic structures from these to T cells, thus eliciting adaptive immune responses (56).

The immunogenicity of an antigen is significantly influenced by its final cellular location (cytoplasmic, secreted, or anchored in or to the cell wall) in the bacterial delivery vector, and several studies have shown that surface anchoring of the antigen results in the highest antigen immunogenicity (3, 51). In the case of lactobacilli, there are several ways to anchor proteins to the extracellular surface, including lipid-mediated N-terminal anchoring to the cell membrane, N-terminal anchoring to the cell membrane mediated by a noncleaved N-terminal signal peptide (SP), C-terminal sortase-mediated covalent anchoring to the cell wall, and noncovalent anchoring through the presence of additional domains that interact with the cell wall, such as LysM domains. While the sortase-mediated route has been explored quite extensively (12, 23, 35, 42), other routes have received less attention (19).

In keeping with the above-mentioned considerations, the goal of the present study was to increase the proinflammatory properties of *L. plantarum* for vaccine delivery by expressing invasin from the enteropathogenic bacterium *Yersinia pseudotuberculosis*. Invasin is a multidomain virulence factor, consisting of a 500-amino-acid transmembrane domain and a 497-residue extracellular 5-domain structure (domains D1 to D5 [D1-D5]), forming an extended rod-like structure (28). The protruding C-terminal domains are important for the interaction of invasin with its receptor (D5 appears at the outer end of the rod and includes the C

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
L. plantarum WCFS1	Host strain	38
E. coli TOP10	Host strain	Invitrogen
Plasmids		
pCR-Blunt II TOPO	3.5-kb cloning vector for PCR fragments; Kan^r	Invitrogen
pLp_2588sAmyA	Em^r; pSip401 derivative (58) containing the inducible P_{sppA} promoter translationally fused to a gene construct encoding the Lp_2588 signal peptide followed by AmyA	45
pEV	Em^r; pLp_2588sAmyA derivative where the Lp_2588-AmyA cassette has been removed (control plasmid, "empty vector")	This study
pLp_1261Inv	Em^r; pLp_2588sAmyA derivative where the Lp_2588-AmyA cassette has been replaced by the lipoanchor sequence from lp_1261 fused to part of the *inv* gene encoding the 492 C-terminal residues of invasin (domains D1 to D5)	This study
pLp_1261InvS	Em^r; like pLp_1261Inv, but encoding only the 190 C-terminal residues of invasin (domains D4 and D5)	This study
pLp_1452Inv	Em^r; like pLp_1261Inv, but with the lipoanchor from Lp_1452 instead of the lipoanchor from Lp_1261	This study
pLp_1452InvS	Em^r; like pLp_1261InvS, but with the lipoanchor from Lp_1452 instead of the lipoanchor from Lp_1261	This study
pLp_1568InvS	Em^r; pLp_2588sAmyA derivative where the Lp_2588-AmyA cassette has been replaced by a cassette encoding C-terminally truncated Lp_1568 fused to domains D4 and D5 of invasin; for N-terminal signal peptide-based anchoring of invasin	This study
pLp_3014Inv	Em^r; pLp_2588sAmyA derivative where the Lp_2588-AmyA cassette has been replaced by a cassette encoding Lp_3014 fused to domains D1 to D5 of invasin; for anchoring through an N-terminal LysM domain	This study
pLp_3014InvS	Em^r; like pLp_3014Inv, but encoding only the 190 C-terminal residues of invasin (domains D4 and D5)	This study

terminus of the protein [28]). Invasin promotes the uptake of *Y. pseudotuberculosis* by M cells in the gut through binding to β_1-integrins exposed on the M cell surface, thereby targeting the bacterium to the lymphatic tissues (11). In addition, invasin is capable of initiating proinflammatory host cell reactions by activation of the innate immune system (7, 21, 26). To study whether invasin could have similar effects in *L. plantarum*, a full-length form (D1-D5, referred to as Inv) and a truncated form (D4-D5, referred to as InvS) of its extracellular domain were expressed and N-terminally anchored to the bacterial surface.

MATERIALS AND METHODS

Bacterial strains, cell lines, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* TOP10 cells (Invitrogen, Carlsbad, CA) were grown in brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, United Kingdom) at 37°C with shaking. *L. plantarum* cells were grown statically in MRS (Oxoid) broth at 37°C. Solid media were prepared by adding 1.5% (wt/vol) agar to the broth. The plasmid constructions were first established in *E. coli* cells and then transformed into *L. plantarum* cells. The antibiotic concentrations used were 5 μg/ml and 200 μg/ml erythromycin for *L. plantarum* and *E. coli*, respectively.

