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Receptor Binding Domain of *Escherichia coli* F18 Fimbrial Adhesin FedF Can Be both Efficiently Secreted and Surface Displayed in a Functional Form in *Lactococcus lactis*

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Adherence of F18 fimbrial *Escherichia coli* to porcine intestinal epithelial cells is mediated by the adhesin (FedF) of F18 fimbriae. In a previous study, we demonstrated the specificity of the amino acid residues between 60 and 109 as the receptor binding domain of FedF. In this study, different expression, secretion, and anchoring systems for the receptor binding domain of the FedF adhesin in *Lactococcus lactis* were evaluated. Two partially overlapping receptor binding domains (42 and 62 amino acid residues) were expressed as fusions with *L. lactis* subsp. *cremoris* protein PrtP for evaluation of secretion efficiency. To evaluate the cell surface display of these FedF-PrtP fusions, they were further combined with different lengths of PrtP spacers fused with either the *L. lactis* AcmA anchor or the PrtP cell wall binding domain. An HtrA-defective *L. lactis* NZ9000 mutant was constructed to determine its effect on the level of secreted or anchored fusion proteins. Recombinant *L. lactis* clones secreting the receptor binding domain of F18 fimbriae as a fusion with the H domains of *L. lactis* protein PrtP were first constructed by using two different signal peptides. FedF-PrtP fusions, directed by the signal sequence of *L. brevis* SlpA, were throughout found to be secreted at significantly higher quantities than corresponding fusions with the signal peptide of *L. lactis* Usp45. In the surface display systems tested, the *L. lactis* AcmA anchor performed significantly better, particularly in the *L. lactis* NZ9000Δ*htrA* strain, compared to the *L. lactis* PrtP anchor region. Of the cell surface display constructs with the AcmA anchor, only those with the longest PrtP spacer regions resulted in efficient binding of recombinant *L. lactis* cells to porcine intestinal epithelial cells. These results confirmed that it is possible to efficiently produce the receptor binding domain of the F18 adhesin in a functionally active form in *L. lactis*.

F18 fimbrial *Escherichia coli* strains adhere to and colonize the microvilli of small intestinal epithelial cells in piglets and are associated with porcine postweaning diarrhea and pig edema disease (6, 18). These infectious endemic porcine diseases are the most widespread causes of death in weaned pigs (6, 15, 18). There are no commercial vaccines available against infections caused by F18 fimbria-carrying *E. coli* strains. Colonization by bacteria is a prerequisite event in the infection process and is initiated by the adherence of bacteria to the host cell surface. Adherence is usually mediated by adhesins (17, 44). Bacterial adhesins, with the same receptor, are found to be highly conserved structures, which makes them attractive candidates for vaccine development (1, 25, 48). The functionality of this approach has been recently demonstrated with model systems (9, 10, 25). Regarding the adhesin of F18 fimbriae, we have shown that *E. coli* strains carrying this fimbrial type adhere to porcine intestinal epithelial cells via the FedF protein (42). Furthermore, we have recently mapped the receptor binding region of FedF, to be used as a putative surface antigen in lactic acid bacteria (LAB), between amino acid (aa) residues 60 and 109 of FedF (43).

During the last decade, the functionality of LAB as potential antigen delivery vehicles has been intensively explored to develop safe, food-grade, and cost-effective mucosal vaccines (29, 47). Several studies describing the production of foreign antigens in *L. lactis* have been reported (4, 8, 29, 31, 46). Furthermore, presentation of antigens to the mucosal immune system with specific responses has been reported with several LAB model systems (29, 32, 33, 34). The outcome of the immune responses with LAB vaccine vectors has been found to be affected not only by the amount of expressed antigen but also by the cellular location of the antigen in the production host (29). Secreted, cell surface displayed, and intracellularly produced antigens have been tested, and especially cell surface display of vaccine antigens has gained much attention as the preferred localization of immunogens in LAB (29, 32, 37).

When bacteria secrete foreign proteins, the challenges often faced are compatibility of the protein produced with the secretion machinery and proteolysis of the products of interest. *L. lactis* HtrA was recently found and described as a unique, stress-inducible extracellular housekeeping protease that is responsible for the degradation of abnormal exported proteins. Expression studies with an HtrA-defective *L. lactis* strain have revealed that expression of heterologous proteins is facilitated by lack of HtrA activity (30, 36).

In this work, we have focused on developing an efficient production system for the receptor binding domain of the FedF adhesin in *L. lactis* by studying different alternatives for cell surface presentation. The *L. lactis* NZ9000 strain was chosen for nisin-controlled expression. An HtrA-defective *L. lactis* mutant was constructed to ensure efficient expression of secreted or anchored fusion proteins. Two signal peptides derived from *Lactobacillus brevis* S-layer protein SlpA (45) and

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from major secretory protein Usp45 of *L. lactis* were compared in secretion constructs in which the receptor binding domain of the F18 fimbrial FedF protein was fused to the H domain of *L. lactis* subsp. *cremoris* cell envelope protease PrtP. For surface display studies, the receptor binding domain of FedF was further fused with spacer regions of various lengths derived from the PrtP protein and anchored to the cell wall via the *L. lactis* AcmA repeats (26) or the *L. lactis* subsp. *cremoris* PrtP (41) anchor region, followed by expression in *L. lactis* NZ9000 or *L. lactis* NZ9000Δ*htrA* cells. Expression analyses revealed that the amounts of secreted or anchored fusion proteins produced by the HtrA-defective strain differed substantially from those produced by wild-type *L. lactis* NZ9000. We were also able to demonstrate that some of the secreted and surface-displayed fusion proteins had the ability to adhere efficiently and in a specific manner to isolated porcine intestinal epithelial cells in vitro.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The strains and plasmids used in this study are listed in Table 1. *Lactococcus* strains were grown at 30°C in M17 (Difco, Detroit, Mich.) containing 0.5% (wt/vol) glucose and 5 μg of chloramphenicol per ml. *E. coli* strains were grown in tryptic soy broth or Luria broth medium complemented with appropriate antibiotics. The B5 BAC *Caulobacter crescentus* strain was cultured as described by the manufacturer of the PurePro Caulobacter Expression system (Invitrogen Corporation, Carlsbad, Calif.).

DNA methods and transformation. Routine molecular biology techniques were used (2, 39). Enzymes were used as recommended by the manufacturers (Promega, Madison, Wis.; New England Biolabs Inc., Beverly, Mass.). Plasmid DNA was isolated from *L. lactis* by using the QIAfilter Plasmid Midi Kit (Qiagen GmbH, Hilden, Germany). *L. lactis* cells were transformed as described by Holo and Nes (20). 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 an *L. lactis* NZ9000Δ*htrA* strain. Primer pairs 1214/1216 and 1217/1224 were used for the amplification of two fragments (about 500 bp in size) spanning the *ywaE* gene and the 3′ end of *htrA* from *L. lactis* NZ9000 chromosomal DNA, respectively. The design of primer pair 1214/1216 was based on the *ywaE* sequence and the genomic organization of *L. lactis* MG1363 (our unpublished data), and the design of primer pair 1217/1244 was based on the sequence of *L. lactis* IL-1403. The two PCR products were ligated together, and the resulting 1.0-kb fragment was amplified from the ligation mixture with primers 1214 and 1228, followed by cloning into pG$^+$HOST4 (Appligene). The resulting construct (pKTH5138) was used to transform *L. lactis* NZ9000 cells, followed by chromosomal integration via a single crossover. The second crossover was obtained by cultivating the integrants essentially as described earlier (3). As a result, strain NZ9000Δ*htrA*, with a chromosomal deletion in the *htrA* promoter and the 5′-end region of *htrA*, was formed. The chromosomal deletion was verified by PCR and DNA sequencing.

