Surface Display of the Receptor-Binding Region of the *Lactobacillus brevis* S-Layer Protein in *Lactococcus lactis* Provides Nonadhesive Lactococci with the Ability To Adhere to Intestinal Epithelial Cells

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Lactobacillus brevis* is a promising lactic acid bacterium for use as a probiotic dietary adjunct and a vaccine vector. The N-terminal region of the S-layer protein (SlpA) of *L. brevis* ATCC 8287 was recently shown to mediate adhesion to various human cell lines in vitro. In this study, a surface display cassette was constructed on the basis of this SlpA receptor-binding domain, a proteinase spacer, and an autolysin anchor. The cassette was expressed under control of the *nisA* promoter in *Lactococcus lactis* NZ9000. Western blot assay of lactococcal cell wall extracts with anti-SlpA antibodies confirmed that the SlpA adhesion domain of the fusion protein was expressed and located within the cell wall layer. Whole-cell enzyme-linked immunosorbent assay and immunofluorescence microscopy verified that the SlpA adhesion-mediating region was accessible on the lactococcal cell surface. In vitro adhesion assays with the human intestinal epithelial cell line Intestine 407 indicated that the recombinant lactococcal cells had gained an ability to adhere to Intestine 407 cells significantly greater than that of wild-type *L. lactis* NZ9000. Serum inhibition assay further confirmed that adhesion of recombinant lactococci to Intestine 407 cells was indeed mediated by the N terminus-encoding part of the *slpA* gene. The ability of the receptor-binding region of SlpA to adhere to fibronectin was also confirmed with this lactococcal surface display system. These results show that, with the aid of the receptor-binding region of the *L. brevis* SlpA protein, the ability to adhere to gut epithelial cells can indeed be transferred to another, nonadhesive, lactic acid bacterium.

Lactobacilli are commonly found microorganisms in nature and have been widely used in dairy and other food fermentations for centuries. Many *Lactobacillus* species are also members of the normal microbiota of the human and animal gastrointestinal and genitourinary tracts. Various *Lactobacillus* species are used commercially as probiotics because of their proposed beneficial effects on the health of the host. Among the requirements commonly suggested for a microorganism to be classified as probiotic are the ability to adhere to gut epithelial tissue and the ability to at least transiently colonize the gastrointestinal tract (10). Compared with the present knowledge of the adhesive mechanisms of many pathogenic bacteria, only limited information is available about the adhesive surface molecules of lactobacilli and their tissue receptors. A recent study suggested fibronectin as one of the eukaryotic receptors that mediate the adhesion of lactobacilli to epithelial cells (16). Lectin-like molecules (25) and lipoteichoic acids (11, 34) have also been shown to function as adhesins in lactobacilli. In several cases, however, the adhesion of lactobacilli has been reported to be protein mediated (1, 7, 8, 22), but thus far, only a few proteinaceous adhesins have been identified, including a protein component of the bacterial ATP-binding cassette (29), an S-layer protein (33, 36), a surface protein that is able to bind

to porcine intestinal mucus and gastric mucin (28), and a Mub protein adhering to mucus components (30).

S-layers are regular paracrystalline surface protein arrays commonly found in species belonging to all major phylogenetic groups of *Bacteria* and are an almost universal feature of *Archaea* (reviewed in reference 32). Most S-layers are composed of a single protein or glycoprotein species that forms the S-layer lattice by an intrinsic self-assembly process. Diverse functions have been proposed for S-layers, like acting as cell-protective coats, molecular sieves, molecular and ion traps, and cell adhesion mediators (reviewed in reference 37).

S-layers are also found in some species of the genus *Lactobacillus* (24, 44). To date, only a few S-layer genes of lactobacilli have been described (4, 6, 36, 41). The functions of lactobacillary S-layers are poorly known. In *Lactobacillus acidophilus*, the S-layer has been proposed to mediate binding to avian intestinal epithelial cells (33), and in *Lactobacillus crispatus*, the S-protein (CbsA) has been shown to mediate binding to collagen (40). Flagellar display experiments with *Escherichia coli* have shown that the S-layer protein (SlpA) of *Lactobacillus brevis* ATCC 8287 mediates adhesion to human intestinal cell lines and fibronectin in vitro via a binding region located within the N-terminal part of the SlpA protein (15).

In this report, we describe the successful transfer and expression of the SlpA receptor-binding region in *Lactococcus lactis* and demonstrate that nonadhesive lactococci can be endowed with the ability to adhere to a human intestinal epithelial cell line and fibronectin with the aid of the SlpA adhesin.