DNA manipulations and plasmid construction. DNA manipulations were performed essentially as previously described (54). The primers used in this study were purchased from Operon Biotechnologies GmbH (Cologne, Germany) and are listed in Table 2. Genomic DNA from *L. plantarum* overnight colonies was obtained by lysing the cells in a microwave oven at maximum intensity for 2 min. The cell lysate was used directly, i.e., without any further DNA purification, as the template in subsequent PCRs using hot start KOD polymerase (Toyobo, Japan). Amplified PCR fragments were separated on 1% agarose gels and purified using the NucleoSpin extract II kit (Macherey-Nagel GmbH & Co., Düren, Germany). PCR fragments were cloned into restriction-digested plasmids using the In-Fusion HD cloning kit (Clontech Laboratories, Mountain View, CA), following the manufacturer's instructions. Plasmid DNA was purified from *E. coli* using the NucleoSpin plasmid kit (Macherey-Nagel GmbH & Co). *L. plantarum* was transformed by electroporation according to a previously described method (33). The DNA sequences of all PCR amplicons were verified by sequence analyses.

All expression constructs used in this study (Table 1 and Fig. 1) are derivatives of pSIP401, a member of the pSIP400 vector series developed for inducible gene expression in lactobacilli (58, 59) and further developed for secretion and C-terminal anchoring of proteins (23, 45). For practical reasons, a previously described pSIP401 variant called pLp_2588AmyA (45) was used as a starting point. Initially, a 1,476-bp fragment from the *inv* gene corresponding to the extracellular part of the invasin protein (492 C-terminal residues, domains D1-D5) was amplified from the *Y. pseudotuberculosis* chromosome (the genomic DNA was a kind gift from G. Kapperud at the Norwegian Institute of Public Health) using the primers TInvF and TInvR. The amplicon was subcloned into the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA) according to the protocol provided by the supplier. The *inv* fragment was reamplified from the pCR-Blunt II-TOPO vector using the primer pair InvF/InvR, and a 225-bp fragment corresponding to the 75 N-terminal amino acids from Lp_1261 was amplified with the primer

TABLE 2 Primers used in this study

Primer	Sequence[a]
1261F	GGAGTATGATTCATATGAATTTCAAAACAGCTGCAA
1261R	**GTCGACCGCCGCAATCGTGCCCCCG**TTCTTACCGAGACGGT
1452F	GGAGTATGATTCATATGAAGAAATGGCTCATTGCC
1452R	**GTCGACCGCCGCAATCGTGCCTTGA**ACCGTGACTTTAGGTTCGT
1568F	GGAGTATGATTCATATGAAATTGTTTAAGAAAATTACGATAAAT
1568R	**CGGTCAGCGTGTCGAC**CGCTGCATAAATTTGCTTAGCA
3014F	GGAGTATGATTCATATGAAAAAACTTGTAAGTACAATCGTAACT
3014R	**CGGTCAGCGTGTCGAC**AAGGGCCCAAGCAGCCAT
TInvF	CATATGAGCGTCACCGTTCAGCAGC
TInvR	GAATTCTTATATTGACAGCGCACAGAGC
InvF	**CGGGGGCACGATTGCGGCGGTCGAC**AGCGTCACCGTTCAGCAGC
InvSF	**CGGGGGCACGATTGCGGCGGTCGAC**ACGCTGACCGGTATTCTGGT
InvR	CCGGGGTACCGAATTCTTATATTGACAGCGCACAGAGC

[a] Underlining indicates 15-bp extensions that are complementary to the ends of the NdeI-EcoRI-digested p2588sAmyA vector. Such overlap is necessary for In-Fusion cloning (In-Fusion HD cloning kit). Boldface indicates primer extensions necessary for fusing anchor and Inv fragments by splicing by overlap extension (SOE)-PCR. NB: there are four nonoverlapping nucleotides in the InvF-1452R pair, but this does not affect the correctness of the final PCR product (as confirmed by sequencing).