Construction of plasmid vectors for secretion studies. A fusion of the signal sequence (SS) of the *L. brevis* S-layer protein gene (*slpA*) and the *L. lactis* nisin promoter (P_{nisA}) was PCR amplified with primers 623 and 1236 (Table 2) by using plasmid pKTH5056 (4) as the template. The adhesin (*fedF*) gene sequence of *E. coli* F18 fimbriae (National Center for Biotechnology Information accession number Z26520) was used to design primers for the amplification of two 126-bp sequences encoding $FedF_{59\text{-}100}$ or $FedF_{130\text{-}171}$ and a 186-bp sequence encoding $FedF_{59\text{-}120}$. The *fedF* sequences were PCR amplified with primer pairs 1237/1242, 1237/1238, and 1333/1335 (Table 2), respectively, with plasmid pIH120 (22) as the template. The proteinase (*prtP*) gene sequence of *L. lactis* subsp. *cremoris* Wg2 (EMBL accession number M24767) was used to design primers for amplification of the 630-bp *prtP* spacer encoding the PrtP H domain (41), with the His tag sequence included at the 3′ terminus. Plasmid DNA of pLP712 (16) was used as the template in the PCR for primers 1239 and 1240.

The P_{nisA}-SS_{slpA} gene fusion, the *fedF* fragments, and the sequence encoding the synthetic propeptide LEISSTCDA (27, 28) were fused together by the recombinant PCR technique as described earlier (19, 23). The recombinant PCR products amplified with primer pairs 623/1238 (for $FedF_{59\text{-}120}$), 623/1242 (for $FedF_{59\text{-}100}$), and 623/1335 (for $FedF_{130\text{-}171}$) and the *prtP* spacer sequence were digested with EcoRI, ligated together, and PCR amplified with primers 623 and 1240. The resulting PCR product was digested with BglII and XbaI and cloned into the BglII-XbaI site of plasmid pNZ8037 (12). The resulting plasmids were named pKTH5141, pKTH5142, and pKTH5155, respectively (Table 1).

For a negative control, a plasmid without *fedF* was constructed by PCR amplifying the DNA fragment encoding the P_{NisA}-SP_{SlpA}-LEISSTCDA propeptide with primer pair 623/1241 with the 623/1242 recombinant PCR product described above as the template. The resulting PCR fragment was digested with EcoRI and ligated to the 630-bp *prtP* sequence. The ligation product was PCR amplified with primers 623 and 1240 and finally inserted into the BglII/XbaI site of pNZ8037. The plasmid lacking the *fedF* fragments was named pKTH5143.

L. lactis NZ9000 and NZ9000Δ*htrA* were transformed with the secretion constructs, resulting in *L. lactis* strains GRS1091 and GRS1095 carrying pKTH5141, GRS1092 and GRS1096 carrying pKTH5142, and GRS1093 and GRS1097 carrying pKTH5143, respectively. Transformation of *L. lactis* NZ9000 with pKTH5155 resulted in GRS1106.

Corresponding expression cassettes were constructed with the signal sequence of the *usp45* gene from *L. lactis* (28) as follows. SS_{usp} was PCR amplified from *L. lactis* NZ9000 chromosomal DNA with primers 1243 and 1244, and the sequence encoding P_{nisA} was PCR amplified from pKTH5056 with primers 623 and 1245. The two *fedF* fragments encoding $FedF_{59\text{-}120}$ and $FedF_{59\text{-}100}$, were PCR amplified with primer pairs 1237/1238 and 1237/1242, respectively, with pIH120 as the template. The *fedF* sequences were fused downstream of P_{nisA} and SS_{usp} by the recombinant PCR technique. The resulting PCR products were inserted into the XbaI/BglII site of pNZ8037, giving rise to plasmids pKTH5144 ($FedF_{59\text{-}120}$) and pKTH5145 ($FedF_{59\text{-}100}$).

The DNA fragment encoding P_{NisA}-SP_{Usp}-LEISSTCDA was PCR amplified with primers 623 and 1241 from one of the recombinant products and inserted into pNZ8037. This plasmid, lacking *fedF*, was named pKTH5146. After transformation of *L. lactis* strains NZ9000 and NZ9000Δ*htrA* with pKTH5144, pKTH5145, and pKTH5146, new *L. lactis* strains GRS1098 and GRS1102 with pKTH5144, strains GRS1099 and GRS1103 with pKTH5145, and strains GRS1100 and GRS1104 with pKTH5146, respectively, were formed (Table 1).

Nisin induction. Nisin induction of recombinant *L. lactis* strains was performed as follows. From overnight cultures, 3% (vol/vol) inoculums were made into fresh growth medium and the bacteria were grown at 30°C until the optical density at 600 nm (OD_{600}) reached 0.3 to 0.4. For titration of an induction level that did not inhibit growth, nisin was added at different concentrations (0.1 to 10 ng/ml), and cell growth was measured by OD_{600} determination every half hour with a Bioscreen device (Labsystems, Helsinki, Finland). For the expression and adhesion assays, cells were propagated at the highest nisin induction level that did not affect growth. The bacterial cells were cultivated for 3 h and then harvested by centrifugation at 5,000 × *g* (5 min at 4°C).

Purification of proteins expressed from pKTH5141, pKTH5142, and pKTH5155. Expression of the $FedF_{59\text{-}120}$-$PrtP_{1399\text{-}1608}$, $FedF_{59\text{-}100}$-$PrtP_{1399\text{-}1608}$, and $FedF_{130\text{-}171}$-$PrtP_{1399\text{-}1608}$ proteins was induced by adding nisin to the medium of growing *L. lactis* GRS1091, GRS1092, and GRS1106 cells. His tag-fused FedF-PrtP proteins were purified from the supernatant with a His Trap column in accordance with the instructions given by Pharmacia (Uppsala, Sweden). Purified His tag fusions were identified by immunoblotting with an anti-His_6 antibody (Roche). The protein concentration was determined against a bovine serum albumin standard, and purified FedF-PrtP protein was used as a standard to quantify secretion of FedF-PrtP proteins.

Immunoblotting. *L. lactis* strains were induced for 3 h, and cells were removed by centrifugation (5,000 × *g*, 5 min, 4°C). A volume of 0.5 ml of each supernatant was directly dotted onto a polyvinylidene difluoride (PVDF) membrane. Immunoblotting was performed essentially as described before (43), with the following modifications. After blocking with 3% blocking reagent (Roche) for 1 h at room temperature, the membrane was incubated with anti-His_6 antibodies (diluted 1:500 in 3% blocking reagent) for 17 h at 4°C.