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TABLE 1. Strains, plasmids, and cell line used in this study

Strain, plasmid, or cell line	Relevant properties[a]	Reference or source
Strains		
Lactobacillus brevis ATCC 8287		ATCC
Lactococcus lactis NZ9000	*pepN::nisR nisK*	9
Lactococcus lactis NCDO712	Lac$^+$ Prt$^+$	NCDO[b]
Escherichia coli DH5α	Transformation host	12
Plasmids		
pUC19	Ap^r; *E. coli* cloning vector	44
pKTH5033	pUC19 derivative containing 1.5-kb *prtP* spacer	This study
pKTH5042	pUC19 derivative containing 0.6-kb *prtP* spacer	This study
pNG101his	Cm^r; 4.6 kb; pNG101 derivative carrying His tag and autolysin anchor transcriptionally fused to *nisA* promoter	K. Leenhouts
pKTH5043	pNG101his derivative carrying 0.6-kb *prtP* spacer fused to *nisA* promoter	This study
pKTH5046	pNG101his derivative carrying 1.5-kb *prtP* spacer fused to *nisA* promoter	This study
pKTH5050	pKTH5043 derivative carrying mature part of *bla* gene and 0.6-kb *prtP* spacer fused to *nisA* promoter	This study
pKTH5051	pKTH5046 derivative carrying mature part of *bla* gene and 1.5-kb *prtP* spacer fused to *nisA* promoter	This study
pKTH5056	pKTH5046 derivative carrying adhesion region from *slpA* gene (coding for amino acids 1–247) and 1.5-kb *prtP* spacer fused to *nisA* promoter	This study
Cell line Intestine 407	Human intestinal epithelial cell line ATCC CCL-6	ATCC

[a] Ap^r, resistance to ampicillin; Cm^r, resistance to chloramphenicol.
[b] NCDO, National Collection of Dairy Organisms.

L. lactis was chosen as the model host because of its amenability to genetic engineering, the availability of a wide variety of genetic tools for this bacterium, and its wide experimental use as a producer of bioactive molecules.

MATERIALS AND METHODS

Bacterial strains, plasmids, cell line, and growth media. The strains and plasmids used in this study are listed in Table 1. *Lactobacillus* strains were grown in MRS (Difco, Detroit, Mich.) at 37°C. *Lactococcus* strains were propagated in M17 (Difco) containing 0.5% (wt/vol) glucose at 30°C without shaking or with gentle shaking (60 rpm) for the fibronectin adhesion assay. *E. coli* strains were grown in Luria-Bertani medium at 37°C under aeration. Antibiotics were used, when appropriate, at the following concentrations: chloramphenicol, 5 μg/ml (for *L. lactis*); ampicillin, 50 μg/ml (for *E. coli*).

The Intestine 407 cell line was obtained from the American Type Culture Collection (ATCC). Intestine 407 cells were routinely grown at 37°C in a 95% air–5% CO$_2$ atmosphere in RPMI 1640 medium (Life Technologies, Paisley, Scotland) supplemented with 2 mM L-glutamine, 1% heat-inactivated (30 min 56°C) fetal calf serum, 50 U of penicillin per ml, 50 μg of streptomycin per ml, and a 1% (vol/vol) minimal essential medium nonessential amino acid solution (Life Technologies). For adhesion assays, Intestine 407 cell monolayers were prepared by inoculating eight-well microscope slides (Knittel, Braunschweig, Germany) with 25 μl of a freshly diluted (in growth medium) cell suspension, incubating them for 1 h at 37°C, and then covering the slides with growth medium and further incubating them for 2 days at 37°C. The slides were washed once with phosphate-buffered saline (PBS) before the adhesion assays.

DNA methods and transformation. Routine molecular biology techniques were used (2). Enzymes were used as recommended by the manufacturers (Promega, Madison, Wis.; New England Biolabs Inc., Beverly, Mass.). Plasmid DNA was isolated from *L. lactis* and *L. brevis* by using the QIAfilter Plasmid Midi Kit (Qiagen GmbH, Hilden, Germany) and 8 mg of lysozyme per ml and from *E. coli* by using the Wizard Minipreps kit (Promega). *L. lactis* and *E. coli* cells were transformed as described by Holo and Nes (14) and Sambrook and Russell (31), respectively. Correct PCR amplification was verified by using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, Calif.) in combination with the DNA sequencing kit for BigDye terminator cycle sequencing (Applied Biosystems).

Construction of plasmid vectors. The proteinase (*prtP*) gene sequence of *L. lactis* subsp. *cremoris* Wg2 (EMBL accessions number M24767) was used to design primers for the amplification of 0.6- and 1.5-kb *prtP* spacers. For both *prtP* spacers, the same downstream primer, 5′-CGAAGAATTCGAGTCGTATCAT

CCGTGC-3′ (*Eco*RI site underlined), was used for PCR amplification. The upstream primers with an *Nco*I site (underlined) for the 0.6- and 1.5-kb *prtP* fragments were 5′-TTACCCATGGGTGCC AATCGAGACC-3′ and 5′-CGAA CCATGGTCAAGCACCCAACG-3′, respectively. Plasmid DNA isolated from strain *L. lactis* NCDO712 was used as the template in PCRs. The resulting PCR products, 0.6- and 1.5-kb *prtP* fragments, were treated with Klenow polymerase to generate blunt ends, digested with *Eco*RI, and cloned into *Eco*RI- and *Sma*I-digested pUC19 (41), resulting in plasmids pKTH5042 and pKTH5033, respectively (Table 1). The *prtP* spacers were isolated as *Nco*I-*Eco*RI fragments from pKTH5042 and pKTH5033 and cloned into the *Nco*I-*Eco*RI sites of pNG101his (Table 1), resulting in plasmids pKTH5043 and pKTH5046, respectively.