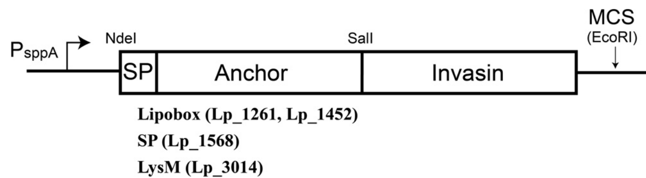

FIG 1 Schematic overview of the expression cassette for N-terminal anchoring of invasin in *L. plantarum*. The vectors are based on previously described secretion vectors (19) in which the cassette is translationally fused to the inducible P_{sppA} promoter. All parts of the cassette are easily exchangeable using the introduced restriction sites: the NdeI site at the translational fusion point, the SalI site between the N-terminal anchor and invasin, and the downstream multiple cloning site (MCS, including EcoRI). Three principally different N-terminal anchoring motifs were used, all containing a signal peptide (SP) for secretion: two different lipoanchors were generated using lipobox fragments from Lp_1261 and Lp_1452, one transmembrane anchor was generated by fusing invasin to C-terminally truncated Lp_1568, which contains an SP but no signal peptide cleavage site, and one LysM anchor was generated by fusing invasin to Lp_3014.

pair 1261F/1261R. These fragments (with 25 overlapping base pairs) were subsequently mixed and fused together in a splicing by overlap extension-PCR (SOE-PCR) reaction (29) using the primers 1261F and InvR. By using this approach, a 7-residue linker (encoded by GGC ACG ATT GCG GCG GTC GAC, corresponding to the amino acid residues GTIAAVD) was introduced between Lp_1261 and Inv. This linker encodes a 5-residue loop naturally found between domains D1 and D2 in the invasin protein followed by a SalI restriction site (encoding VD). The Lp1261-Inv fragment was subsequently In-Fusion cloned into NdeI-EcoRI-digested pLp_2588AmyA (45), yielding the plasmid pLp_1261Inv. A plasmid encoding a shorter version of Inv (190 C-terminal residues, domains D4-D5), pLp_1261InvS, was constructed using the same strategy, except that the InvS fragment was amplified with the primer pair InvSF/InvR. Using the same strategy, the Inv and InvS fragments were also fused downstream to a fragment from lp_1452 (corresponding to the 142 N-terminal residues of the protein, amplified using the primers 1452F and 1452R). The resulting 1452-Inv and 1452-InvS fragments were subsequently In-Fusion cloned into NdeI-EcoRI-digested pLp_2588AmyA, yielding the plasmids pLp_1452Inv and pLp_1452InvS, respectively.

The Inv and InvS fragments were further fused to Lp_3014 from *L. plantarum* for noncovalent, N-terminal anchoring. This gene product is a putative transglycosylase that binds the peptidoglycan in the cell wall through an N-terminal LysM domain (65). All 612 bp from the lp_3014 open reading frame (corresponding to 204 residues) were amplified with the primer pair 3014F/3014R and In-Fusion cloned into NdeI-SalI-digested pLp_1452InvS, resulting in the plasmid pLp_3014InvS. To construct pLp_3014Inv, the pLp_1452Inv vector was digested with SalI and EcoRI, and the resulting Inv fragment was cloned into SalI-EcoRI-digested pLp_3014InvS.

Vectors encoding Inv and InvS anchored by an N-terminal transmembrane anchor were constructed using Lp_1568, an N-terminally anchored penicillin binding protein from *L. plantarum* with a putative uncleaved signal peptide. A 2,013-bp fragment from lp_1568 (corresponding to the complete protein with a 7-residue C-terminal truncation) was amplified with the primer pair 1568F/1568R and subsequently In-Fusion cloned into the NdeI-SalI-digested pLp_1452InvS vector, resulting in the plasmid pLp_1568InvS. Despite numerous attempts, a variant of this plasmid with the full-length invasin was not obtained.

Finally, a plasmid lacking an open reading frame was constructed for use as a negative control. The sticky ends of the NdeI-EcoRI-digested pLp_2588sAmyA plasmid were made blunt by incubation with Phusion polymerase (New England BioLabs, Inc., Ipswich, MA) and 10 mM deoxynucleoside triphosphate for 10 s at 72°C. The blunted, linearized plasmid was recircularized by incubation with T4 DNA ligase (Invitrogen, Carlsbad, CA) overnight at 15°C, yielding the plasmid pEV. All plasmids were transformed into *E. coli* TOP10 cells before electroporation into *L. plantarum* WCFS1.

Antisera. Polyclonal antibodies against invasin were ordered from ProSci (Poway, CA) and generated by immunizing rabbits with synthetic invasin peptides ($_{804}$NGQNFATDKGFPKT$_{817}$ and $_{942}$YSSDWQSGEYWVKK$_{955}$). Three production bleeds were taken after the immunization of the animals, of which the third bleed was used in all experiments.

Harvesting of invasin-expressing cells. To analyze invasin production, *L. plantarum* cells harboring the constructed plasmids were diluted in MRS to a cell density with an optical density at 600 nm (OD_{600}) of ~0.1 from an overnight culture and incubated for approximately 2 h at 37°C, after which invasin expression was induced with 25 ng/ml peptide pheromone as described elsewhere (27). Two hours after induction, 8×10^9 CFU of bacterial cells were harvested by centrifugation at $7,000 \times g$ for 2 min at 4°C, and the resulting cell pellets were washed once in phosphate-buffered saline (PBS).