Construction of plasmid vectors for anchoring of FedF-PrtP to the cell wall. To anchor the FedF-PrtP fusion proteins to the cell wall of *L. lactis* NZ9000 and NZ9000Δ*htrA*, three different sets of constructs were made. (i) The DNA sequence encoding the *L. lactis* autolysin (AcmA) anchor (26) was inserted downstream of *prtP* in plasmids pKTH5141, pKTH5142, and pKTH5143 as follows. The 0.6-kb autolysin (*acmA*) repeat domain sequence was PCR amplified with primers 1330 and 1331 with pNG101His (K. Leenhouts) as the template. The PrtP H-domain-encoding sequence was amplified with primers 1239 and 1332. The two PCR products were fused by the recombinant PCR technique. The recombinant DNA fragment was digested with EcoRI and XbaI and cloned into the EcoRI/XbaI sites of pKTH5141, pKTH5142, and pKTH5143, resulting in pKTH5156, pKTH5157, and pKTH5158, respectively. *L. lactis* NZ9000 and NZ9000Δ*htrA* were transformed with pKTH5156 (GRS1107 and GRS1112, re-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
L. lactis strains		
NZ9000	Plasmid-free strain with chromosomal genes *nisR* and *nisK* needed for nisin induction	11
NCDO 712	*lac*$^+$ *prt*$^+$ *L. lactis* strain	16
GRS1091	NZ9000 derivative with pKTH5141	This work
GRS1092	NZ9000 derivative with pKTH5142	This work
GRS1093	NZ9000 derivative with pKTH5143	This work
GRS1095	NZ9000Δ*htrA* derivative with pKTH5141	This work
GRS1096	NZ9000Δ*htrA* derivative with pKTH5142	This work
GRS1097	NZ9000Δ*htrA* derivative with pKTH5143	This work
GRS1098	NZ9000 derivative with pKTH5144	This work
GRS1099	NZ9000 derivative with pKTH5145	This work
GRS1100	NZ9000 derivative with pKTH5146	This work
GRS1102	NZ9000Δ*htrA* derivative with pKTH5144	This work
GRS1103	NZ9000Δ*htrA* derivative with pKTH5145	This work
GRS1104	NZ9000Δ*htrA* derivative with pKTH5146	This work
GRS1106	NZ9000 derivative with pKTH5155	This work
GRS1107	NZ9000 derivative with pKTH5156	This work
GRS1108	NZ9000 derivative with pKTH5157	This work
GRS1109	NZ9000 derivative with pKTH5158	This work
GRS1112	NZ9000Δ*htrA* derivative with pKTH5156	This work
GRS1113	NZ9000Δ*htrA* derivative with pKTH5157	This work
GRS1114	NZ9000Δ*htrA* derivative with pKTH5158	This work
GRS1119	NZ9000 derivative with pKTH5165	This work
GRS1120	NZ9000 derivative with pKTH5166	This work
GRS1121	NZ9000 derivative with pKTH5167	This work
GRS1125	NZ9000Δ*htrA* derivative with pKTH5165	This work
GRS1126	NZ9000Δ*htrA* derivative with pKTH5166	This work
GRS1127	NZ9000Δ*htrA* derivative with pKTH5167	This work
GRS1129	NZ9000 derivative with pKTH5169	This work
GRS1130	NZ9000 derivative with pKTH5170	This work
GRS1131	NZ9000 derivative with pKTH5171	This work
GRS1133	NZ9000Δ*htrA* derivative with pKTH5169	This work
GRS1134	NZ9000Δ*htrA* derivative with pKTH5170	This work
GRS1135	NZ9000Δ*htrA* derivative with pKTH5171	This work
E. coli strains		
107/86	Clinical isolate with F18 fimbriae	21
One Shot TOP10F′	Chemically competent cells	Invitrogen
C. crescentus strains		
One Shot B5 BAC	Electrocompetent cells	Invitrogen
PEL43	B5 BAC *C. crescentus* derivative with pCX-TOPO	This work
PEL44	B5 BAC *C. crescentus* derivative with pKTH5153	This work
Plasmids		
pCX-TOPO	5.4 kb; vector for high-level expression of RsaA fusion proteins	Invitrogen
pG$^+$HOST4	Em^r; thermosensitive derivative of pWV01 replicon	Appligene-Oncor
pIH120	Amp^r; pUC18 derivative carrying the entire F18 fimbrium—encoding region	22
pLP712	Plasmid carrying *lac prt*	16
pNG101his	Cm^r; 4.6 kb; pNG101 derivative carrying His tag and autolysin anchor	K. Leenhouts
pNZ8037	Cm^r; pSH71; 4.1 kb	11
pKTH5056	Cm^r; pNG101 derivative carrying 1.5-kb *prtP* spacer and *acmA* anchor sequence	4
pKTH5138	pG$^+$HOST4 derivative carrying *L. lactis ywaE* fused to 3′ end of *L. lactis htrA*	This work
pKTH5141	Cm^r; pNZ8037 (Bg/II/XbaI::P_{nisA}-SS_{slpA}-Pro_{leiss}-*fedF*$_{aa59–120}$-*prtP*$_{aa1399–1608}$-Tag_{his6})	This work
pKTH5142	Cm^r; pNZ8037 (Bg/II/XbaI::P_{nisA}-SS_{slpA}-Pro_{leiss}-*fedF*$_{aa59–100}$-*prtP*$_{aa1399–1608}$-Tag_{his6})	This work
pKTH5143	Cm^r; pNZ8037 (Bg/II/XbaI::P_{nisA}-SS_{slpA}-Pro_{leiss}-*prtP*$_{aa1399–1608}$-Tag_{his6})	This work
pKTH5144	Cm^r; pNZ8037 (Bg/II/XbaI::P_{nisA}-SS_{usp}-Pro_{leiss}-*fedF*$_{aa59–120}$-*prtP*$_{aa1399–1608}$-Tag_{his6})	This work
pKTH5145	Cm^r; pNZ8037 (Bg/II/XbaI::P_{nisA}-SS_{usp}-Pro_{leiss}-*fedF*$_{aa59–100}$-*prtP*$_{aa1399–1608}$-Tag_{his6})	This work
pKTH5146	Cm^r; pNZ8037 (Bg/II/XbaI::P_{nisA}-SS_{usp}-Pro_{leiss}-*prtP*$_{aa1399–1608}$-Tag_{his6})	This work
pKTH5153	pCX-TOPO expressing *fedF*$_{aa59–100}$-*rsaA*	This work
pKTH5155	Cm^r; pNZ8037 (Bg/II/XbaI::P_{nisA}-SS_{slpA}-Pro_{leiss}-*fedF*$_{aa131–170}$-*prtP*$_{aa1399–1608}$-Tag_{his6})	This work
pKTH5156	Cm^r; pKTH5141 (EcoRI/XbaI::*prtP*$_{aa1399–1608}$-Tag_{his6}-*acmA*)	This work
pKTH5157	Cm^r; pKTH5142 (EcoRI/XbaI::*prtP*$_{aa1399–1608}$-Tag_{his6}-*acmA*)	This work
pKTH5158	Cm^r; pKTH5143 (EcoRI/XbaI::*prtP*$_{aa1399–1608}$-Tag_{his6}-*acmA*)	This work
pKTH5165	Cm^r; pNZ8037 (BgIII/XbaI::P_{nisA}-SS_{slpA}-Pro_{leiss}-*fedF*$_{aa59–120}$-*prtP*$_{aa1399–1716}$-Tag_{his6})	This work
pKTH5166	Cm^r; pNZ8037 (BgIII/XbaI::P_{nisA}-SS_{slpA}-Pro_{leiss}-*fedF*$_{aa59–100}$-*prtP*$_{aa1399–1716}$-Tag_{his6})	This work
pKTH5167	Cm^r; pNZ8037 (BgIII/XbaI::P_{nisA}-SS_{slpA}-Pro_{leiss}-*prtP*$_{aa1399–1716}$-Tag_{his6})	This work
pKTH5169	Cm^r; pKTH5056 (Bg/II/XbaI::P_{nisA}-SS_{slpA}-Pro_{leiss}-*fedF*$_{aa59–120}$-Tag_{his6})	This work
pKTH5170	Cm^r; pKTH5056 (Bg/II/XbaI::P_{nisA}-SS_{slpA}-Pro_{leiss}-*fedF*$_{aa59–100}$-Tag_{his6})	This work
pKTH5171	Cm^r; pKTH5056 (Bg/II/XbaI::P_{nisA}-SS_{slpA}-Pro_{leiss}-Tag_{his6})	This work

TABLE 2. Oligonucleotides used in the study

Oligonucleotide	Nucleotide sequence[a]
623	5′-CCAAGATCTAGTCTTATAACTATACTG-3′
1214	5′-TCTTTCTAGAATCAGCCACTTCCTCTGAT-3′
1216	5′-ATTTGGATCCCAATGAGGATAAGTGGATA-3′
1217	5′-GCCGGGATCCGTTCCCTCGATGTTTTACT-3′
1224	5′-GTTGAAGCTTGAGAAGGTCAACCATACGA-3′
1228	5′-GTTGCTGCAGGAGAAGGTCAACCATACGAA-3′
1236	5′-CAAGTCGACGATATTTCGAGAGCTGAAGCAGTCGTTGAAACG-3′
1237	5′-CGAAATATCGTCGACTTGTGATGCACCAGGAACTTTGACATGCCAGGCT-3′
1238	5′-AAGAGAATTCTGGACCAGTAAATCGACA-3′
1239	5′-TCCTGAATTCTTGCAGGCAGCTAAGCAGG-3′
1240	5′-CGCTTCTAGATTAATGATGATGATGATGAACTGTCTTGGCCGCTT-3′
1241	5′-TCAATCTAGATGCATCACAAGTCGACGAT-3′
1242	5′-CAGTTCTAGATGGCCCCCACTGAGATT-3′
1243	5′-GGAGGCACTCAAAATGAAAAAAAAGATTATCTC-3′
1244	5′-ACGATATTTCGAGAGCGAAAACACCTGACAACGG-3′
1245	5′-TCTTTTTTTTCATTTTGAGTGCCTCCTTATAATTTATTTTG-3′
1330	5′-CAGTTCATCATCATCATCATACTACTTATACCGTCA-3′
1331	5′-TGGATCTAGAGAACCGACCCGTTC-3′
1332	5′-GTCTTCTAGAATGATGATGATGATGAACTGTCTTGGCCGCTTCAACG-3′
1333	5′-CGAAATATCGTCGACTTGTGATGCACCTGGTAATGCTCAGACATATCCCCTTTC-3′
1335	5′-CTGGTCTAGATGGTCCCGTGTCTAGTCTATAAGATTTTGC-3′
1341	5′-TGATGAATTCCAATTGCAGGCAGC-3′
1342	5′-GCTGTCTAGACTAATGATGATGATGATGGTGTTCTTCACGTTGTTTCC-3′
1343	5′-CTGCTCTAGATCTGGCCCCCACTG-3′
1344	5′-CTGCTCTAGATCTGGTCCCGTGTCTA-3′
1345	5′-CTGCTCTAGATCTGGACCAGTAAATCG-3′
1346	5′-CTGCTCTAGATCTGCATCACAAGTCGA-3′

[a] Recognition sites of restriction enzymes are underlined.

spectively), pKTH5157 (GRS1108 and GRS1113, respectively), and pKTH5158 (GRS1109 and GRS1114, respectively).

