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The mucosal adhesion properties of the probiotic *Lactobacillus rhamnosus* GG SpaCBA and SpaFED pilin subunits

Running title: Mucosal adhesion of *L. rhamnosus* GG pilin subunits

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ABSTRACT

Lactobacillus rhamnosus GG is a well-established Gram-positive probiotic strain, whose health-benefiting properties are in part dependent upon a prolonged residency in the gastrointestinal tract and likely dictated by an adherence to the intestinal mucosa. Previously, we identified two pilus gene clusters (*spaCBA* and *spaFED*) in the genome of this probiotic, each of which contained the predicted genes for three pilin subunits and a single sortase. We also confirmed the presence of SpaCBA pili on the cell surface and attributed an intestinal mucus-binding capacity to one of the pilin subunits (SpaC). Herein, we now report the cloning of the remaining pilin genes (*spaA*, *spaB*, *spaD*, *spaE*, and *spaF*) in *Escherichia coli*, production and purification of the recombinant proteins, and an assessment of their adherence to human intestinal mucus. Our findings indicate that the SpaB and SpaF pilin subunits also exhibit substantial mucus binding, which can be inhibited competitively in a dose-related manner. Moreover, the binding between SpaB pilin subunit and the mucosal substrate appears to operate through electrostatic contacts and is not related to a recognized mucus-binding domain. We conclude from these results that it is conceivable two pilin subunits (SpaB and SpaC) in the SpaCBA pilus fiber play a role in binding to intestinal mucus, but for the uncharacterized and putative SpaFED pilus fiber only a single pilin subunit (SpaF) is potentially responsible for mucus adhesion.

INTRODUCTION

The human intestinal microbiota is comprised of more than 1000 species of commensal and probiotic bacteria, including several members of the Gram-positive genus *Lactobacillus* (42, 52). Many strains of lactobacilli display a variety of health-promoting effects in humans and consequently are commercialized for use as probiotics in foods and nutritional supplements (reviewed in 48). Often a necessary precondition for probiotic bacteria colonization of the human gastrointestinal (GI) tract is a preferential adherence to the intestinal mucosa, which in turn prolongs and stabilizes intestinal residency, possibly then triggering a variety of defensive host cell immune responses and excluding pathogenic bacteria by competitive inhibition or steric hindrance (48). The outermost layer of the intestinal mucosa, a secreted and hydrated mucus gel acting as a protective barrier and filter, consists primarily of a heterogeneous mix of highly glycosylated membrane-associated and secreted glycoproteins called mucins (36). Although many studies have demonstrated that various probiotic *Lactobacillus* spp. adhere initially to the mucus gel layer relatively few details are known about the overall molecular mechanism of mucosal adhesion (reviewed in 23). Nonetheless, several studies have reported that the adherence of *Lactobacillus* cells to the mucosal barrier is frequently a surface protein-mediated interaction. For example, Rojas et al. (44) determined that the ability of *Lactobacillus fermentum* 104R (since reclassified as *Lactobacillus reuteri* 104R) to bind porcine small intestinal mucus and gastric mucin was facilitated by the cell surface-localized mucus adhesion promoting protein (MapA). Similarly, Macías-Rodríguez et al. (25) described the presence of two adhesion-associated proteins specific for porcine intestinal mucus-related substrates that are attached non-covalently to the cell surface of *Lactobacillus fermentum* BCS87. Roos and Jonsson (45) also

demonstrated the adherence between the surface-associated Mub (mucus binding) protein from *Lactobacillus reuteri* 1063 and intestinal mucus components derived from porcine and poultry sources. As well, Pretzer et al. (38) identified a large multidomain surface protein in *Lactobacillus plantarum* WCFS1 with a binding specificity for the mannose moieties in mucins. Interestingly, Kinoshita et al. (19) discovered that glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme associated normally with glycolysis, is localized on the surface of *L. plantarum* LA318 cells and adheres tightly to human colonic mucin.

Until quite recently, only indirect or circumstantial evidence suggested that pilus-like structures extend from the surface of probiotic lactobacilli (28, 39). However, in our previous study (18) we demonstrated that *Lactobacillus rhamnosus* GG, a well studied and widely used probiotic strain (48), is a piliated microbe. Pili are slender elongated heteromeric proteinaceous surface appendages that exist in numerous other Gram-positive bacteria and often mediate adherence between both pathogenic and nonpathogenic species and their host cell targets (reviewed in 20, 26, 40, 49), but now emerge as possible adhesion facilitators for probiotic lactobacilli colonization of the GI tract (18). Prototypically, the pilus fiber is composed of one major pilin that builds up the pilus backbone and two additional minor pilins (26, 40, 49), one subunit that has a role in signaling the cessation of pilus polymerization (27, 39) and is deposited at the pilus base and at irregular intervals along the pilus backbone, and another subunit with an adhesive character that is often localized at the pilus tip (1, 41). The current model of pilus assembly in *Corynebacterium diphtheriae* (27) suggests that each of these pilin subunits are connected covalently to one another through isopeptidyl bonds by a membrane-bound transpeptidase (pilin-specific sortase) to produce polymerized pili, which are then attached covalently to the cell wall by a different transpeptidase (the housekeeping

sortase) capable of recognizing all C-terminal LPXTG-like substrates. The genes encoding these pilus proteins, as well as the pilin-specific sortase, are clustered at the same locus within the genome (54).