Flow cytometry and indirect immunofluorescence microscopy of invasin-expressing *L. plantarum*. Approximately 1×10^8 harvested cells were resuspended in 300 μl PBS containing 1% bovine serum albumin (PBS-B) and 40 μl rabbit antiserum (containing anti-invasin polyclonal antibodies). After incubation at room temperature (RT) for 30 to 60 min, the bacteria were centrifuged at $7,000 \times g$ for 2 min at 4°C and washed five times with 500 μl PBS five times. The cells were subsequently incubated with goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch, PA) diluted 1:200 in PBS-B for 30 to 60 min at room temperature. After collecting the bacteria by centrifugation at $7,000 \times g$ for 2 min and washing with 500 μl PBS-B five times, staining was analyzed by flow cytometry using a MACSQuant analyzer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), following the manufacturer's instructions. For indirect immunofluorescence microscopy, the bacteria were visualized under a Leica SP5 confocal scanning laser microscope using a 488-nm argon laser (FITC photomultiplier tube [PMT]) and a bright field (BF) PMT for transmitted light (Leica Microsystems, GmbH, Wetzlar, Germany).

Cell lines. U937 cells stably transfected with the NF-κB reporter plasmid 3x-κB-*luc* (9) (a kind gift from Rune Blomhoff) were grown in RPMI 1640 medium (PAA Laboratories GmbH, Pasching, Austria) containing 1% nonessential amino acids, 1 mM sodium pyruvate, 50 μM thioglycerol, 25 μg/ml gentamicin (Garamycin), and 10% fetal calf serum (Gibco Life Technologies, Paisley, United Kingdom). Cells were maintained in a humidified incubator at 37°C and 5% CO_2.

NF-κB reporter assay. U937 cells were cultivated in the presence of 75 μg/ml hygromycin for 24 h before seeding. Amounts of 3×10^4 cells in 80 μl of medium were seeded per well in a white 96-well plate (Nunc, Rochester, NY). *L. plantarum* cells harboring the invasin expression plasmids were grown, induced, and harvested as described above. Approximately 8×10^9 cells were resuspended in 300 μl PBS and exposed to UV light for 15 min at RT, after which the attenuated bacteria were collected by centrifugation at $7,000 \times g$ for 1.5 min at RT. Subsequently, 2×10^7 bacteria or 1 μg/ml lipopolysaccharide (LPS) was added to the wells. After 6 h of incubation in a humidified incubator at 37°C, the luciferase activity of the cells was assayed by using the Bright-Glo luciferase assay system according to the instructions of the manufacturer (Promega, WI).

Statistical analyses. Quantitative experimental data come from triplicate experiments and are presented as the means ± standard deviations (SD). Where relevant, statistically significant differences ($P < 0.01$) were determined by using unpaired *t* tests.

RESULTS

Surface display of invasin in *L. plantarum*. To display invasin from *Y. pseudotuberculosis* on the surface of *L. plantarum*, the pSIP vector system previously developed for protein secretion and C-terminal cell wall anchoring (23, 44, 45) was further developed for N-terminal anchoring, as shown schematically in Fig. 1. N-terminal anchoring is necessary to orient the C-ter-

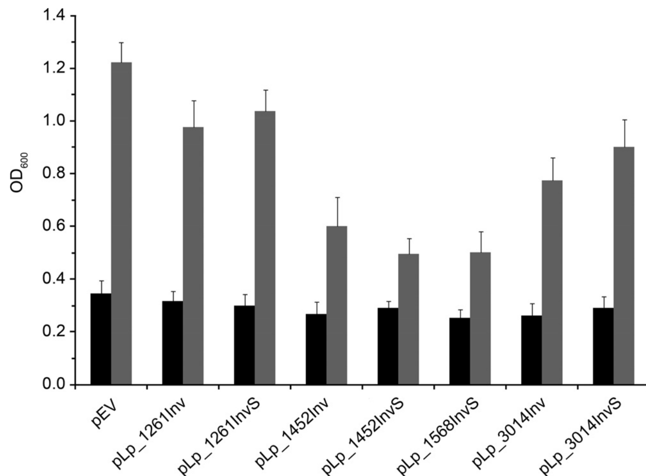

FIG 2 Cell growth of invasin-expressing *L. plantarum* strains. OD_{600} values were measured at the induction point (black bars) and 2 h after induction (gray bars). The data are means from triplicate experiments and presented as the means ± SD.