(ii) The *prtP-acmA* fragment in pKTH5156, pKTH5157, and pKTH5158 was removed by EcoRI/XbaI digestion and replaced with a 954-bp fragment of the *L. lactis prtP* gene encoding the putative helix (H), cell wall (W), and anchor (AN) domains. The *prtP* sequence of *L. lactis* subsp. *cremoris* Wg2 (EMBL accession number M24767) was used to design primers 1341 and 1342 for amplification of the *prtP* fragment from pLP712 (16). The PCR products were digested with EcoRI/XbaI and inserted into pKTH5156, pKTH5157, and pKTH5158. The resulting plasmids were designated pKTH5165, pKTH5166, and pKTH5167, respectively.

(iii) In the third set, fragments encoding P_{NisA}-SP_{SlpA}-LEISSTCDA-$FedF_{59-120}$, P_{NisA}-SP_{SlpA}-LEISSTCDA-$FedF_{59-100}$, and P_{NisA}-SP_{SlpA}-LEISSTCDA were PCR amplified with primer pairs 623/1345, 623/1343, and 623/1346, respectively, with plasmids pKTH5141, pKTH5142, and pKTH5143 as the templates, respectively. The PCR fragments were cloned into BglII/XbaI-digested pKTH5056 (4) upstream of the fragment encoding the $PrtP_{1153-1668}$ (516-aa) spacer and *acmA* anchor sequences. Plasmids, pKTH5169, pKTH5170, and pKTH5171 were cloned into *L. lactis* NZ9000 and NZ9000Δ*htrA*, resulting in HtrA-positive strains GRS1129(pKTH5169), GRS1130(pKTH5170), and GRS1131(pKTH5171) and HtrA-negative strains GRS1133(pKTH5169), GRS1134(pKTH5170), and GRS1135 (pKTH5171). All constructs were DNA sequenced to verify the correct open reading frames.

Cloning of *fedF* into pCX-TOPO. A fragment spanning the receptor binding domain of FedF (aa 60 to 100) was amplified with primer pair 1327/1328 from *E. coli* 107/86 chromosomal DNA. The fragment was cloned into expression vector pCX-TOPO as a fusion to a truncated *C. crescentus* S-layer protein gene (*rsaA*) (Invitrogen Corporation), and the vector was used to transform *E. coli* TOP10F′ cells. The resulting construct, pKTH5153, was verified by DNA sequencing. Transformation of B5 BAC *C. crescentus* cells with pKTH5153 resulted in recombinant *C. crescentus* strain PEL44. Intact pCX-TOPO was transferred into B5 BAC *Caulobacter* cells, which were used as a control expression strain (PEL43).

Expression of $FedF_{60-100}$ fused to RsaA. Secreted $FedF_{60-100}$-RsaA fusions (expressed by PEL44 at 30°C [90 rpm, 2 to 3 days]) and RsaA proteins (expressed by control strain PEL43) were purified in accordance with the instructions given by the manufacturer (Invitrogen). Purified $FedF_{60-100}$-RsaA fusions were further dialyzed against phosphate-buffered saline (PBS; 4°C, overnight) and used for antibody production in rabbits. Immunization was carried out as described earlier (42).

Whole-cell enzyme-linked immunosorbent assay (ELISA) for detection of cell surface-exposed polypeptides. Recombinant *L. lactis* cells were harvested after nisin induction and resuspended in PBS to an OD_{600} of 1. Aliquots of 750 μl were withdrawn, and the cells were pelleted at 5,000 × *g* and washed twice in PBS, followed by resuspension in 200 μl of anti-FedF-RsaA antiserum, which was first diluted 1:10 in PBS, and incubation for 1 h (unspecific binding to *L. lactis* cells was first removed by incubating the serum with *L. lactis* GRS1119, GRS1121, or GRS1131 cells for 4 h at 4°C). The cells were then washed twice in PBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Richmond, Calif.), and diluted 1:100 in PBS, followed by one washing step with PBS and one with the substrate buffer (40 mM Na acetate, 40 mM Na citrate [pH 4.4]). After the washing steps, the cells were resuspended in 1 ml of substrate buffer. Cells were further diluted 1:5 to 1:25 in substrate buffer, resulting in cell suspensions with OD_{600} values of 0.15 to 0.03. Microtiter plate wells were loaded with 100 μl of each cell suspension, after which the plates were developed essentially as described earlier (24).

In vitro adhesion of secreted fusion proteins to porcine epithelial cells. HiTrap purified fusion proteins (50 μg/ml) from the supernatants of induced GRS1091, GRS1092, and GRS1106 were incubated with isolated porcine jejunal epithelial cells essentially as described before (43). Anti-His_6 antibodies (dilution of 1:50, incubation for 1 h at room temperature [Roche]) and fluorescein isothiocyanate-labeled anti-rabbit antibodies (Dako) were used as the primary and secondary antibodies, respectively. The adhesion was evaluated by immunofluorescence microscopy with Fluoprep (BioMérieux) as the mounting medium.

In vitro adhesion of recombinant *L. lactis* to porcine epithelial cells. Bacteria were harvested after 3 h of nisin induction, washed twice in PBS, and resuspended in PBS to an OD_{600} of 0.5. Adhesion of bacteria to intestinal epithelial cells from 8-week-old pigs was performed essentially as described before (43). The statistical significance of the adherence capabilities of the bacterial cells was evaluated by one-way analysis of variance, and pairwise differences between 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). Data were considered significant when *P* values were less than 0.01.

RESULTS

Construction of expression vectors for secretion of FedF-PrtP fusions. When developing cell surface display systems for heterologous proteins, evaluation of secretion capacity is a straightforward way to determine the level of gene expression and export compatibility of a target protein. In addition, secreted forms of a vaccine candidate protein may also be tested as an alternative antigen presentation system along with surface-exposed or intracellularly produced immunogens. Therefore, to test the ability of *L. lactis* to express the FedF adhesin of *E. coli* F18 fimbriae, we first constructed plasmid vectors for extracellular expression of three truncated forms of the FedF protein, including the adhesive 42 and 62 aa residues or a nonadhesive part (42 aa residues) of FedF (43) (Fig. 1). The secretion cassettes were constructed in such a way that they could also be directly used for the surface display constructs, after addition of cell wall anchors, by cloning the sequence encoding the H domain of *L. lactis* subsp. *cremoris* Wg2 protein PrtP (41) in frame downstream of the *fedF* fragments. Furthermore, for the secretion of hybrid proteins $FedF_{59-100}$-PrtP, $FedF_{59-120}$-PrtP, and $FedF_{130-171}$-PrtP, the signal peptide of either *L. brevis* S-layer protein SlpA or *L. lactis* major secreted protein Usp45 was used (Fig. 1). In addition, to enhance the