In our recent study (18), we discovered that in the *L. rhamnosus* GG genome the pili-encoding genes for two different pilus fibers are arranged as the *spaCBA* and *spaFED* gene clusters and, based on a genomic comparison with another *L. rhamnosus* strain (LC705), that the *spaCBA* cluster was confined to only *L. rhamnosus* GG. Moreover, in our previous work (18) each of the predicted genes for the major pilin subunit forming the pilus backbone (SpaA and SpaD), one ancillary minor pilin subunit (SpaB and SpaE) that based on a model for pilus biogenesis is likely positioned at the pilus base and decorating the pilus backbone (27), and another larger sized minor pilin subunit considered adherent in nature (SpaC and SpaF) were identified in *L. rhamnosus* GG according to a shared amino acid identity with pilins from two enterococcal species. In addition, we also detected within the sequences of the predicted *spaCBA* and *spaFED* gene products the anticipated consensus motifs and domains characteristic of a pilin primary structure, including the Sec-dependent secretion signal, sortase recognition site, YPKN pilin-like motif, and E box (18). Subsequently, the expression and localization of intact SpaCBA pili on the cell surface of *L. rhamnosus* GG was confirmed in immunoblots and immunogold-labeled electron micrographs using antiserum specific for the SpaC pilin (18). In earlier studies, adhesion interactions between the *L. rhamnosus* GG strain and intestinal mucosal surfaces have been reported and well characterized (15, 31, 33, 46, 55-57). However, in our recent study (18) the influence of SpaCBA pilus fibers in mediating the binding between *L. rhamnosus* GG cells and human intestinal mucus was revealed in adhesion experiments using both *L. rhamnosus* GG pretreated with SpaC

antiserum and a *L. rhamnosus* GG *spaC* insertion mutant. More specifically, we demonstrated significant binding between recombinant SpaC pilin protein and intestinal mucus, thereby identifying a mucus-binding capacity for one of the minor pilin components localized at the tip and along the backbone of the SpaCBA pilus (18). To expand further upon these findings, we now present a study in which each of the remaining predicted pilin subunits (SpaA, SpaB, SpaD, SpaE, and SpaF) in the *spaCBA* and *spaFED* gene clusters are over-produced in recombinant form, purified to apparent homogeneity, and characterized for adherence to human intestinal mucus.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids, and genomic DNA. *Escherichia coli* strains TOP10 [F- *mcr*A D(*mrr-hsd*RMS-*mcr*BC) F80*lac*ZDM15 D*lac*X74 *rec*A1 *ara*D139 D(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] and BL21(DE3)pLysS [F^- *omp*T *hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3) pLysS (Cam^R)] were used for cloning and expression, respectively. *E. coli* was cultivated in Luria-Bertani (LB) medium with agitation at 37ºC and supplemented with 50 μg/ml kanamycin as required. The pET28b+ expression vector (Novagen) was used for cloning the *Lactobacillus rhamnosus* GG *spaA*, *spaB*, *spaD*, *spaE*, and *spaF* genes. Genomic DNA was isolated from *L. rhamnosus* GG (ATCC 53103) as described earlier (18). Established protocols were employed in all DNA manipulations, including PCR amplifications, restriction endonuclease digests, ligations, and transformations, as described previously (47).

Cloning of the *L. rhamnosus* GG SpaCBA and SpaFED pilin genes. The coding sequence of the SpaCBA and SpaFED pilin genes was obtained from the *L. rhamnosus* GG genomic sequence (18). Each of the *spaA*, *spaB*, *spaD*, *spaE*, and *spaF* genes, without the region encoding the N-terminal secretion signal and the C-terminal sortase recognition site, was PCR amplified from *L. rhamnosus* GG genomic DNA using pairs of flanking 5'- and 3'-end oligonucleotide primers (Oligomer, Finland) with an *Eco*RI site (a *Sac*I site for *spaF*) or a *Xho*I site (see Table 1). The respective PCR products were digested with *Eco*RI (or *Sac*I for *spaF*) and *Xho*I restriction endonucleases, ligated into the similarly digested pET28b+ expression vector, and then transformed into *E. coli* TOP10. The resultant expression plasmids (pKTH5319 for *spaA*, pKTH5320 for *spaB*, pKTH5324 for *spaD*, pKTH5379 for *spaE*, and pKTH5341 for *spaF*) were isolated and propagated in *E. coli* BL21(DE3)pLysS for intracellular production of C-terminal hexahistidine-tagged SpaCBA and SpaFED pilin proteins. For each of the recombinant pilins, seven residues (nine residues for SpaF) at the N-terminus and two residues preceding the C-terminal hexahistidine-tag originate from amino acids encoded by the expression vector. Cloning of the *spaC* gene has been described elsewhere (18).

Production and purification of recombinant SpaCBA and SpaFED pilin proteins. For production and purification of recombinant SpaA, SpaB, SpaD, SpaE, and SpaF pilins, the procedure is essentially the same as reported for the isolation of the SpaC minor pilin (18). In brief, *E. coli* cells were grown at 37ºC to mid-log phase in LB medium containing 50 μg/ml kanamycin, the protein expression induced for 3 h by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cells recovered by centrifugation. The pelleted cells were resupended in lysis buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM

imidazole] and the cell suspensions then disrupted using sonication followed by centrifugation and filtration (0.45 μm) to produce clarified cell-free lysates. The hexahistidine-tagged pilin proteins (SpaA, SpaB, SpaD, SpaE, and SpaF) were then purified by using Ni^{2+}-chelating affinity chromatography. Each of the cell-free lysates was passed through a Ni-NTA agarose (Qiagen) column, which was rinsed extensively with wash buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole] and the pilin proteins then removed with elution buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 250 mM imidazole]. Elution fractions containing purified proteins were pooled and buffer-exchanged to 10 mM Tris-HCl (pH 8.0) for the SpaA, SpaD, SpaE, and SpaF pilins and to 50 mM sodium acetate (pH 5.1) for the SpaB pilin by using EconoPac 10 DG desalting columns (BioRad). The recombinant pilins were concentrated using 10- or 30-kDa Microsep filters (Pall Life Sciences), the protein purity assessed by SDS-PAGE, and the protein concentration approximated by A_{280} measurements.