minal β_1-integrin binding domains of invasin distal to the *L. plantarum* cell surface. To achieve N-terminal anchoring, signals from two lipoproteins (Lp_1261 and Lp_1452), one protein with an uncleaved signal peptide acting as an N-terminal transmembrane anchor (Lp_1568), and a cell surface protein with a LysM anchoring motif (Lp_3014) were exploited. Lp_1261 and Lp_1452 are predicted to encode the oligopeptide ABC transporter OppA and the peptidylprolyl isomerase PrsA, respectively (65), which are anchored to the outer leaflet of the plasma membrane through an N-terminal, lipid-modified cysteine residue (30). The Inv-encoding gene fragments were fused to 75-residue and 142-residue N-terminal fragments, respectively, approximately corresponding to the regions upstream of the catalytic domains as predicted by Pfam (22). Lp_1568 is predicted to encode the penicillin binding protein 2B, which is inserted into the plasma membrane through its signal peptide, which lacks a signal peptidase (SPase) cleavage site (65). Lp_3014 is a putative extracellular transglycosylase with a cleavable signal peptide and an N-terminal LysM domain according to SignalP (52) and Pfam (22). The LysM domain is a widespread protein motif in Gram-positive bacteria, which mediates noncovalent binding to the peptidoglycan in the cell wall (8). In the case of Lp_3014 and Lp_1568, the invasin fragments were fused to the essentially complete *L. plantarum* protein.

Two versions of invasin were fused to the anchor sequences. The longer version, referred to as Inv, comprises all five extracellular domains, D1 to D5, of this protein (28), whereas the shorter version, InvS, comprises the C-terminal D4 and D5 domains only. It was anticipated that InvS might have a lower probability of causing secretion problems in *L. plantarum*, because of its smaller size. This strategy led to the construction of a total of seven plasmids encoding invasin destined for surface display in *L. plantarum* (the eighth construct, pLp_1568Inv, was never obtained; see Materials and Methods for details).

The presence of constructs for invasin expression affected the growth rate of the *L. plantarum* host strain to various extents (Fig. 2). Strains harboring plasmids with Lp_1452- or Lp_1568-derived anchors showed clearly reduced growth, indicating that overproduction of these chimeric proteins confers significant stress on the production host. On the other hand, strains harboring Lp_1261- or Lp_3014-derived anchors showed only slightly reduced growth.

Flow cytometry analyses showed a clear shift in the fluorescence signal compared to that of the control strain, confirming that all tested anchors mediate surface expression of invasin (Fig. 3A). Immunofluorescence microscopy (Fig. 3B) confirmed surface localization and revealed an uneven distribution of the fluorescence signal, which appears strongest at the septum and at distinct focal patches (Fig. 3C).

Invasin displayed on the *L. plantarum* surface stimulates NF-κB-activated luciferase expression in human monocytes. Seven invasin-producing *L. plantarum* strains were then evaluated for their capacity to activate NF-κB in the monocytic cell line U937. This cell line is stably transfected with an NF-κB reporter plasmid, meaning that the production of luciferase is induced upon activation of NF-κB (9). The innate immune response is the first line of defense against invading microorganisms. NF-κB regulates a wide variety of proinflammatory genes, including cytokines, chemokines, and adhesion molecules which play critical roles in innate immune responses (40). Previous studies on invasin from *Yersinia enterocolitica*, which shows 73% sequence identity with invasin from *Y. pseudotuberculosis*, have shown that activation of NF-κB is mediated by invasin's ability to bind to β_1-integrins on the surface of epithelial cells (55). The presence of β_1-integrins on the U937 cells was verified by flow cytometry using β_1-integrin-recognizing anti-CD29-FITC antibody (CD29 corresponds to β_1-integrin), and this experiment showed that virtually all U937 cells were CD29 positive (results not shown).

The activation experiments were performed by coincubation of invasin-producing bacteria, harvested 2 h after induction, and the monocytic cell line U937, followed by detection of NF-κB-activated luciferase expression by chemiluminescence (Fig. 4). This revealed clear differences between the various strains. Compared to U937 cells alone or U937 cells incubated with bacteria harboring the empty vector, bacteria harboring pLp_1261Inv, pLp_1261InvS, pLp_1452Inv, pLp_1452InvS, and pLp_1568InvS induced statistically significant higher levels of chemiluminescence. Of these, bacteria harboring pLp_1261Inv and pLp_1452Inv induced levels that were significantly higher than the levels induced by the other positive strains, pLp_1261InvS, pLp_1452InvS, and pLp_1568InvS, and that were similar to the level obtained with the positive control, bacterial LPS. Both of these best-performing strains produce the complete 5-domain extracellular domain of invasin. In contrast, incubation of U937 cells with *L. plantarum* harboring pLp_3014Inv or pLp_3014InvS did not yield chemiluminescence signals that were distinguishable from the background.