For expression of the mature β-lactamase protein (Bla), its gene (*bla*) was PCR amplified from pUC19 by using primers 5′-TACTTCTAGACCACCCAG AAACGCTGG-3′ and 5′-ATACTCTAGAGCTTACCAATGCTTAATCAG-3′ with an *Xba*I site (underlined). The approximately 0.8-kb PCR fragment obtained was digested with *Xba*I and cloned into the *Xba*I sites of pKTH5043 and pKTH5046, resulting in plasmids pKTH5050 and pKTH5051, respectively.

To create an exact joint between the *nisA* promoter and the signal sequence of the *L. brevis slpA* gene, the recombinant PCR technique was applied essentially as described earlier (18). Briefly, the *nisA* promoter fragment was amplified from pNG101his with primers 5′-CCAAGATCTAGTCTTATAACTATACTG (P1; *Bgl*II site underlined) and 5′-TAAACTTGATTGCATGGTGAGGTGCCTC (P2), resulting in an approximately 0.2-kb fragment. The DNA region encoding the SlpA signal peptide and the SlpA receptor-binding region (amino acids 31 to 247) were synthesized by using primers 5′-GAGGCACTCACCATGCAATCA AGTTTAAAG-3′ (P3) and 5′-CACATCTAGACTGTTATCGTTGGTTGC-3′ (P4; *Xba*I site underlined), resulting in an approximately 0.7-kb fragment. The 0.2- and 0.7-kb PCR fragments were hybridized, and the hybrid formed was extended by DNA polymerase in a PCR without primers, followed by a further PCR amplification in the presence of the primer pair P1-P4. The resulting 0.9-kb fragment was purified, digested with *Bgl*II and *Xba*I, and ligated into *Bgl*II-*Xba*I-digested pKTH5046, resulting in plasmid pKTH5056 (Fig. 1).

Nisin induction. For controlled expression, nisin induction of recombinant *L. lactis* strains was performed as follows. From a culture grown overnight, a 3% (vol/vol) inoculum was transferred to fresh medium and the bacteria were grown at 30°C until the optical density at 600 nm was 0.2 to 0.3. Nisin was added at different concentrations (0.8 to 10 ng/ml), and cells were propagated for 4 to 20 h before harvesting.

Whole-cell ELISA for detection of cell surface-exposed polypeptides. Recombinant *L. lactis* cells harboring pKTH5050, pKTH5051, or pKTH5056 were harvested after nisin induction or overnight incubation. Cell surface-displayed polypeptides were assayed with a whole-cell enzyme-linked immunosorbent as-
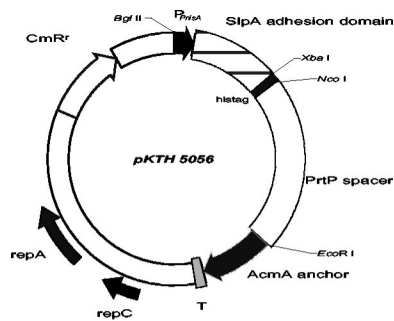
FIG. 1. Schematic drawing of vector pKTH5056, which was constructed for the controlled expression of the surface-anchored adhesion domain of *L. brevis* SlpA in *L. lactis*. P$_{nisA}$, promoter sequence of the *nisA* gene of *L. lactis*; AcmA anchor, anchor peptide from the *L. lactis acmA* gene; PrtP spacer, 515-amino-acid-encoding region of the *L. lactis* subsp. *cremoris* Wg2 *prtP* gene; T, transcription terminator sequence; CmR^r, chloramphenicol resistance gene.

say (ELISA) essentially as described earlier (2a). The primary antibodies used were anti-SlpA serum (41) or anti-Bla serum (kindly provided by Matti Sarvas, National Public Health Laboratory).

Protein analysis. The total protein extracts were prepared from cells harvested after 4 h of nisin induction. The bacteria were resuspended in 500 µl of digestion buffer (50 mM HEPES [pH 7.0], 20% [wt/vol] sucrose, 5 mM MgCl$_2$, 5 mM CaCl$_2$, 10 mg of lysozyme per ml, 42 U of mutanolysin per ml). The digestion reactions were allowed to proceed for 90 min at 37°C in a water bath before the mixtures were chilled and disrupted by sonication for 30 s, resulting in total protein extracts that were mixed with Laemmli buffer (19).

The cell wall-associated polypeptides were extracted as follows. Recombinant *L. lactis* cells harboring pKTH5056 were harvested after nisin induction (4 h) or from overnight culture. The bacteria were resuspended in 1 ml of 50 mM HEPES (pH 7.0) and centrifuged. Lactococcal cells were disrupted by homogenization with glass beads in a cell mill (Bühler Vibrogen Cell Mill VL4; Edmund Bühler GmbH, Bodelshausen, Germany) for 60 s and resuspended in 500 µl of 50 mM HEPES (pH 7.0). After centrifugation (1,000 × *g*, 5 min, 4°C), the supernatant containing the cell walls and membranes was recovered and centrifuged again (18,500 × *g*, 30 min, 4°C) in order to pellet the cell wall-associated material. The pellet was resuspended in digestion buffer and incubated for 90 min at 37°C in a water bath for solubilization of the cell walls, followed by addition of Laemmli buffer.