Generation of SpaA and SpaB pilin-specific antibodies. Antisera specific for *L. rhamnosus* GG SpaA and SpaB pilin proteins were generated using the same method described for producing SpaC antiserum (18). In summary, rabbits received 1 ml subcutaneous (SC) injections containing 400 μg purified recombinant SpaA or SpaB pilins mixed 1:1 with Freud's complete adjuvant, and over three-week intervals a set of three subcutaneous booster injections (1 ml) of 200 μg pilin protein in Freud's incomplete adjuvant (1:1 mix). Two weeks after the final booster injections, blood was collected for preparation of the SpaA and SpaB antiserum.

Protein and whole cell radiolabeling. Radiolabeling of pilin proteins with iodine-125 (^{125}I) was carried out as described previously (18). Pierce Iodination Reagent following the

manufacturer's protocol (Pierce) was used to radiolabel purified SpaA, SpaB, SpaC, SpaD, SpaE, and SpaF pilin proteins and ovalbumin (Sigma), and residual unbound radiolabel was removed chromatographically by using D-Salt™ Polyacrylamide Desalting Columns (Pierce). Metabolic radiolabeling of *L. rhamnosus* GG cells with tritiated thymidine was performed essentially as described earlier (58). Briefly, *L. rhamnosus* GG was grown overnight in MRS broth containing 10 µl/ml [5'-^{3}H] thymidine (16.7 Ci/nmol), the cells recovered by centrifugation, washed and resuspended in phosphate-buffered saline (PBS; pH 7.2), and the optical density of the culture suspension adjusted (A_{600} = 0.25) to normalize the number of cells.

Isolation of human intestinal mucus. Surgically removed tissue acquired from patients suffering from operable colorectal cancer and undergoing colonic resection was the source of intestinal mucus used for binding studies (32). The recovery and use of resected human intestinal tissue was sanctioned by the ethics committee for the Hospital District of Southwest Finland and with the prior informed written consent of participating patients. Intact mucosa-containing resected tissue from noncancerous segments was chosen and the mucus layer extracted according to the method described earlier (58).

Binding of radiolabeled SpaCBA and SpaFED pilins to intestinal mucus. Saturating amounts of intestinal mucus were added to Maxisorp™ microtiter plate wells (Nunc), allowed to incubate overnight at 4ºC, washed extensively with PBS to remove unbound mucus, and incubated in blocking solution (0.5% bovine serum albumin in PBS) for 1 h at room temperature. The blocking solution was aspirated away and 50 pmol radiolabeled SpaA, SpaB, SpaD, SpaE, or SpaF pilin subunits (each dissolved in blocking solution) were then

added to wells containing immobilized mucus and incubated for 1 h at 37ºC. After the wells were washed three times with PBS, the amount of protein-bound radioactivity (^{125}I) was quantified using a Wallac 1480 WIZARD® 3" automatic gamma counter (PerkinElmer). The mucus binding experiment was also performed with 50 pmol radiolabeled SpaC pilin (positive control) and ovalbumin (background control)

Competitive inhibition of mucus binding by the SpaB and SpaF pilins. Competitive binding of radiolabeled SpaB and SpaF pilins to mucus was carried using the procedure described earlier (18). Using microtiter plate wells containing immobilized intestinal mucus (see above), 50 pmol radiolabeled SpaB or SpaF pilins (dissolved in blocking solution) were added to wells also containing 0, 100, 300, and 900 pmol competing amounts of unlabeled SpaB or SpaF pilins and allowed to incubate for 1 h at 37ºC. The wells were then washed three times with PBS and the amount of bound radioactive (^{125}I) protein measured with an automatic gamma counter. As controls, 50 pmol radiolabeled SpaC pilin with competing protein (positive) and 50 pmol radiolabeled ovalbumin without competing protein (background) were also included in the competitive inhibition assay.

Inhibition of binding between SpaB minor pilin and mucus. Radiolabeled (^{125}I) SpaB pilin (50 pmol) was pretreated with either 2.5 µg/µl lysozyme (Sigma) or near saturating quantities (~2.5 µg/µl) of unbound intestinal mucus and tested for binding to immobilized intestinal mucus according to the method described previously (see above). As a control, the inhibition assay was also performed with 50 pmol radiolabeled SpaC pilin.

Antibody-mediated inhibition of *L. rhamnosus* GG mucosal adhesion. *Lactobacillus rhamnosus* GG mucosal adhesion was inhibited by antiserum specific for SpaA and SpaB pilins according to the method described previously for SpaC antiserum-mediated inhibition of mucus-binding (18). Briefly, a metabolically radiolabeled (^{3}H) *L. rhamnosus* GG cell suspension was preincubated with each of the SpaCBA pilin-specific antiserum diluted 1:100, a 100 µl volume added to microtiter plate wells coated with human colonic mucus (49), and allowed to incubate for 1 h at 37°C. Each of the wells was then washed twice with PBS to rinse away cells adhering weakly to the mucosal substrate. The cells remaining bound to the immobilized mucus were resuspended in a 1% SDS/0.1N NaOH solution, incubated for 1h at 60°C, and the lysed cell suspension then assessed for radioactivity by liquid scintillation counting. Mucosal adhesion was estimated as a percent ratio of the measured radioactivities for the lysed cell suspension and the cell suspension added initially to the wells.