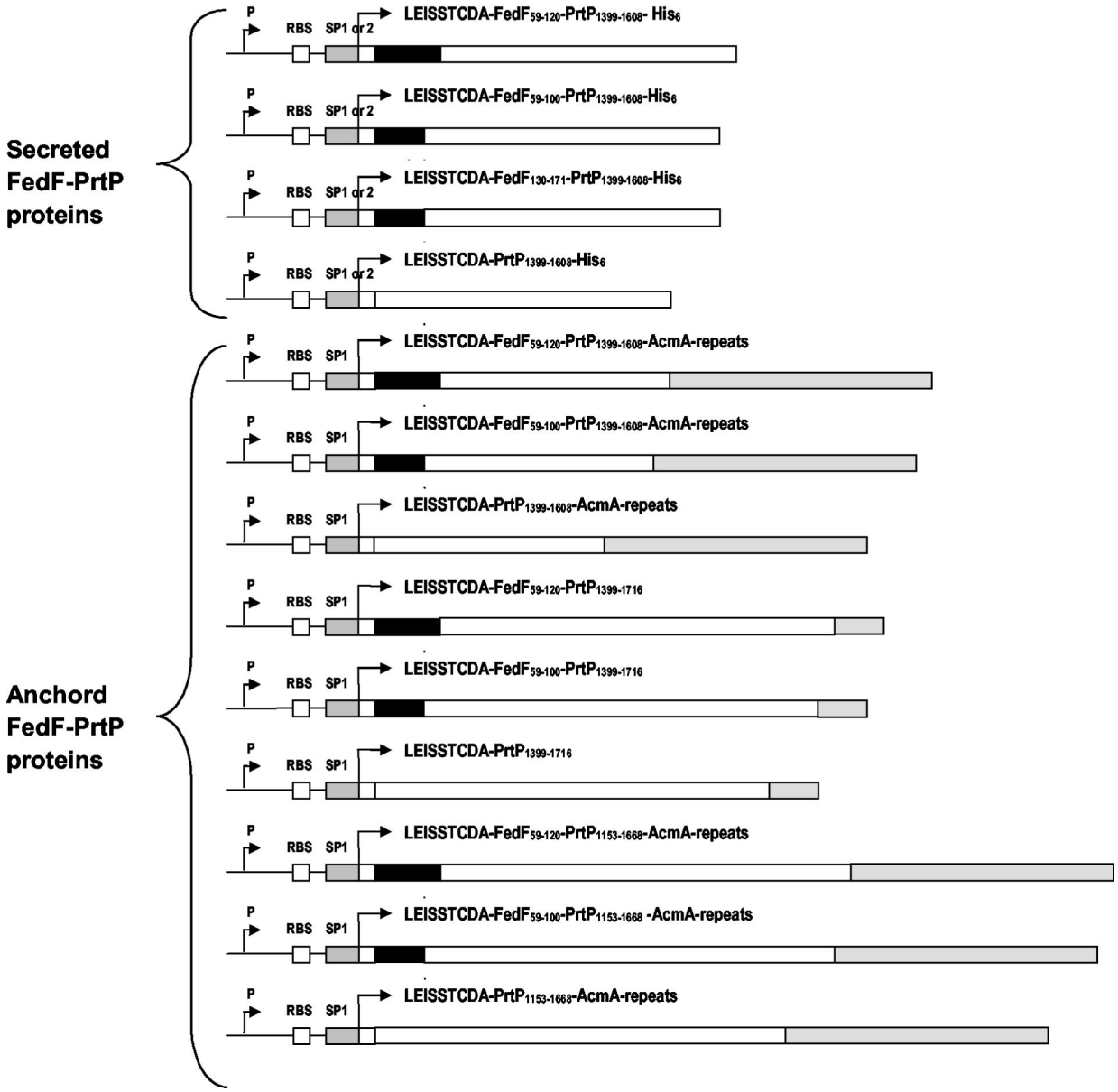

FIG. 1. Schematic representation of the different expression cassettes constructed for controlled expression of the secreted and cell surface-anchored receptor binding region of the FedF adhesin of *E. coli* F18 fimbriae. P, promoter sequence of the *nisA* gene of *L. lactis*; RBS, ribosome binding site; SP1, signal peptide of *L. brevis* S-layer protein (SP_{SlpA}); SP2, signal peptide of *L. lactis* Usp45 protein (SP_{Usp}); LEISSTCDA, a synthetic propeptide; AcmA repeats, anchor domain of *L. lactis* AcmA protein; PrtP, 210-, 270-, or 516-amino-acid region of the *L. lactis* subsp. *cremoris* Wg2 PrtP protein; His_6, histidine tag with six repeats.

processing and secretion capacity of fusion protein FedF-PrtP, a synthetic sequence encoding the propeptide LEISSTCDA (27) was inserted between the appropriate signal sequence and *fedF*. The sequences encoding the dipeptide Gly-Pro were inserted on both sides of *fedF* to increase the flexibility of the FedF fragment in the fusion protein. The *L. lactis* nisin promoter (P_{nisA}) in pNZ8037 was used for inducible expression of the hybrid proteins. To enable purification and detection of the protein, a His tag sequence was included at the 3′ end of each construct. For expression of FedF-PrtP, two plasmids with SS_{slpA}, pKTH5141 and pKTH5142 (for $FedF_{59\text{-}120}$-PrtP and $FedF_{59\text{-}100}$-PrtP, respectively), and two with SS_{usp}, pKTH5144 and pKTH5145 (for $FedF_{59\text{-}120}$-PrtP and $FedF_{59\text{-}100}$-PrtP, respectively), were introduced into two *L. lactis* strains, NZ9000 and its derivative NZ9000Δ*htrA*, devoid of the extracellular housekeeping protease HtrA (see Materials and Methods). For negative controls, three plasmids were constructed, one plasmid, pKTH5155, carrying the *slpA* signal sequence and a fragment encoding a nonadhesive FedF peptide of 42 aa residues, and two plasmids, pKTH5143 (with SS_{slpA}) and pKTH5146 (with SS_{usp}), carrying the same expression cassettes but lacking *fedF*.

Secretion of FedF-PrtP fusion proteins. The ability of *L. lactis* to secrete FedF-PrtP fusion proteins into the culture

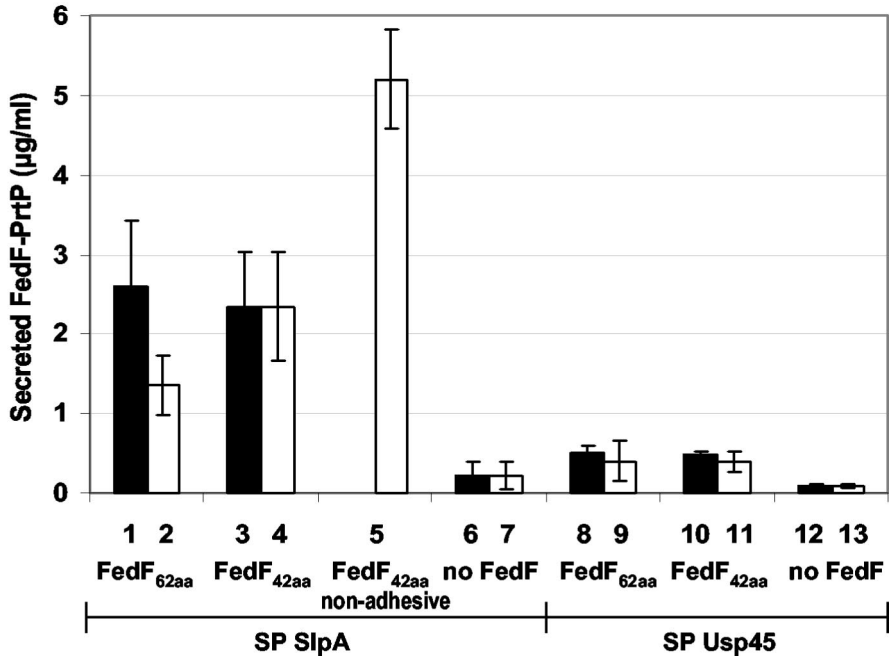

FIG. 2. Secretion yields of FedF-PrtP fusion proteins from induced *L. lactis* NZ9000 (white bars) and NZ9000Δ*htrA* (black bars) strains with the signal peptide of the *L. brevis* S-layer protein (SP SlpA) or the *L. lactis* Usp45 protein (SP Usp45). The NZ9000Δ*htrA* strains correspond to bars 1 (GRS1095), 3 (GRS1096), 6 (GRS1097), 8 (GRS1102), 10 (GRS1103), and 12 (GRS1104). The NZ9000 derivatives correspond to bars 2 (GRS1091), 4 (GRS1092), 5 (GRS1106), 7 (GRS1093), 9 (GRS1198), 11 (GRS1099), and 13 (GRS1100). $FedF_{62aa}$ and $FedF_{42aa}$ correspond to the strains expressing aa 59 to 120 and 59 to 100 of *E. coli* F18 fimbrial adhesin FedF, respectively; $FedF_{42aa}$ non-adhesive corresponds to a strain expressing FedF aa 130 to 171, which are outside of the receptor binding region in FedF. The means and standard deviations of two separate tests are shown.