The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted (MiniProtean II; Bio-Rad) onto nitrocellulose membranes. Polyclonal rabbit anti-SlpA antibodies (40) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) were used as primary and secondary antibodies, respectively. Bands were detected by addition of 4-chloro-1-naphthol (horseradish peroxidase color development reagent; Bio-Rad). Semiquantitation of the S-layer and fusion protein (from plasmid pKTH5056) bands was carried out by using a Multi-Analyst (Bio-Rad). Intensity values (numbers of pixels per unit of band area) were adjusted with cell amounts loaded onto the SDS-PAGE gel.

Immunofluorescence assay for detection of peptides on the cell surface of *L. lactis*. Detection of SlpA on the cell surface by immunofluorescence assay was performed essentially as described previously (2a). The slides were incubated for 1 h with undiluted or diluted (1:5 to 1:20 in PBS) anti-SlpA antibody (unspecific binding to *L. lactis* cells was first removed by incubating the serum with *L. lactis* NZ9000 cells for 4 h at 4°C) in a moist chamber at 37°C, washed twice with PBS, and finally incubated with fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin G serum (dilution, 1:10; DAKO A/S, Glostrup, Denmark) for 1 h in a moist chamber at 37°C.

Bacterial adhesion assays. Bacteria were harvested from overnight cultures or after nisin induction. For the assay of adhesion to epithelial cells, bacteria were resuspended in RPMI medium and the optical densities at 600 nm of these suspensions were adjusted to approximately 0.8. From these cell suspensions, 10 µl was added to each well of the Intestine 407 monolayer in microscope slides and incubated for 1 h at 37°C in a moist chamber. After treatment, the monolayers were washed five times with PBS at room temperature for 10 min with gentle agitation and fixed for 10 min with methanol. Air-dried slides were stained with Giemsa (Merck Eurolab, Darmstadt, Germany) and examined by light microscopy.

The statistical significance of the adherence capabilities of the bacterial cells was evaluated by one-way analysis of variance, and pairwise differences between the means of groups were determined by the Tukey HSD test for post-analysis-of-variance pairwise comparisons (available at http://faculty.vassar.edu/lowry /VassarStats.html). Differences were considered significant when *P* values were less than 0.01.

The ability of anti-SlpA serum to prevent the bacterial adherence to Intestine 407 cells was examined by incubating cells with diluted (1:20 in PBS) anti-SlpA serum (or with PBS as a negative control) for 2 h at room temperature. The bacterial cells were washed with PBS after treatment, resuspended in RPMI medium, and applied to Intestine 407 monolayer as described earlier. Bacterial adherence to Intestine 407 cells was scored as the average number of bacteria attached to one Intestine 407 cell (approximately 100 Intestine 407 cells per sample were counted). Student's *t* test for unpaired values was used to evaluate the statistical significance of the differences in adhesion of lactococcal cells after anti-SlpA antibody treatment compared with the control (PBS treatment). Differences were considered significant when *P* values were less than 0.01.

Bacterial adhesion to human plasma fibronectin (Becton Dickinson, Bedford, Mass.) was performed as described before (43), with the following modifications. Fibronectin was used at a surface concentration of 46 fmol/mm^2, and the slides were blocked with 1% (wt/vol) blocking reagent (Roche) in PBS and washed twice with 0.25% blocking reagent in PBS and once with PBS. The time of incubation with bacteria in PBS was 3 h, and nonadherent bacteria were removed by washing 3 × 10 min in PBS.

The statistical significance of differences between the abilities of bacteria to adhere to fibronectin was analyzed as described for bacterial adherence to Intestine 407 cells.

RESULTS

Construction of expression vectors for surface display. A derivative of the pNG101his vector (Table 1), allowing expression of surface-located proteins, was constructed. The pNG101his vector carries, under control of the *nisA* promoter, the signal sequence of the *L. lactis acmA* gene, a multicloning site, and a gene fragment encoding the cell wall-anchoring regions of the lactococcal AcmA protein (5). To increase the surface accessibility of hybrid proteins to be expressed, we used parts of the *L. lactis* subsp. *cremoris* Wg2 *prtP* gene (17) to construct spacers 0.6 and 1.5 kb in size. The 0.6- and 1.5-kb *prtP* inserts were cloned into pNG101his in frame with the AcmA cell wall-anchoring gene fragment under the *nisA* promoter, resulting in plasmids pKTH5043 and pKTH5046, respectively (Table 1).