DISCUSSION

In recent years, substantial research efforts on LAB as *in situ* vaccine delivery vehicles have generated promising results. These vehicles are regarded as safe for human use but tend to suffer from insufficient immune responses directed toward the antigen, especially in oral delivery (39). Ways to circumvent this include codelivery of adjuvants (4, 34) or targeting of the bacteria to relevant cells of the immune system, e.g., using dendritic cell-binding peptides (48). Another option is to target so-called antigen-sampling microfold (M) cells, which cover underlying mucosal lymphoid

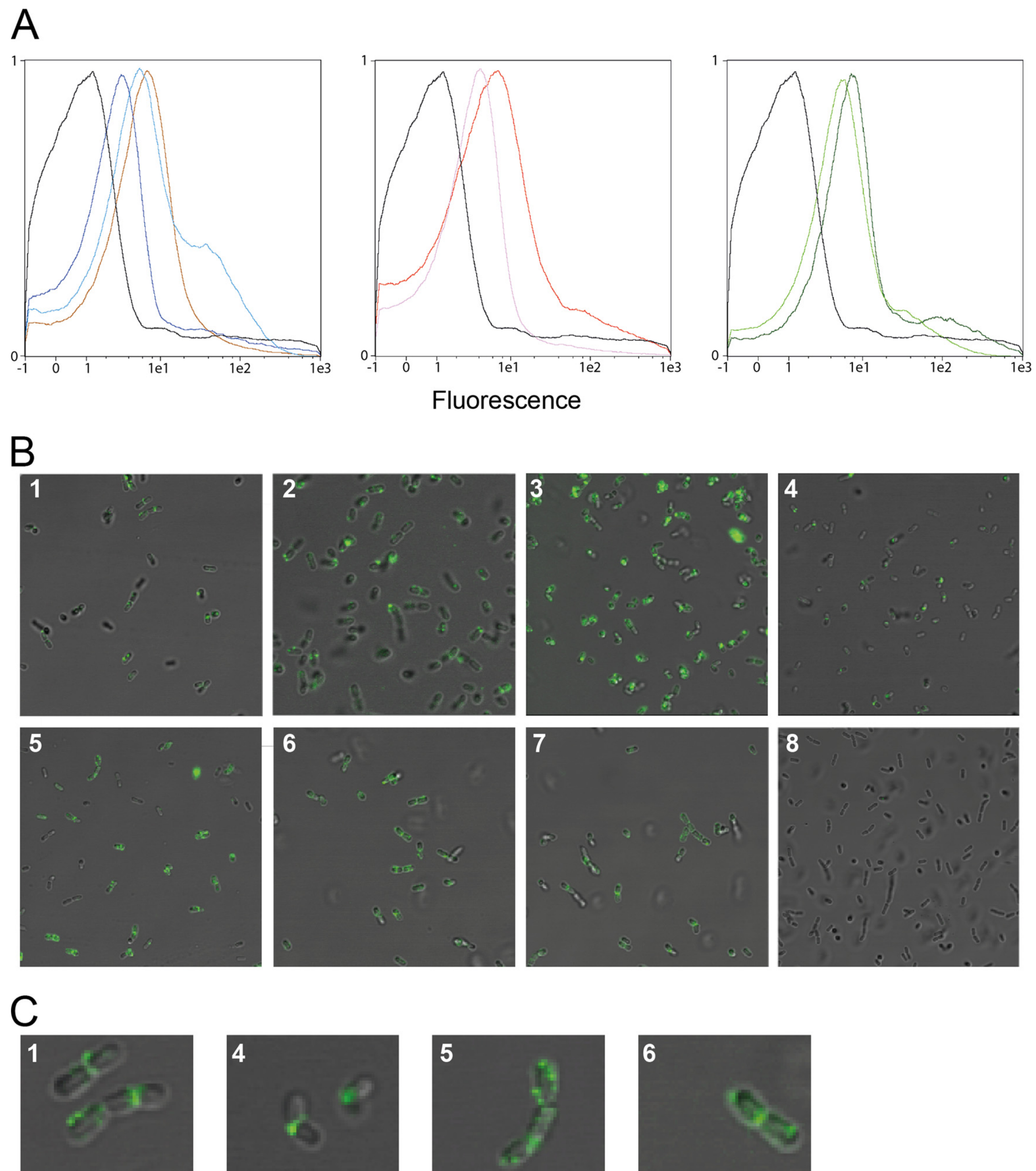

FIG 3 Surface localization of invasin in modified *L. plantarum* cells. (A and B) Representative flow cytometric (A) and microscopic (B) analyses of *L. plantarum* cells harboring plasmids designed for N-terminal surface anchoring of invasin. The cells were probed with rabbit anti-invasin polyclonal antibody and, subsequently, FITC-conjugated anti-rabbit IgG antibodies. The *L. plantarum* strains are denoted by different colors in the flow cytometry histograms and different numbers in the micrographs. *L. plantarum* harboring a vector without the *inv* gene was used as negative control (pEV, black, 8) and is depicted in all three flow cytometry histograms. *L. plantarum* strains harboring the following invasin-encoding plasmids are shown: pLp_1261Inv (light blue, 1), pLp_1261InvS (dark blue, 2), pLp_1452Inv (pink, 3), pLp_1452InvS (red, 4), pLp_1568InvS (brown, 5), pLp_3014Inv (green, 6), and pLp_3014InvS (dark green, 7). (C) Magnifications of representative cells depicted in panel B: pLp_1261Inv (1), pLp_1452InvS (4), pLp_1568InvS (5), and pLp_3014Inv (6).

tissue and represent a promising portal for mucosal vaccine delivery (32). In contrast to intestinal epithelial cells, M cells can take up microorganisms or antigens from the intestinal lumen and deliver them to the mucosal immune system in the lamina propria, leading to an enhanced immune response (2). Some pathogens, such as the human pathogen *Y. pseudotuberculosis*, exploit this route to more efficiently enter the body while at the same time suppressing the immune response. *Y. pseudotuberculosis* targets M cells by expressing invasin that binds β_1-integrins exposed on the surface of M cells (31, 43). Interestingly, invasin binding to