Statistical Analysis. The linear association between the amount of unlabeled pilin protein (0, 100, 300, and 900 pmol) and the measured amount (fmol) of radiolabeled pilin protein (^{125}I) was tested to assess the dose-dependent pattern of inhibition for mucus binding by means of the Pearson product-moment correlation coefficient ($t = -4.05$ and $n = 16$ for SpaB, $t = -2.88$ and $n = 14$ for SpaF, and $t = -6.42$ and $n = 16$ for SpaC). The unequal variance t-test and the Bonferroni multiple comparison test were used to evaluate the significant difference in amounts of bound radiolabeled pilin protein (^{125}I) or *L. rhamnosus* GG cells (^{3}H) between measured samples.

RESULTS

Production and purification of recombinant SpaCBA and SpaFED pilins. The *spaA*, *spaB*, *spaD*, *spaE*, and *spaF* genes were each amplified by PCR from *L. rhamnosus* GG using the cloning primers listed in Table 1 and the respective PCR products cloned in a pET28b+ expression vector as described in Materials and Methods. Recombinant pilins with C-terminal hexahistidine-tagging were produced intracellularly in *E. coli* and purified by Ni^{2+}-agarose affinity chromatography. Purified SpaA and SpaB pilins were used to produce SpaA- and SpaB-specific antibodies in rabbits (see Materials and Methods for details), and all purified SpaCBA and SpaFED pilin proteins were analyzed for adherence to intestinal mucus. The cloning, production, and purification of the SpaC minor pilin as well as its use to generate SpaC antiserum have been described previously (18). The molecular weights for each of the purified recombinant pilin proteins (SpaA, SpaB, SpaC, SpaD, SpaE, and SpaF) corresponding to bands detected by SDS-PAGE (Fig. 1) were in agreement with calculated molar masses (Table 2). An examination of the additional properties of the pilin subunits (Table 2) also revealed the highest isoelectric point (pI ~8) for the SpaB pilin. Interestingly, SDS-PAGE of purified recombinant SpaB pilin (Fig. 1; lane 2) indicated the presence of a weakly stained high molecular weight protein band of unknown origin, but possibly representing an aggregate form of SpaB caused by SDS-induced precipitation. Since strongly alkaline proteins (e.g., pI ~11) tend to precipitate in the presence of SDS at a pH below their isoelectric point, the extent of which is related to how positive charged the protein is at a particular pH (29) and that sometimes manifests as an anomalous SDS-PAGE migration pattern (7), a moderately alkaline SpaB protein might be prone to similar behavior. As well, the reason for a doublet band of purified SpaA pilin (Fig. 1; lane 1) that would appear

occasionally on SDS-gels is unknown, but might be related to intramolecular isopeptide bonds identified previously for maintaining rigidity in the pilin structure (6, 16, 17) and whose altered formation possibly causes pilin proteins to migrate aberrantly when analyzed by SDS-PAGE (41).

Mucus-binding properties of the SpaCBA and SpaFED pilins. In our previous work, we established binding between the SpaC minor pilin and human intestinal mucus using radiolabeled protein (18). In this present study, we used the same approach to assess the mucus-binding properties of the predicted major pilin (SpaA and SpaD) and minor pilin (SpaB, SpaE, and SpaF) subunits associated with the two pilus gene clusters in *L. rhamnosus* GG. Recombinant forms of the pilin subunits were purified, radiolabeled with iodine-125 (^{125}I), and screened for binding to human colonic mucus. The SpaC minor pilin was included as a positive control in the binding analysis. As shown in the mucus-binding profile for the SpaCBA and SpaFED pilins (Fig. 2), only the SpaB, SpaC, and SpaF subunits exhibited significant binding to the mucosal substrate. Since the remaining three pilin subunits (SpaA, SpaD, and SpaE), all of which are predicted to contribute to the structural integrity of the pilus and lack discernible homology to recognized mucus-specific adhesins had displayed mucus binding below the background level, the absence of appreciable adherence to mucus was not completely unexpected. However, the two minor pilin subunits (SpaC and SpaF), which were predicted to be localized at the tip of different pilus fibers and prototypically adhesive in nature, had demonstrated an anticipated mucus-binding profile. Since the SpaB minor pilin subunit shares similar predicted roles with the SpaA, SpaD, and SpaE pilins (see above), binding between the SpaB subunit and intestinal mucus was an unforeseen result. Moreover, the amount of mucus binding by the SpaB subunit was approximately seven-fold

more than measured for the SpaF and SpaC pilins. Since of the six SpaCBA and SpaFED pilin subunits only the SpaB minor pilin is a positive charged protein (Table 2), the binding between the SpaB pilin and negative charged mucosal moieties might be mediated simply by electrostatic interactions and independent of a specific mucus-binding domain. Nonetheless, the two minor pilins (SpaB and SpaF) with measurable adherence to mucus were included in a competitive mucosal adhesion assay for additional characterization.