Characterization of surface accessibility with a β-lactamase reporter. To establish a reporter system with which to study the utility of the spacers, the β-lactamase gene (*bla*) was cloned into pKTH5043 and pKTH5046, resulting in plasmids pKTH5050 and pKTH5051, respectively. Thus, pKTH5050 and pKTH5051 encode a fusion protein consisting of the AcmA signal peptide, the mature β-lactamase (Bla), one of the PrtP spacers, and the AcmA cell wall anchor. The surface accessibility of Bla in these chimeric fusion proteins was assessed by whole-cell ELISA with anti-Bla antibodies. The color response obtained with nisin-induced *L. lactis* strain NZ9000 harboring pKTH5051 was clearly more intense than that of *L. lactis* NZ9000 carrying pKTH5050 and those of uninduced cells (data not shown). The result indicated that the PrtP$_{1153-1668}$ spacer encoded by the 1.5-kb *prtP* fragment present in pKTH5051 gave better surface accessibility of Bla than did the PrtP$_{1453-1668}$ gene product encoded by the 0.6-kb *prtP* fragment of pKTH5050.

Surface display vector for expression of the SlpA adhesion domain in lactococci. On the basis of the results obtained with the whole-cell ELISA of *L. lactis* cells carrying pKTH5051, the SlpA adhesion-mediating region (amino acids 31 to 247) was
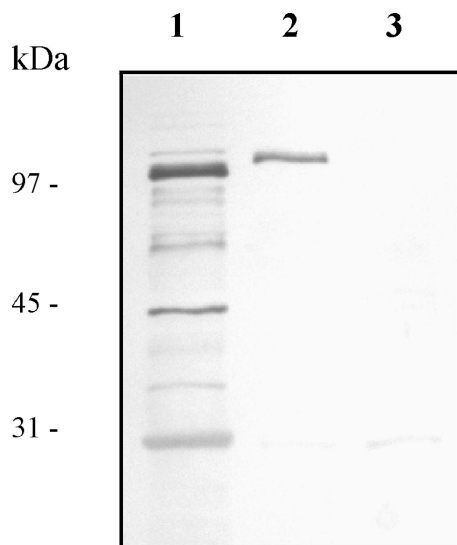
FIG. 2. Western blot analysis of the SlpA adhesion-mediating region. Total cellular proteins and cell wall-associated polypeptides were extracted from *L. lactis* NZ9000 cells harboring pKTH5056. The protein samples were prepared from equal amounts of cells as described in Materials and Methods, and samples were separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane, and incubated with polyclonal SlpA antiserum diluted 1:1,000. Lanes: 1, total cellular proteins from nisin-induced recombinant cells; 2, cell wall-associated polypeptides from nisin-induced recombinant cells; 3, cell wall-associated polypeptides from noninduced recombinant cells. Sizes of molecular mass marker proteins are indicated on the left.

cloned into pKTH5046, which carries the same 1.5-kb PrtP spacer as pKTH5051 (Table 1). To maintain a homologous signal sequence and a mature protein structure, the *acmA* signal sequence was replaced with that of the *L. brevis slpA* gene, resulting in the vector pKTH5056 (Fig. 1).

Cellular localization of the SlpA adhesion domain fused to PrtP and AcmA. The expression and cellular location of the SlpA-PrtP-AcmA fusion protein were studied with nisin-induced *L. lactis* NZ9000 cells harboring pKTH5056 by Western blot analysis of total protein extracts and cell wall-associated polypeptides. After separation by SDS-PAGE, blots were probed with anti-SlpA antibodies. As illustrated in Fig. 2 (lane 2), a band corresponding to the expected molecular mass (111 kDa) of the fusion protein was detected among the cell wall-associated polypeptides extracted from *L. lactis* with pKTH5056. A strong fusion protein band was also detected in the total protein extracts (Fig. 2, lane 1) from induced recombinant *L. lactis* cells, indicating efficient gene expression from pKTH5056. In contrast, no band corresponding to the fusion protein was detected in the cell wall-associated polypeptide extracts from uninduced *L. lactis* with pKTH5056 (Fig. 2, lane 3). This assay thus suggested that *L. lactis* cells transformed with pKTH5056 expressed SlpA adhesion-mediating molecules within its surface structure.

Surface accessibility of the SlpA receptor-binding region. The surface accessibility of the SlpA adhesion-mediating region was studied by whole-cell ELISA and immunofluorescence microscopy. For the whole-cell ELISA, recombinant *L. lactis* NZ9000 cells harboring pKTH5056 were harvested after nisin induction and from an uninduced culture. The cells were

incubated with anti-SlpA antibodies and assayed as described earlier for the Bla construct. The ELISA absorbance values of the induced recombinant lactococcal cells were clearly higher in every cell dilution than those of uninduced cells (Fig. 3). This result demonstrated that the pKTH5056-encoded SlpA receptor-binding region of the fusion protein was successfully displayed on the recombinant *L. lactis* cell surface.