medium was determined by immunoblotting. The supernatants of induced *L. lactis* strains GRS1091, GRS1092, GRS1193, GRS1095, GRS1096, GRS1097, GRS1098, GRS1099, GRS1100, GRS1102, GRS1103, GRS1104, and GRS1106 were blotted onto a PVDF membrane, followed by detection with anti-His tag (His_6) antibody and quantification with purified FedF-PrtP standard (Fig. 2). A substantially higher level of fusion proteins was secreted by clones possessing the SlpA signal peptide than by those with the Usp45 signal peptide (Fig. 2). Compared to the strains with the Usp45 signal peptide, the level of secreted FedF-PrtP was four- to sixfold higher with the SlpA signal peptide, depending on the length of the FedF adhesion domain and whether the protein was expressed from NZ9000 or the NZ9000Δ*htrA* derivative (Fig. 2). The highest secretion yields with the adhesive FedF regions were obtained with NZ9000Δ*htrA* strains GRS1095 ($FedF_{62aa}$) (approximately 2.6 μg/ml) and GRS1096 ($FedF_{42aa}$) (approximately 2.3 μg/ml) and NZ9000 strain GRS1092 ($FedF_{42aa}$) (approximately 2.3 μg/ml). Among the HtrA-positive strains, the strain expressing $FedF_{42aa}$-PrtP (GRS1092) secreted 1.7-fold more into the culture medium than did GRS1091 expressing $FedF_{62aa}$ (Fig. 2). Thus, the fusion protein with $FedF_{62aa}$ seemed to be more susceptible to degradation by HtrA than did its counterpart with Fed_{42aa}. Surprisingly, the construct carrying 42 aa of a nonadhesive part of FedF (GRS1106) secreted twice as much FedF-PrtP fusion protein as did the receptor binding domain-carrying construct GRS1092.

Adhesion of secreted FedF-PrtP proteins to porcine epithelial cells. Secreted FedF-PrtP proteins carrying a histidine tag were collected from the supernatants of induced *L. lactis* GRS1091 ($FedF_{59-120}$-PrtP), GRS1092 ($FedF_{59-100}$-PrtP), and GRS1106 ($FedF_{130-171}$-PrtP) cells with a His Trap column, concentrated, and incubated with isolated porcine epithelial cells, followed by detection by fluorescence microscopy after incubation with rabbit anti-FedF-RsaA antiserum and fluorescein isothiocyanate-labeled anti-rabbit antibodies. Epithelial cells incubated in the presence of $FedF_{59-120}$-PrtP and $FedF_{59-100}$-PrtP exhibited bright fluorescence (Fig. 3A and C), whereas epithelial cells incubated with $FedF_{130-171}$-PrtP were only weakly fluorescent (Fig. 3E). The result demonstrated that the receptor binding domain of FedF, when secreted as a fusion protein with PrtP in *L. lactis*, had retained its ability to bind porcine intestinal epithelial cells in vitro.

Construction of expression vectors for cell surface display. For surface display, the secreted FedF-PrtP proteins were anchored to the cell wall of *L. lactis* NZ9000 and NZ9000Δ*htrA* with the cell wall anchoring region of either the lactococcal AcmA protein (26) or the lactococcal PrtP protein (41). Three groups of expression vectors, with *prtP* spacer sequences of 0.6, 0.8, and 1.5 kb, were constructed to test the surface accessibility of the hybrid proteins to be expressed. In the first group, the *acmA* repeats were inserted into pKTH5141, pKTH5142, and pKTH5143 downstream of and in frame with the 630-bp *prtP* fragment encoding the *L. lactis* subsp. *cremoris* PrtP H domain of 210 aa. The new plasmids were pKTH5156, pKTH5157, and pKTH5158, respectively. In the second group, the 954-bp fragment encoding the PrtP helix (H), wall (W), and anchor (AN) domains was PCR amplified and placed in frame with the *fedF* fragments in expression vectors pKTH5141, pKTH5142, and pKTH5143, giving rise to pKTH5165, pKTH5166, and

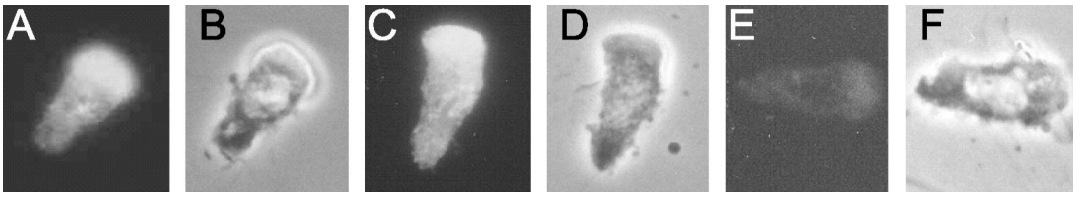

FIG. 3. Indirect immunofluorescence assay of porcine jejunal epithelial cells after incubation with secreted and purified FedF-PrtP fusion proteins produced in induced *L. lactis*. Adhesion of $FedF_{59-120}$-$PrtP_{1399-1608}$ (A), $FedF_{59-100}$-$PrtP_{1399-1608}$ (C), and $FedF_{130-171}$-$PrtP_{1399-1608}$ (E) proteins from recombinant *L. lactis* NZ9000 strains GRS1091, GRS1092, and GRS1093, respectively, is shown. Anti-FedF-RsaA antibodies and fluorescein isothiocyanate -conjugated secondary antibodies were used. (B, D, and F) Light microscopic fields corresponding to panel A, C, and D, respectively. Magnification, ×4,500.

pKTH5167, respectively. In the third group, the fragments encoding P_{NisA}-SP_{SlpA}-LEISSTCDA-$FedF_{59-120}$, P_{NisA}-SP_{SlpA}-LEISSTCDA-$FedF_{59-100}$, and P_{NisA}-SS_{SlpA}-LEISSTCDA cassettes were inserted into pKTH5056 in frame with the 1,548-bp *prtP* fragment, encoding the 516-aa PrtP spacer, and the AcmA autolysin anchor sequence. The resulting plasmids were pKTH5169, pKTH5170, and pKTH5171, respectively. All three of the expression constructs from the three different anchoring systems were used to transform *L. lactis* strains NZ9000 and NZ9000Δ*htrA*, giving rise to the 18 new strains listed in Table 1.

Surface accessibility of the FedF receptor binding region. The surface accessibility of the FedF adhesion-mediating region, expressed by the 18 *L. lactis* clones, was assayed by whole-cell ELISA with anti FedF-RsaA antibodies. For the whole-cell ELISA, recombinant *L. lactis* NZ9000 and NZ9000Δ*htrA* cells harboring pKTH5156, pKTH5157, pKTH5158, pKTH5165, pKTH5166, pKTH5167, pKTH5169, pKTH5170, and pKTH5171 were harvested from induced and uninduced cultures.