Competitive mucosal adhesion of the SpaB and SpaF minor pilins. To assess whether the mucosal adhesion interaction for the SpaB and SpaF pilin subunits can be inhibited in a dose-related manner as reported for the SpaC minor pilin (18), we performed *in vitro* competitive binding experiments (Fig. 3) using radiolabeled proteins and intestinal mucus as described in Materials and Methods. Both radiolabeled SpaC pilin (positive) and ovalbumin (background) were included as controls in the competitive binding assay. Each of the radiolabeled pilin subunits (SpaB, SpaF, and SpaC) bound to the mucus substantially more than the background control (Figs. 3A, 3B, and 3C). Moreover, supported by a statistical analysis of the binding data for all three pilin subunits, competing amounts of unlabeled proteins (100, 300, and 900 pmol) caused an apparent dose-related inhibition of mucus binding, although the SpaB subunit demonstrated the most convincing dose-related reduction in adhesion (Fig. 3A). The results we obtained here are in agreement with competitive adhesion binding.

The influence of electrostatic interactions on the mucus-binding specificity of the SpaB minor pilin. To determine whether electrostatic interactions influence the mucus-binding specificity of the basic SpaB pilin protein, we used positive charged lysozyme (pI ~11) to inhibit the mucosal adhesiveness of SpaB pilin. In a previous study (14), it was reported that

lysozyme can crosslink mucus by electrostatic means and, in doing so, contribute partially to the build-up of the macromolecular gel-like nature of mucus. As controls, we included the effect of adding unbound mucus and performed the binding experiment with the SpaC minor pilin. Binding of SpaB pilin to intestinal mucus was diminished by approximately 50% in the presence of lysozyme (2.5 µg/µl), indicating that lysozyme competes with SpaB pilin for mucus and electrostatic interactions may play some part in SpaB-mediated mucosal adhesion (Fig. 4). As expected near saturating quantities of unbound mucus abolished a significant portion of binding between the SpaB pilin and bound mucus (Fig. 4). For the SpaC minor pilin, mucus binding was reduced by about two-fold in the presence of additional mucus and unaffected by large amounts of lysozyme.

Inhibition of *L. rhamnosus* GG mucosal adhesion by pilin-specific antibodies. Previously, the presence of cell surface-localized intact pili, encoded by the *spaCBA* gene cluster, including their capacity for mucus binding and the inhibition thereof have been demonstrated in *L. rhamnosus* GG using antiserum specific for the SpaC minor pilin (18). As a means to characterize the mucosal adherence properties of SpaCBA pili further, we examined the effect of pretreating *L. rhamnosus* GG cells with SpaB antiserum. As controls, untreated cells and cells pretreated with SpaA- and SpaC-specific antibodies were also included. Mucosal adhesion by the untreated cells was detected as anticipated and the pretreatment of cells with SpaC antiserum confirmed our earlier findings (18).

Pretreatment with SpaB antiserum had surprisingly failed to block mucosal adhesion (Fig. 5) despite the demonstration of significant mucus binding by the recombinant SpaB pilin (Fig. 2) and the functionality of the SpaB-specific antibodies as indicated by their use in identifying

surface-localized SpaCBA pilus fibers by immunogold-labeled electron microscopy (J. Reunanen et al., to be published). The *spaB* gene in the *spaCBA* pilus gene cluster has been predicted previously as an ancillary minor pilin (18) and, according to a current model of pilus assembly (27), likely corresponds to the same type of pilin subunit deposited at the pilus base and sparingly along the pilus backbone and implicated in signaling the cessation of pilus polymerization. Consequently, the binding of SpaB-specific antibodies to SpaB localized similarily in the SpaCBA pilus structure may only prevent SpaB-mediated mucus adhesion and thus impose little steric hindrance that would affect the mucus-binding capacity of the SpaC pilin. This rationale was borne out when cells pretreated with both SpaB and SpaC antiserum could no longer adhere to mucus (Fig. 5). Mucus binding appears to have been blocked for the most part by the SpaC-specific antibody, reflecting the possibility that the SpaB pilin in assembled pilus fibers contributes less to *L. rhamnosus* GG mucosal adhesion than deduced from the high level of mucus binding for the individual SpaB pilin subunit (Fig. 2). Moreover, since each type of pilin is situated primarily in a different location within the pilus structure, a SpaC pilin at the tip of a pilus fiber (18) may be more accessible to mucosal substrates than a SpaB pilin in its predicted position at the pilus base.

Another unexpected result was the five-fold reduction in mucus binding by the cells pretreated with SpaA antiserum (Fig. 5) even though the SpaA pilin subunit did not adhere appreciably to intestinal mucus (Fig. 2). However, because the SpaA major pilin is the predicted predominant subunit comprising the pilus backbone (18), the pili-bound SpaA-specific antibodies may simply hinder sterically the SpaC pilin subunits shown to be deposited at the pilus tip and intermittently throughout the SpaCBA pilus (18), and therefore cause obstruction of SpaC-mediated mucosal adhesion.

DISCUSSION

The continuous shedding of intestinal epithelial cells and the peristaltic movement of food and water through the intestine create a dynamic environment with considerable challenges for stable bacterial colonization of the gastrointestinal tract (52). Since a sizeable portion of the intestinal microbiota coexists symbiotically with the host, it is advantageous that the intestine encompass an environmental niche favoring localized commensal growth. The mucus gel layer, which spans the epithelial cell lining of the inner intestinal walls, is naturally the first region for possible microbial contact and attachment, and can provide an environment comprised of a conserved glycan-based matrix for localizing microbes, binding sites to prevent microbial washout from the intestine, and a carbohydrate-rich source of nutrients (34, 52). Within the intestinal microbiota, a diverse range of commensal, probiotic, and pathogenic species have adapted to a mucosal milieu by developing unique surface-associated features with mucus-related specificity (2).