The result obtained with the whole-cell ELISA was further verified with immunofluorescence microscopy by testing the reactivity of nisin-induced and uninduced *L. lactis* NZ9000 cells carrying pKTH5056 and of wild-type *L. lactis* NZ9000 cells with anti-SlpA antibodies. The nisin-induced cells carrying pKTH5056 exhibited positive fluorescence when reacted with anti-SlpA antibodies (Fig. 4A), while no fluorescence was obtained with the wild-type *L. lactis* NZ9000 cells (Fig. 4B). No fluorescence was observed either when uninduced recombinant cells with pKTH5056 were incubated with anti-SlpA antibodies (data not shown). The immunofluorescence assay thus confirmed the surface accessibility of the SlpA adhesion-mediating region of the pKTH5056-encoded fusion protein.

Adhesion of recombinant lactococci to Intestine 407 cells in vitro. The ability of pKTH5056 carrying *L. lactis* NZ9000 cells to adhere to human intestinal epithelial cells was assayed by an in vitro adhesion test using Intestine 407 cells. The adhesion assay, testing nisin-induced recombinant *L. lactis* cells with pKTH5056 or pKTH5046 and wild-type *L. lactis* NZ9000 cells, indicated that *L. lactis* NZ9000 cells with pKTH5056 possessed an approximately sevenfold greater ability to adhere to Intestine 407 cells than wild-type NZ9000 cells, whereas an only three- to fourfold increase could be obtained compared to NZ9000 carrying pKTH5046 (Fig. 5). The binding of *L. lactis* cells with pKTH5056 to Intestine 407 cells was found to be
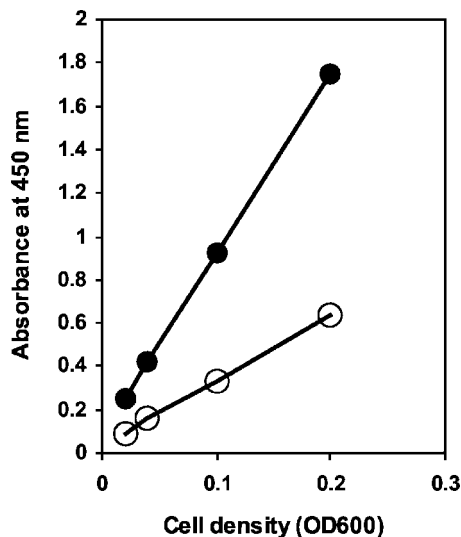

FIG. 3. Whole-cell ELISA for detection of the surface-exposed SlpA adhesion-mediating region encoded by pKTH5056. Anti-SlpA antibody was allowed to bind to lactococcal cells harvested from nisin-induced or uninduced cultures, and after the addition of a horseradish peroxidase conjugate, different lactococcal cell densities were incubated with a chromogenic substrate. Symbols: ●, nisin-induced NZ9000 (pKTH5056); ○, uninduced NZ9000(pKTH5056). OD600, optical density at 600 nm.
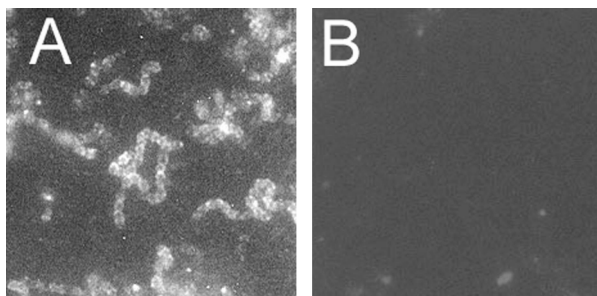
FIG. 4. Immunofluorescence microscopy of recombinant *L. lactis* NZ9000 cells harboring pKTH5056 (A) and wild-type *L. lactis* NZ9000 (B). Recombinant cells were harvested after overnight nisin induction, and wild-type cells were harvested after overnight incubation. Both cell types were treated with anti-SlpA antibodies (diluted 1:20 in PBS) and a fluorescein isothiocyanate-conjugated secondary antibody. Both pictures were taken after 16-s exposures. Magnification, ×4,300.

FIG. 6. Adhesion of recombinant *L. lactis* NZ9000 cells carrying pKTH5056 (A) and wild-type *L. lactis* NZ9000 (B) to Intestine 407 cells. Recombinant lactococcal cells were incubated with PBS for 2 h before the adhesion assay. Magnification, ×4,500.

statistically significantly ($P < 0.01$) greater than that of the negative control cells (wild-type NZ9000 and induced NZ9000 with pKTH5046).

To obtain even more definitive evidence that the adhesion was indeed mediated by the N-terminal part of the *L. brevis* SlpA protein expressed by pKTH5056, a serum inhibition study was carried out. Nisin-induced recombinant NZ9000 cells with pKTH5056 were incubated with either anti-SlpA antibodies or PBS before use in adhesion assays with Intestine 407 cells. Pretreatment with SlpA antiserum inhibited the binding of induced NZ9000 cells with pKTH5056 to the level of negative controls (wild-type NZ9000 and induced NZ9000 with pKTH5046), and a statistically significant ($P < 0.01$) difference between the numbers of PBS-treated cells and cells treated with anti-SlpA

antibodies bound to Intestine 407 cells was found (Fig. 5 and 6). Thus, the adhesion inhibition tests confirmed that the adherence to Intestine 407 cells of *L. lactis* NZ9000 harboring pKTH5056 was indeed mediated by the SlpA receptor-binding region encoded by pKTH5056.