The ELISA absorbance values of the induced recombinant lactococcal cells were distinctly higher than those of uninduced cells, which remained at the level of wild-type NZ9000 (data not shown). Of the three anchoring types tested, the *L. lactis* PrtP spacer region of 210 aa ($PrtP_{1399-1608}$), in combination with the AcmA anchor, allowed the greatest surface display of FedF epitopes in both *L. lactis* NZ9000 and NZ9000Δ*htrA*, with only a relatively small difference between the two hosts (Fig. 4). Further, the level of $FedF_{62aa}$ surface expression was significantly higher than that of $FedF_{42aa}$ (Fig. 4). In contrast, with the 516-aa PrtP spacer ($PrtP_{1153-1668}$) and the AcmA anchor, the level of surface expression of FedF epitopes could not be distinguished from that of the negative control in the wild-type NZ9000 background. However, when expressed in NZ9000Δ*htrA*, both FedF constructs were equally surface dis-

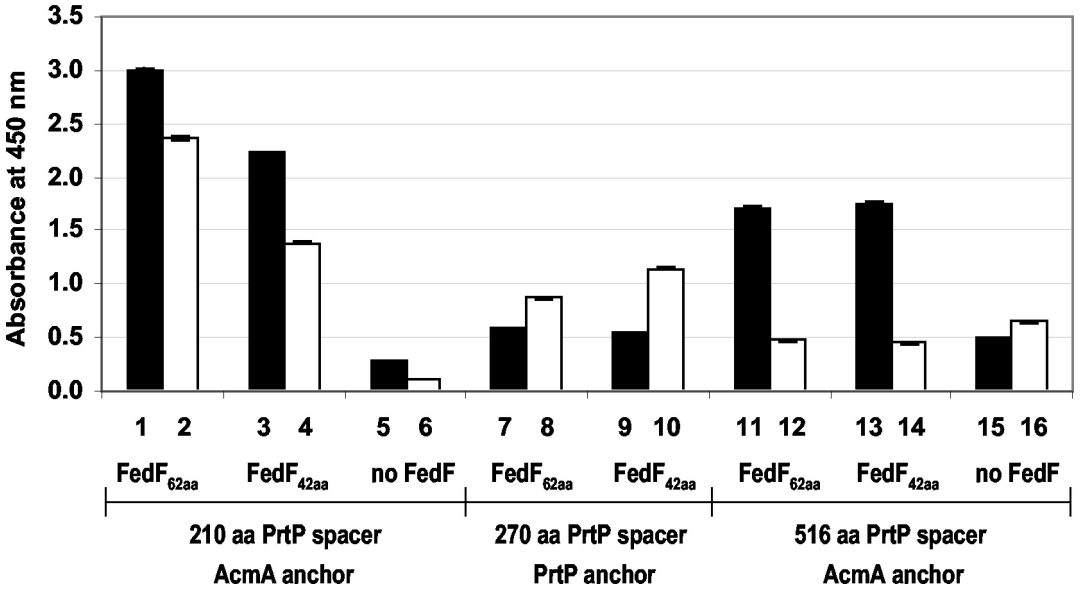

FIG. 4. Whole-cell ELISA for detection of cell surface exposure of the FedF receptor binding regions $FedF_{62aa}$ ($FedF_{59-120}$) and $FedF_{42aa}$ ($FedF_{59-100}$) as fusions with PrtP spacers of 210, 270, and 516 aa residues and the Acm and PrtP anchors in *L. lactis* NZ9000 and NZ9000Δ*htrA*. The NZ9000Δ*htrA* derivatives are shown as black bars numbered as follows: 1, GRS1112; 3, GRS1113; 5, GRS1114; 7, GRS1125; 9, GRS1126; 11, GRS1133; 13, GRS1134; 15, GRS1135. *L. lactis* NZ9000 derivatives are shown as white bars numbered as follows: 2, GRS1107; 4, GRS1108; 6, GRS1109; 8, GRS1119; 10, GRS1120; 12, GRS1129; 14, GRS1130; 16, GRS1131. The mean and standard deviation of three parallel tests are shown. The absolute difference between any two sample means was significant at the 0.01 level according to the Tukey HSD test (http://faculty.vassar.edu/lowry/VassarStats.html).

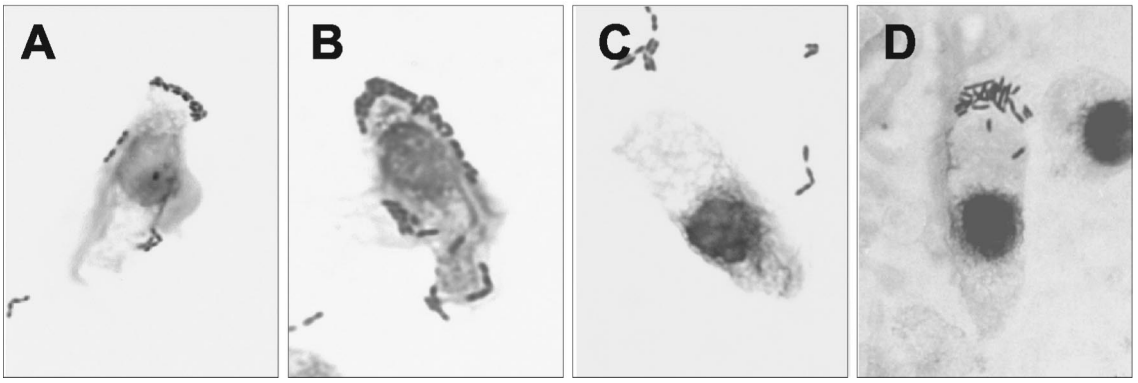

FIG. 5. Adhesion of *L. lactis* strains surface displaying FedF-PrtP proteins to porcine jejunal cells in vitro. Recombinant *L. lactis* NZ9000Δ*htrA* derivatives displaying $FedF_{59-120}$ (GRS1133) (A) or $FedF_{59-100}$ (GRS1134) (B) on the cell surface via the 516-aa PrtP spacer and the Acm anchor are shown. Panels C and D show the negative and positive control strains, *L. lactis* GRS1135, expressing only PrtP without FedF (C), and *E. coli* ERF2055, expressing whole F18 fimbriae, respectively. Magnification, ×4,500.

played at a high level, approaching the amount of the two previous constructs with the 210-aa PrtP spacer (Fig. 4). With the last group of FedF constructs, with the native PrtP anchor and the PrtP spacer of 270 aa ($PrtP_{1399-1668}$), the poorest overall performance was observed. In this group, surface expression remained at a low level in the NZ9000Δ*htrA* background but was, surprisingly, somewhat higher in the NZ9000 background (Fig. 4).

Adhesion of recombinant lactococci to porcine intestinal epithelial cells. For the adhesion assay, the best-performing constructs in the NZ9000Δ*htrA* background, GRS1112 ($FedF_{59-120}$-$PrtP_{1399-1608}$), GRS1113 ($FedF_{59-100}$-$PrtP_{1399-1608}$), GRS1133 ($FedF_{59-120}$-$PrtP_{1153-1668}$), and GRS1134 ($FedF_{59-100}$-$PrtP_{1153-1668}$), expressing AcmA autolysin-anchored FedF-PrtP fusion proteins, were chosen. Control strains in the adhesion assay were F18 fimbrial *E. coli* and *L. lactis* strains GRS1114 and GRS1135, expressing fusion proteins without FedF (Fig. 5 and 6). The adhesiveness of the shorter FedF peptide of 42 aa (GRS1134) was clearly better than that of the longer FedF peptide of 62 aa (GRS1133) (Fig. 5). Surprisingly, neither of the NZ9000Δ*htrA* strains (GRS1112, GRS1113) surface displaying FedF protein fragments as fusions with the H domain of *L. lactis* PrtP was adhesive (Fig. 6), even though the amount of these fusion proteins at the lactococcal cell surface was the highest measured by ELISAs in this study (Fig. 2). This might suggest that the receptor binding region of FedF was not fully accessible for the receptor, even though it was accessible for the FedF antibodies.

DISCUSSION

In this work, our aim was to develop an efficient cell surface display system in *L. lactis* for the receptor binding domain of the *E. coli* F18 fimbrial adhesin FedF, to be used in further immunization experiments and in the development of a mucosal vaccine against porcine postweaning diarrhea and edema disease. Our preliminary attempts to express the entire FedF adhesin as a fusion protein in *L. lactis* suggested both inefficient secretion and degradation of the end product (data not shown). Therefore, in this study, we started the optimization of the expression and secretion efficiencies of FedF by using only that region of the adhesin which is essential for the specificity of its binding to porcine intestinal epithelial cells, to maximize the secretion capacity of the FedF fragments and to minimize unwanted proteolysis. On the basis of the secretion results, the gene constructs allowing the most efficient extracellular production of FedF derivatives were further modified for surface display by adding different spacers and anchor regions.