The prevailing consensus about what constitutes an effective probiotic follows the general rule that these types of bacteria must have an inherent ability for maintaining persistent growth in the GI tract (9, 59). However, several studies using various probiotic strains have reported that rather than becoming one of the fortified species of the intestinal microbiota, the probiotics are allochthonous, existing transiently and requiring constant replenishing to persist for extended periods in the intestine (3, 5, 10, 59). Moreover, studies have also revealed variations in the level of intestinal persistence between many probiotic bacteria (13, 18). Although some debate continues whether persistent colonization is essential for the beneficial

immunomodulating effects of probiotics (9), lactobacilli bacteria must still rely on specific adherence mechanisms for displacing pathogens and preventing their own immediate washout from the intestinal tract. Nonetheless, a plethora of studies have demonstrated binding between a variety of probiotic *Lactobacillus* spp. and mucus-related substrates (23), but very few have pinpointed either by genomic or proteomic means those cell surface components directly responsible for mucosal adhesion. In our recent study (18) we reported the presence of elongated pilus fibers on the cell surface of the probiotic *L. rhamnosus* GG strain. Moreover, we attributed a mucus-binding capacity to these pili (encoded by the *spaCBA* gene cluster), and in particular, to one of the individual pilin subunits (SpaC) constituting the pilus fiber. Such pili, by virtue of their long and slender appendaged architecture, an observed propensity to tether around the cell exterior, and a binding specificity for mucus are ideal surface structures for ensuring microbes remain attached and embedded within a mucosal matrix. Consequently, the *L. rhamnosus* GG strain, which has been characterized as an effective probiotic is expected to have a distinct advantage over nonpiliated probiotic lactobacilli for maintaining an extended or stable residency in the GI tract.

The structural constituents of the two pilus fibers (SpaCBA and SpaFED) encoded within the genome of *L. rhamnosus* GG include one major pilin and two minor pilin subunits, all of which resemble prototypically those structural components of pili found in many other Gram-positive bacteria (26, 40, 49). So far, only the SpaC minor pilin subunit from the SpaCBA pilus has been assessed for a binding specificity (18), leaving five predicted pilus components, the SpaA and SpaD major pilin and the SpaB, SpaE, and SpaF minor pilin subunits, uncharacterized. In this study, we cloned the corresponding genes (s*paA*, *spaB*, *spaD*, *spaE*, and *spaF*), produced and purified recombinant forms of pilin protein, and undertook a

systematic assessment of their respective binding specificity for human intestinal mucus. Following the initial screen for mucus-binding properties, we observed no appreciable mucosal adhesion with the SpaA, SpaD, and SpaE pilin subunits, a somewhat expected result with hindsight when the predicted roles of these components in an assembled pilus fiber and the lack of mucosal-related binding domain homologs in each of their primary structures are taken into account (18). Although pilin subunits from some Gram-positive bacteria show adherence to the extracellular matrix (ECM) proteins underlying the intestinal epithelium (11,21,51), our own attempts to screen for similar binding specificities with the three pilin subunits unable to adhere to mucus were largely unsuccessful (data not shown). At present, major pilin subunits comprising the pilus backbone and minor pilin subunits located at the pilus base of other Gram-positive pili have not demonstrated specific adhesion properties (20, 26, 40, 49). However, for the SpaB and SpaF minor pilins, each showed significant adherence to the mucosal substrate, and in a competitive adhesion assay the inhibition of mucus binding was dose-related, with the best dose-dependent reduction in adhesion displayed by the SpaB pilin. Considering that SpaF pilin is one of the two large-sized pilus components (see Table 2) and correspondingly a predicted adherent tip pilin, a mucus binding capacity could be conceivably anticipated. Previously, we described the presence of a von Willebrand factor-like domain with possible lectin-type binding character in the primary structure of the SpaC pilin (18), but for the SpaF pilin similar or related domain homologies were not observed. Although pilus fibers encoded by the *spaFED* gene cluster have yet to be identified on the surface of *L. rhamnosus* GG cells, our results suggest that only one of the three pilin subunits (SpaF) in an assembled pilus structure would possess a mucus-binding specificity.

The detection of binding between the SpaB minor pilin and intestinal mucus, which was considerably more than measured for the SpaF and SpaC pilins was a rather surprising outcome in the absence of any homology to established mucus-specific adhesins (18). When the physical and chemical properties of the six pilin subunits were compared, we found that the isoelectric point for the SpaB pilin (pI ~8) was higher than the five other pilins (pI ~5), and resultingly, we investigated whether electrostatic interactions have a role in SpaB-mediated mucosal adhesion. Several reports in the literature have described the use of high ionic strength (1M NaCl) to disrupt charge-dependent bonds and thereby establish charge mechanisms for a varied array of protein-mediated interactions (4, 35, 50). However, we instead chose to use large amounts of a positive charged protein to inhibit binding between the SpaB pilin and mucus. Lysozyme, a highly positive charged enzyme (pI ~11) that is found normally at basal levels throughout the intestinal tract (37) and possibly forms crosslinks with mucus by electrostatic bonding (14), was able to compete with the SpaB pilin for the negative charged mucosal substrate. Since SpaB pilin-mediated mucosal adhesion was inhibited effectively by lysozyme, the binding determinants between SpaB pilin and mucus may likely be governed by electrostatic contacts, and therefore, as proposed for lysozyme, the SpaB pilin may have the same capacity to crosslink with mucus. Moreover, given that the SpaC tip pilin is also deposited occasionally throughout the pilus fiber (18) as is predicted for SpaB, their frequency of incorporation within the SpaCBA pilus fiber will have a direct influence on the overall strength of *L. rhamnosus* GG mucosal adhesion. Previously, we had established the contribution of the SpaC component to SpaCBA pili-mediated mucosal adhesion by pretreating *L. rhamnosus* GG cells with SpaC-specific antiserum (18). In our present study, the results from a similar experiment using SpaB antiserum were inconclusive and did not