β_1-integrins induces proinflammatory responses in epithelial cells, including activation of NF-κB and production of proinflammatory chemokines (36, 55, 61). Based on this knowledge, the idea behind the present study was that display of invasin on the surface of *L. plantarum* could mimic early infection characteristics of *Y. pseudotuberculosis* and thereby confer increased adjuvant proper-

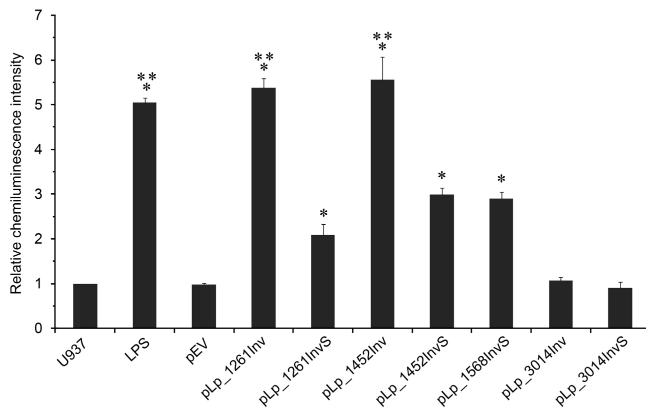

FIG 4 NF-κB-directed luciferase expression in monocytes. Various *L. plantarum* cells were coincubated with U937 monocytes stably transfected with a plasmid (3x-κB-*luc*) encoding a NF-κB-inducible luciferase gene (9). The intensities of the resulting luciferase-generated chemiluminescence signals were measured, and relative values are shown as the ratio of results for individual samples and the negative control (U937). The data come from triplicate experiments and are presented as the means ± SD. Statistically significant differences ($P < 0.01$) compared to the results for *L. plantarum*/pEV are denoted by an asterisk (*). Signals labeled with two additional asterisks (**) are significantly stronger ($P < 0.01$) than the signals labeled with only one asterisk. LPS, bacterial lipopolysaccharides (positive control).

ties on this probiotic organism. It has previously been shown that the D4 and D5 domains are sufficient for invasin binding to β_1-integrins, albeit with a reduced efficiency compared to the 5-domain D1-D5 version (17).

Anchoring of heterologous proteins to LAB surfaces using LPXTG-type C-terminal anchors for covalent linkage to the cell wall has been quite extensively explored (12, 20, 23, 35, 42). Likewise, the use of LysM domains to promote noncovalent association of proteins to LAB cell walls is well documented (1, 46). However, only a very few reports exist on N-terminal anchoring of heterologous proteins in lactobacilli, and these are often based on the use of non-self anchoring signals (16, 41). Interestingly, work on *Bacillus subtilis* has shown that a cellulase could be anchored by fusing it to PrsA, which is the homologue of Lp_1452. This strategy resulted in efficient targeting of the cellulase to the cytoplasmic membrane, but in contrast to the results presented here, the enzyme was not exposed to the extent that it could be detected by flow cytometry of intact cells (37).