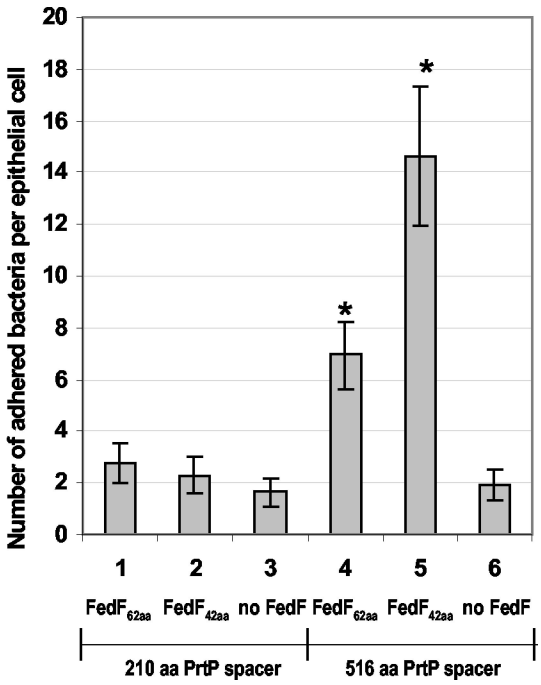

FIG. 6. Adherence of recombinant *L. lactis* NZ9000Δ*htrA* strains to porcine intestinal epithelial cells. The mean number of adherent lactococcal cells per porcine jejunal cell was determined from 50 cells in 50 randomly chosen microscopic fields and is illustrated for *L. lactis* GRS1112 (bar 1), GRS1113 (bar 2), and GRS1114 (bar 3) expressing $FedF_{62aa}$, $FedF_{42aa}$, or no FedF as fusions with the 210-aa PrtP spacer, respectively, and GRS1133 (bar 4), GRS1134 (bar 5), and GRS1135 (bar 6) expressing the corresponding constructs as fusions to the 516-aa PrtP spacer. The results shown are group means with 95% confidence intervals. *, $P < 0.01$.

Controlled expression of heterologous proteins in bacteria may overcome obstacles due to accumulation, aggregation, or degradation, which may occur in high-level production of foreign antigens with constitutive promoters (34). Furthermore, it is well established that a maximal secretion yield does not necessarily correlate with a maximal expression level. Instead, suboptimal expression levels may allow a maximal secretion yield. For this reason, the FedF-PrtP fusion proteins were expressed under control of the inducible *L. lactis* nisin promoter (P_{nisA}), which has been widely used for controlled gene expression in *L. lactis* (4, 11, 30). In our study, it was found that the recombinant *L. lactis* cells tolerated substantially different nisin induction levels. Therefore, the level of nisin used in further experiments was based on the use of the maximal amount of nisin not affecting the growth rate of a given strain.

For optimization of FedF secretion, three different parameters, the signal peptide, the length of the FedF region, and the *L. lactis* host background, were studied. The signal peptide of the *L. lactis* Usp45 protein (28) has been reported to be one of the most effective secretion signals among the known *L. lactis* signal peptides (34). We have demonstrated earlier that the *L. brevis* SlpA signal peptide (3, 45) very efficiently directs the secretion of a reporter in *L. lactis* (40). Here, both signal peptides allowed secretion of all of the FedF-PrtP fusions tested but the quantity of fusion proteins found in the culture medium was throughout substantially higher in expression systems possessing the *L. brevis* S-layer protein signal peptide, confirming the superiority of SP_{SlpA} over SP_{Usp}, at least with these constructs. Alternatively, the P_{nisA}-SS_{usp}-fusions in these constructs may result in an unfavorable mRNA conformation, affecting the translation initiation frequency. In the secretion constructs, the length of the FedF region played a minor role in the HtrA-negative background (NZ9000Δ*htrA*) whereas in wild-type strain NZ9000, the increase of the FedF fragment length from 42 to 62 aa residues reduced the secretion yield almost twofold. This difference may be due to the increased sensitivity of the $FedF_{62}$-PrtP fusion protein to proteolysis. It is also feasible that the longer FedF construct increased secretion stress in NZ9000 and thus resulted in activation of HtrA.

The synthetic propeptide LEISSTCDA has been reported to enhance the processing and secretion efficiencies of secreted heterologous proteins in *L. lactis* (27, 28, 38). We used LEISSTCDA in all of the FedF constructs in the downstream position of the signal sequences. The effect of this propeptide on secretion was, however, not separately evaluated. Thus, its role in the secretion efficiency of the FedF constructs remains to be elucidated. Adhesion assays with purified FedF-PrtP fusion proteins showed their specific and efficient binding to porcine intestinal epithelial cells in vitro. This suggested that, as in *E. coli* (43), the binding domain of FedF could also be produced in *L. lactis* in a receptor binding competent conformation, which was a prerequisite for further development of surface display systems for FedF in *L. lactis*.

On the basis of the results obtained with the secretion constructs, both FedF fragments and *L. brevis* SS_{slpA} were chosen for the surface display constructs for which the lengths of the PrtP spacers and two different anchoring systems were tested as new parameters. With the AcmA anchor (26)-based constructs, the two PrtP spacer lengths of 210 and 516 aa residues allowed high expression levels in NZ9000Δ*htrA* hosts. The most pronounced surface display with these constructs was attained with the shorter spacer. Instead, in wild-type NZ9000 cells, the expression level of the FedF-PrtP fusion with the longer spacer was remarkably decreased, in accordance with increased protease susceptibility or increased HtrA activity, possibly caused by the more complex construct structure. With the PrtP anchor (41), none of the constructs reached a surface display level as high as that obtained with the constructs based on the AcmA anchor in strain NZ9000Δ*htrA*. It has been reported that covalent binding of PrtP to the lactococcal cell wall is not efficiently formed with high-level expression of exported heterologous proteins (26, 31). We have not further characterized whether the low yield obtained is due to release of FedF-PrtP fusion proteins into the culture medium or to a lower expression-translocation efficiency of these constructs.

On the basis of the ELISA results, the NZ9000Δ*htrA* clones with the two best-performing PrtP spacers, with the AcmA anchoring system, and with both FedF fragment sizes were chosen for adhesion studies. The binding assays of the surface-displayed FedF fusions with porcine epithelial cells surprisingly revealed that the two NZ9000Δ*htrA* clones with the shorter PrtP spacer (210 aa) were unable to adhere, even though they were most efficiently recognized by the FedF antibodies in a whole-cell ELISA. Thus, these results suggest that with relatively short peptides, this PrtP spacer is not sufficient to allow receptor binding competent exposure of the FedF adhesin fragments. The longer PrtP spacer (516 aa), instead, allowed the surface presentation of the FedF-PrtP fusion proteins that were effectively recognized by the F18 receptors on porcine intestinal cells. Surprisingly, the $FedF_{42aa}$-PrtP-AcmA fusion was clearly more adhesive than the identical construct carrying the $FedF_{62aa}$ fragment, although neither the immunofluorescence assays performed with purified $FedF_{62aa}$-PrtP and $FedF_{42aa}$-PrtP fusion proteins nor surface display assays showed any significant difference.

Our previous results obtained with MBP-$FedF_{60-123}$ fusion protein produced in *E. coli* suggest an essential function of a putative disulfide bridge between Cys-63 and Cys-83 of FedF in its ability to adhere to porcine epithelial cells (43). The formation of disulfide bonds is catalyzed by thiol-disulfide oxidoreductases. They may also be spontaneously formed in vitro, but this process is believed to be time consuming (14, 35). Disulfide bridges are unusual in extracellular proteins from gram-positive bacteria. In the gram-positive model *Bacillus subtilis*, three proteins were recently described as thiol-disulfide oxidoreductases. Two of these proteins (BdbD and BdbC) were shown to be required for the activity and stability of disulfide-containing secretory reporter proteins (7, 13). Putative thiol-disulfide oxidoreductases from LAB have not been characterized so far, but the positive binding results obtained in the present study with the FedF constructs suggest, although they do not confirm, that the formation of the S-S bridge may also take place in *L. lactis* export systems. This presumption is further supported by a recent work in which a biologically active form of interleukin-12, with two disulfide bonds essential for its activity, was successfully secreted by recombinant *L. lactis* cells in a functional form (5).

The FedF constructs of this study were found to be effectively expressed in *L. lactis* and to possess the ability to bind to porcine intestinal epithelial cells. Furthermore, the 42-aa FedF

fragment functioned as an efficient antigen when fused to RsaA. A further approach is to use these constructs for immunization experiments and for FedF expression under the control of constitutive promoters in other selected LAB. The immunogenicity of the surface-displayed FedF fragments in LAB orally given will be studied. In addition, the need to apply LAB strains with strong adjuvant properties or coexpressing, e.g., cytokines, for enhancing the immune responses against FedF, remains to be elucidated.

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