confirm directly an involvement of the SpaB pilin mucus-binding capacity in the assembled SpaCBA pilus fibers.

We have now given a further insight into the binding specificities of the major and minor pilin subunits for the pilus fibers encoded by the *L. rhamnosus* GG *spaCBA* and *spaFED* gene clusters. In a continuation of our earlier work, we have now shown that two pilin components binding to mucus, one possibly in a lectin-type manner (SpaC) (18) and another through electrostatic contacts (SpaB) are conceivably involved in SpaCBA pili-mediated adherence to intestinal mucus. Moreover, we have established that the SpaF minor pilin would be the only mucus-binding component in the putative SpaFED pilus fiber. Several studies have reported that various Gram-positive pathogens use mucosal adhesion for facilitating their invasive colonization of the GI tract (reviewed in 53). Our report of pilus fibers in the *L. rhamnosus* GG strain with such an evident mucus-binding predisposition lends support to the well-recognized mechanism by which probiotics displace pathogenic bacteria using competitive elimination or steric hindrance (8, 12, 24, 43). Since the intestinal mucosal barrier is a nonhomogeneous collection of various protein- and carbohydrate-rich components, some might represent potential receptor-binding sites that adhere specifically to the pilus fibers in *L. rhamnosus* GG. By using the three recombinant mucus-binding pilin subunits (SpaB, SpaC, and SpaF), we plan to identify, isolate, and characterize the equivalent host cell receptors located within the intestinal mucosa as part of our future studies. Presently, three-dimensional (3-D) structure determination of the major and minor pilin subunits has been described for four piliated Gram-positive species (6, 16, 17, 22), and so the availability of soluble and functional *L. rhamnosus* GG pilin proteins will now facilitate obtaining additional structural insight into the assembly and function of pilus fibers from a probiotic strain.

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FIGURE LEGENDS

FIG. 1. Purified recombinant SpaCBA and SpaFED pilin proteins. Purified recombinant SpaA (lane 1), SpaB (lane 2), SpaC (lane 3), SpaD (lane 4), SpaE (lane 5), and SpaF (lane 6) pilin proteins were separated using 12% SDS-PAGE followed by staining with coomassie brilliant blue R-250. Molecular weight standards (kDa) and the location of the pilin protein bands are as indicated.

FIG. 2. Adherence of SpaCBA and SpaFED pilin proteins to human intestinal mucus. The determination of binding between the 50 pmol radiolabeled (^{125}I) SpaCBA and SpaFED pilins and immobilized mucus, and the corresponding statistical analysis are described in Materials and Methods. The background level of mucus binding was based on 50 pmol radiolabeled (^{125}I) ovalbumin adherence to mucus. Binding data from three to four measurements are given as mean ± SD and the dataset comparisons are considered significant ($P \leq 0.05$).

FIG. 3. Competitive mucus-binding of the SpaB and SpaF minor pilins. The inhibition of mucus binding by 50 pmol radiolabeled (^{125}I) SpaB **(A)**, SpaF **(B)**, and SpaC **(C)** pilins was carried out with competing amounts of the respective unlabeled pilin subunit (0, 100, 300, and 900 pmol) as described in Materials and Methods. Binding between 50 pmol radiolabeled (^{125}I) ovalbumin and immobilized mucus indicated the level of background mucus binding. Binding data are mean values ± SD from three to four measurements. The dataset comparisons and dose-related inhibition of mucus binding are considered significant ($P \leq$

0.01) for each SpaB, SpaF, and SpaC pilin subunit. Further details on the statistical analysis are provided in Materials and Methods.

FIG. 4. Inhibition of binding between SpaB minor pilin and mucus.

The effect of 2.5 ug/ml lysozyme or unbound intestinal mucus on the adherence of 50 pmol radiolabeled (^{125}I) SpaB and SpaC pilins to immobilized mucus was determined using the procedure provided in Materials and Methods. Binding data obtained from three to four measurements are presented as mean ± SD and the dataset comparisons are considered significant ($P \leq 0.05$). Further information on the statistical analysis is provided in Materials and Methods.

FIG. 5. SpaCBA pilin antibody-mediated inhibition of *L. rhamnosus* GG mucosal adhesion.

Radiolabeled (^{3}H) *L. rhamnosus* GG cells were pretreated with SpaA-, SpaB-, and SpaC-specific antiserum and examined for mucus binding as described in Materials and Methods. Binding data from six measurements are given as mean ± SD and the dataset comparisons are considered significant ($P \leq 0.05$). Additional information on the statistical analysis is described in Materials and Methods.