Adhesion of recombinant lactococci to human plasma fibronectin in vitro. As the adherence of *L. brevis* ATCC 8287 SlpA to human fibronectin has recently been reported (15), we also tested the binding of induced and uninduced recombinant *L. lactis* carrying pKTH5056 or pKTH5046 and wild-type *L. lactis* NZ9000 cells to human fibronectin immobilized on glass slides. The results indicated that recombinant *L. lactis* with pKTH5056 adhered to fibronectin whereas wild-type *L. lactis* NZ9000 and recombinant *L. lactis* with pKTH5046, as well as all of the uninduced counterparts, showed no adherence over the background (Fig. 7). The difference in fibronectin binding between the recombinant *L. lactis* strain with pKTH5056 and the control strains was approximately sevenfold, further confirming the specificity of the SlpA region-mediated binding of lactococci.

DISCUSSION

The probiotic research field has produced numerous studies in which the adhesion capabilities of lactobacilli have been tested. However, only a few lactobacillary adhesins have been characterized in detail, including the collagen-binding S-layer protein of *Lactobacillus crispatus* (36), a surface protein of *Lactobacillus fermentum* that is able to bind to porcine intestinal mucus and gastric mucin (28), a Mub protein adhering to mucus components (29), and the fibronectin-binding *L. brevis* SlpA protein (15) that was also further studied in this work. It is likely, however, that *Lactobacillus* strains express several different adhesins (15), and their detailed role in complex adhesion processes may be difficult to reveal, especially if genetic tools and gene-disrupting methods are lacking for the strain in question. A way to study, e.g., the receptor specificity of these binding proteins is to express the putative adhesin in a heterologous host lacking the adhesion property. So far, among lactobacilli, only expression of the *L. crispatus* collagen-binding S-layer protein (36) has been studied in another *Lactobacillus* species (23), whereas in lactococci, some heterologous adhesins of non-lactic acid bacterial origin have been
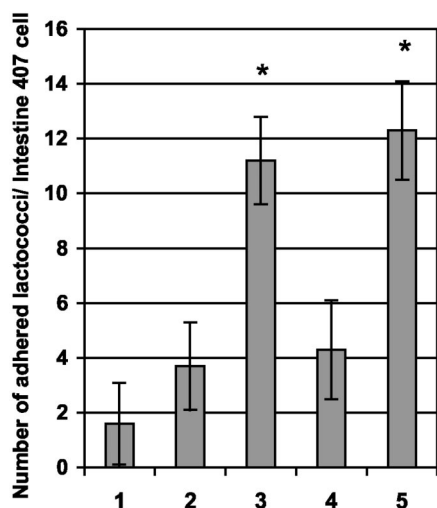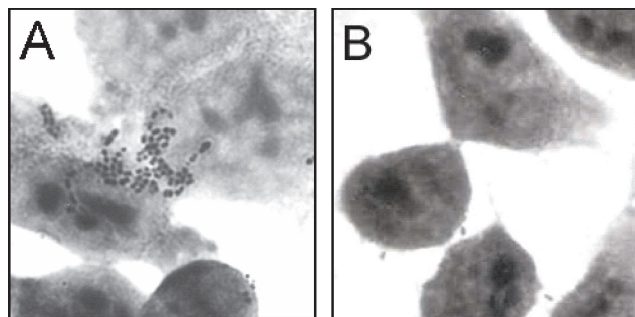

FIG. 5. Adherence of recombinant and wild-type *L. lactis* NZ9000 cells to Intestine 407 cells. The mean number of adherent lactococcal cells per Intestine 407 cell was determined from 16 randomized microscopic fields and is illustrated for *L. lactis* NZ9000 cells harboring no plasmid (bar 1), pKTH5046 (nisin induced) (bar 2), pKTH5056 (nisin induced) (bar 3), pKTH5056 (nisin induced and incubated with anti-SlpA antibodies before the adhesion assay) (bar 4), or pKTH5056 (nisin induced and incubated with PBS before the adhesion assay) (bar 5). The results shown are group means with 95% confidence intervals.*, $P < 0.01$.
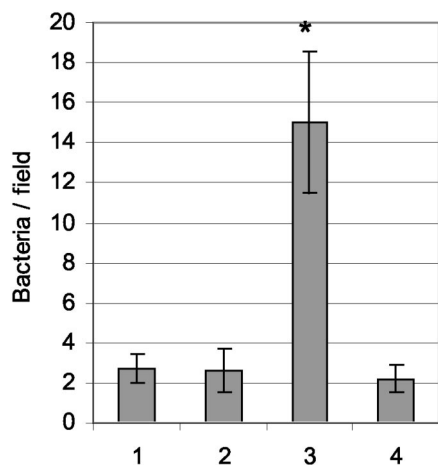
FIG. 7. Binding of recombinant and wild-type *L. lactis* NZ9000 to immobilized fibronectin. Means and standard deviations of bacterial numbers in 20 randomly chosen 4×10^2-μm^2 microscopic fields are shown for *L. lactis* NZ9000 harboring no plasmid (bar 1), pKTH5046 (nisin induced) (bar 2), pKTH5056 (nisin induced) (bar 3), or pKTH5056 (uninduced) (bar 4). The concentration of bacteria was 5×10^8 ml^{-1}. The results shown are group means with 95% confidence intervals. *, $P < 0.01$.

tested (13, 27). Another rationale for transferring adhesin molecules in lactic acid bacteria is to provide nonadhesive mucosal delivery vehicles with a specific adhesion capability to test whether more efficient delivery of bioactive molecules of interest can be obtained.