All N-terminal anchors tested in this study, comprising two lipoproteins (Lp_1261 and Lp_1452), one protein containing an uncleaved signal peptide functioning as an N-terminal transmembrane anchor (Lp_1568), and one LysM-containing protein (Lp_3014), led to surface display of invasin as shown by flow cytometry and immunofluorescence microscopy (Fig. 3). Interestingly, the immunofluorescence microscopy data showed that invasin is unevenly distributed on the *L. plantarum* cell surface. This is not uncommon for surface proteins, and it has been shown that such distinct expression patterns could be directed by the nature of the signal peptide (10). All signal peptides used for surface localization of invasin are predicted to direct secretion through the SecYEG channel (50), but no experimental evidence for signal peptide-mediated localized protein secretion in lactobacilli is currently available.

The invasin-expressing *L. plantarum* strains differed with respect to their ability to activate NF-κB-induced luciferase expression in β_1-integrin-expressing monocytes (Fig. 4), despite the fact that invasin was detected on the surface of all strains (Fig. 3). Bacteria expressing Inv surface targeted by the lipoproteins Lp_1261 and Lp_1452 were clearly the most potent NF-κB activators, followed by InvS anchored by Lp_1261, Lp_1452, and Lp_1568. The data for the Lp_1261 and Lp_1452 constructs clearly show that InvS is less efficient than Inv when it comes to NF-κB activation. Importantly, this difference shows that NF-κB activation is most likely mediated by the invasin molecule and not by other components on the *L. plantarum* surface, such as overproduction of the Lp_1261 and Lp_1452 lipoprotein fragments themselves. In contrast to InvS, Inv contains the D2 domain, which is important for invasin functionality and efficient β_1-integrin binding (17). Somewhat surprisingly, Inv anchored by Lp_3014 did not significantly activate NF-κB. Notably, the Lp_3014 constructs create a fundamentally different way of anchoring, where Inv is associated with the cell wall rather than linked to the cell membrane via an inserted lipid or transmembrane peptide anchor. More generally, it should be noted that while similar amounts of bacterial cells were used in the experiments and while all cells clearly express invasin (Fig. 3), there still may be differences between the experiments due to variation in the number of exposed proteins per cell.

It is generally recognized that activation of NF-κB in monocytes promotes differentiation into activated macrophages, so-called M1 macrophages, which are associated with a proinflammatory T helper 1 immune response (24, 57). Interestingly, the activation of NF-κB observed for the *L. plantarum* strain expressing Lp_1261 lipoanchored invasin was quantitatively similar to the NF-κB activation by LPS from Gram-negative bacteria, which is known to induce differentiation of monocytes into M1 macrophages (49). Thus, *L. plantarum* expressing lipoanchored Inv seems to have acquired the desired proinflammatory properties. Attempts to demonstrate the ability of invasin-expressing *L. plantarum* cells to invade Caco-2 cells were not successful (data not shown; the usefulness of Caco-2 cells as a model system for M cells has been demonstrated in previous studies on invasin-expressing *E. coli* [15]). Dersch and Isberg (17) have previously shown that multimerization of invasin promotes efficient cell internalization. It is conceivable that that the multimerization efficiency of invasin on the *L. plantarum* surface was too low, either due to too-low protein densities or sterical hindrances.

In conclusion, the current study shows that *L. plantarum* surface proteins with three fundamentally different N-terminal anchoring motifs are able to target invasin from *Y. pseudotuberculosis* to the *L. plantarum* cell surface. Furthermore, the data show that the displayed invasin had adjuvant activity in several of the engineered strains. Although the study does not present a systematic study of the suitability of various N-terminal anchoring options, the results so far do indicate that hitherto hardly explored lipoanchoring is both feasible and promising. For the particular purpose of this study, the Lp_1261 lipoanchor worked best, since the strain containing pLp_1261Inv yielded maximum NF-κB activation (Fig. 4) while being among those with the lowest impairment in growth rate (Fig. 2). Lipoanchors like Lp_1261 may provide an important expansion of the toolbox for surface targeting of proteins. Surface expression of invasin by *L. plantarum* could provide the tipping point for skewing a tolerogenic immune response in a proinflammatory direction, thereby increasing antigen immuno-

genicity. Due to their adjuvant properties, the *L. plantarum* strains constructed in this study represent promising mucosal vaccine delivery vehicles. Studies on coexpression of invasin and antigens are currently in progress.

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