TABLE 1. Oligonucleotide primers used for PCR cloning [a]

Gene	ORF	Oligonucleotide primer sequences [b]
spaA	LGG_00442	*f*-5'-TCGGGTTCA**GAATTC**TACGAATGATACGAC
		r-5'-TGCCAGTACCACC**CTCGAG**TGGCAGAATAC
spaB	LGG_00443	*f*-5'-GCAGACACA**GAATTC**AACTGTGCCGACC
		r-5'-CAACTGTATCACC**CTCGAG**TGGCAACAATTGACG
spaD	LGG_02370	*f*-5'-ACCCGTACA**GAATTC**GACAACGACTGTG
		r-5'-GTCCGATTCCGCC**CTCGAG**CGGCAATAATTG
spaE	LGG_02371	*f*-5'-CCACATTGGGTTCA**GAATTC**TGATCAAACTG
		r-5'-TGCGCCAATCGGA**CTCGAG**CGGCAAATAAC
spaF	LGG_02372	*f*-5'-GCAAATTGGCAG**GAGCTC**GGTCCCGGTAG
		r-5'-CCGCTACCACC**CTCGAG**CGGTAGGAGTG

[a] Oligonucleotide primers were designed using the *L. rhamnosus* GG genome sequence (18).

[b] Sequences for restriction endonucleases to facilitate cloning, *Eco*RI (and *Sac*I for *spaF*) in the forward (*f*) and *Xho*I in the reverse (*r*) oligonucleotide primers, are underlined and in boldfaced type.

TABLE 2. Properties of the recombinant SpaCBA and SpaFED pilin proteins

Pilin	Molar mass (kDa)	Amino acid length	Isoelectric point (pI)
SpaA	31	283	5.1
SpaB	21	188	8.0
SpaC	91	836	5.0
SpaD	51	465	5.5
SpaE	45	400	5.9
SpaF	104	943	5.4

Fig. 1

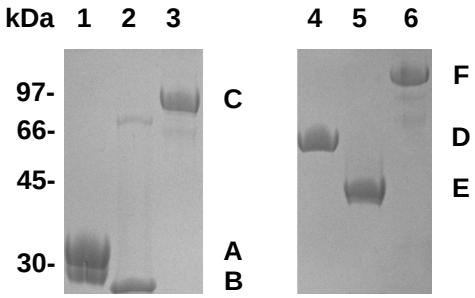

Fig. 2

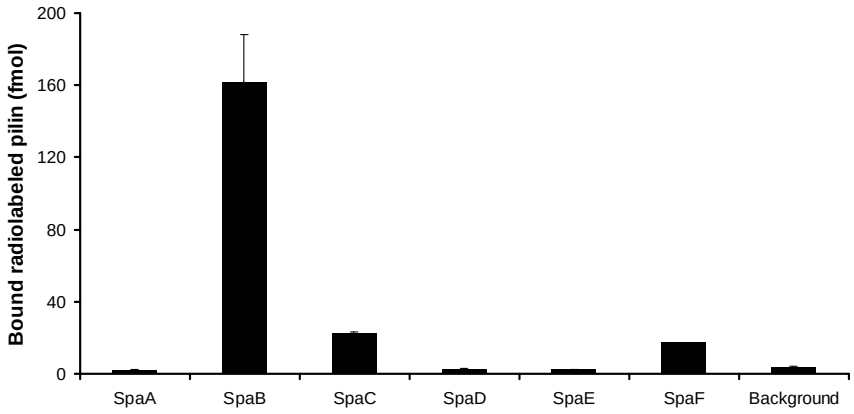

Fig. 3

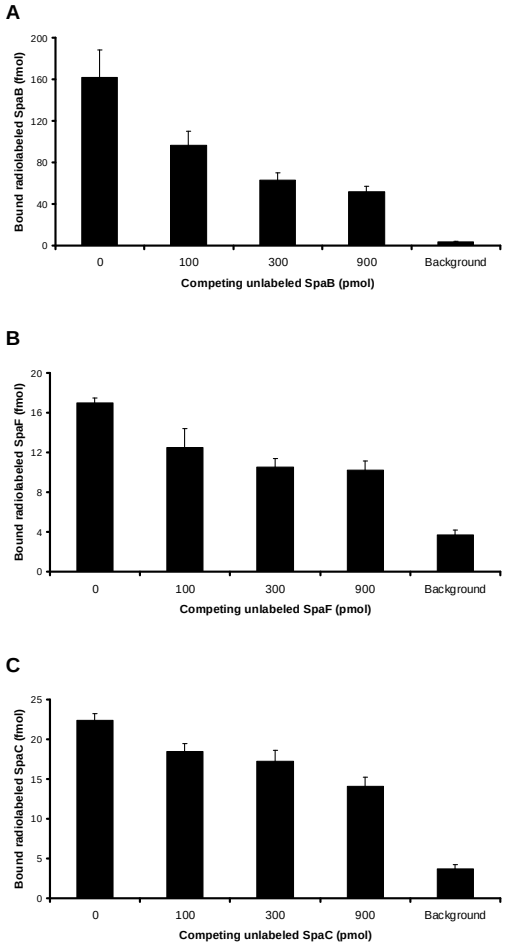

Fig. 4

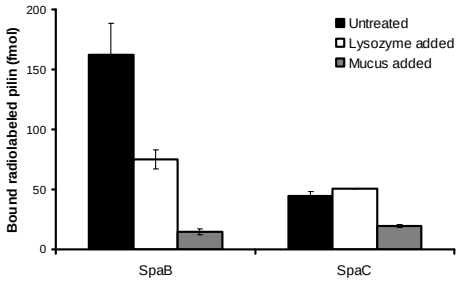

Fig. 5