In this work, we have demonstrated that the N-terminal region of the S-layer protein of *L. brevis* can be used to transform a naturally nonadhesive lactic acid bacterium into an adhesive one. This was achieved by constructing a surface display vector, pKTH5056, encoding the SlpA receptor-binding region as a fusion protein with the spacer and cell wall-anchoring regions of PrtP and AcmA, respectively. We used *L. lactis* as the host because this organism does not adhere to Intestine 407 cells (A. Palva et al., unpublished results), a wide range of genetic tools with which to study it are available, it has been successfully used for surface display of heterologous proteins (13, 26, 39), and it is being developed as a mucosal delivery vehicle (38, 42).

The cell wall attachment of the receptor-binding region of SlpA was accomplished by using an expression vector that has the cell wall-anchoring sequences of the *L. lactis acmA* gene (5). The AcmA repeat cell wall anchor has been previously used for surface expression of the *Bacillus licheniformis* α-amylase and the *E. coli* β-lactamase (5). The mechanism by which the *acmA*-encoded attachment domains interact with the cell wall components is unknown, but the interaction has been suggested to be noncovalent in nature (reviewed in reference 21).

A 515-amino-acid PrtP$_{1153-1668}$-encoding region of the *prtP* gene of *L. lactis* subsp. *cremoris* Wg2, tested to allow efficient surface expression with the Bla reporter protein in pKTH5051, was used as the spacer protein to properly extend the SlpA receptor-binding region out of the cell surface. Surprisingly, with the shorter 215-amino-acid PrtP$_{1453-1668}$ spacer tested, no surface expression of β-lactamase could be demonstrated. In

addition to the length and conformation of a spacer, the nature of the protein to be surface displayed and its interactions with the cell wall molecules also affect the design of the spacer. The 515-amino-acid spacer amplified from the C terminus of the *prtP* gene spans the putative H domain and part of the W domain determined from PrtP of *L. lactis* strain SK11 (35). The function of the putative H domain has not been determined, but it has been suggested to act as a spacer directing other PrtP domains farther from the cell wall (35). In putative H domains, alpha-helixes are the dominant secondary structure. Spacers consisting of alpha-helixes of different lengths and origins have recently been used to enhance the expression of M6 protein on the cell surface of *Streptococcus gordonii* (3).

We chose a 247-amino-acid-long N-terminal region, including the signal peptide of 30 residues, from the *L. brevis* SlpA protein for surface expression and adhesion studies with *L. lactis*. This region was previously shown to include the receptor-binding region of SlpA, which reacts with fibronectin and several human epithelial cell types, by using a flagellar display system in *E. coli* (15). With flagellar display experiments, the binding domain of SlpA could be limited to 81 amino acid residues representing residues 96 through 176 in the unprocessed SlpA protein (15).

In the cell adhesion tests (Fig. 5), some adhesion background with NZ9000 carrying the control vector (pKTH5046) was observed. This is likely due to some aggregation observed both with the control and test vector-carrying strains. However, in the fibronectin assay (Fig. 7), where the lactococci were subjected to gentle agitation during propagation, the aggregation was not significant. Accordingly, the difference between the binding backgrounds with the control strains was almost nonexistent, demonstrating the specificity of the fibronectin binding.

Even though a significant ability to adhere to human epithelial Intestine 407 cells could be provided to *L. lactis* by the aid of the SlpA adhesion-mediating region, the epithelial cell-binding efficiency of *L. lactis* harboring pKTH5056 was substantially lower than that of the wild type *L. brevis* cells synthesizing a surface layer estimated to consist of approximately one-half million SlpA subunits (Palva et al., unpublished; 15). One obvious explanation for this is that the amount of the pKTH5056-encoded region of SlpA in *L. lactis* is only a few percent of the SplA subunits present in the native S-layer in *L. brevis*. We cannot, however, rule out the possibility that the receptor-binding domain of SlpA is more effective when displayed at a high density in the S-layer lattice. The assumption that entire S-layers possess more efficient binding capacity than their protein subunits is also supported by the results of studies of the expression of the collagen-binding CbsA protein of *L. crispatus* in *Lactobacillus casei*. CbsA was anchored with the cell wall-sorting signal of PrtP to the cell wall and successfully expressed in *L. casei*, but no S-layer was formed and the amount of collagen bound was smaller than that observed with wild-type *L. crispatus* (23).

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