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Lactobacillus jensenii Surface-Associated Proteins Inhibit *Neisseria gonorrhoeae* Adherence to Epithelial Cells▽

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High numbers of lactobacilli in the vaginal tract have been correlated with a decreased risk of infection by the sexually transmitted pathogen *Neisseria gonorrhoeae*. We have previously shown that *Lactobacillus jensenii*, one of the most prevalent microorganisms in the healthy human vaginal tract, can inhibit gonococcal adherence to epithelial cells in culture. Here we examined the role of the epithelial cells and the components of *L. jensenii* involved in the inhibition of gonococcal adherence. *L. jensenii* inhibited the adherence of gonococci to glutaraldehyde-fixed epithelial cells like it inhibited the adherence of gonococci to live epithelial cells, suggesting that the epithelial cells do not need to be metabolically active for the inhibition to occur. In addition, methanol-fixed *L. jensenii* inhibited gonococcal adherence to live epithelial cells, indicating that *L. jensenii* uses a constitutive component to inhibit gonococcal interactions with epithelial cells. Proteinase K treatment of methanol-fixed lactobacilli eliminated the inhibitory effect, suggesting that the inhibitory component contains protein. Released surface components (RSC) isolated from *L. jensenii* were found to contain at least two inhibitory components, both of which are protease sensitive. Using anion-exchange and size exclusion chromatography, an inhibitory protein which exhibits significant similarity to the enzyme enolase was isolated. A recombinant His_6-tagged version of this protein was subsequently produced and shown to inhibit gonococcal adherence to epithelial cells in a dose-dependent manner.

Neisseria gonorrhoeae (gonococcus) is an obligate human pathogen that causes the sexually transmitted infection gonorrhea. Gonorrhea is one of the most commonly reported infectious diseases and is second only to chlamydia, with 336,742 cases reported in 2008 in the United States according to the Centers for Disease Control and Prevention (CDC). Gonorrhea is readily treated with antibiotics; however, historically, *N. gonorrhoeae* has developed resistance to each antibiotic used, including penicillins, tetracyclines, spectinomycin, and, most recently, fluoroquinolones. In 2007, the CDC recommended only one class of antibiotics to treat all types of gonorrhea, the cephalosporins (2). Furthermore, women infected with gonorrhea often have asymptomatic infections, providing a significant reservoir for transmission. Undiagnosed, and therefore untreated, gonococcal infections can lead to permanent damage of the female reproductive system, resulting in infertility or ectopic pregnancy. Gonorrhea has also been reported to increase susceptibility to HIV infections (36). Therefore, a method to reduce the number of gonococcal infections that does not involve antibiotics would be beneficial for public health.

Adherence to the host epithelia is the first and most critical step in a gonococcal infection; thus, this step is a target of interest for the development of new therapeutics. After inhibition of gonococcal adherence to epithelial cells, the pathogen could be washed away by the flow of vaginal or menstrual fluid or killed by the antimicrobial agents found in the vaginal mucus, so the pathogen could not establish an infection (49). One potential source for antiadherence treatment is probiotic bacteria. Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (1). The bacterial genus most studied for its probiotic properties is *Lactobacillus*. Lactobacilli naturally inhabit both the gastrointestinal tract and the female reproductive tract of healthy humans. In the female reproductive tract, lactobacilli make up the majority of the indigenous vaginal and endocervical microbiota (49, 50). Lactobacilli have been shown to play a role in protecting women from infection by incoming pathogens, including HIV (35), and epidemiological evidence suggests that women with high numbers of vaginal lactobacilli have reduced susceptibility to gonorrhea and chlamydia following exposure (48). Clearly, lactobacilli are a key component of the human defense against colonization by sexually transmitted pathogens.

Lactobacilli utilize several mechanisms to prevent colonization by incoming pathogens. These mechanisms include direct killing of the organisms by hydrogen peroxide, bacteriocins, and lowering the pH of the vaginal tract to ~4 by production of lactic acid (5, 6, 9). Lactobacilli can inhibit adherence by production of biosurfactants (31, 45, 47), receptor competition (8, 11), or coaggregation with the pathogen, which allows the pathogen to be swept away by the host's bodily fluids (7, 22). Lactobacilli can also inhibit pathogen colonization by causing the host cells to become more resistant to adherence (15, 20, 23, 29) or by suppressing the expression of virulence factors in the pathogen (25).

Lactobacillus jensenii, one of the most prevalent species of lactobacilli indigenous to the human vaginal tract, has been shown to inhibit gonococcal adherence to and invasion of epithelial cells (38). The mechanism used by *L. jensenii* to inhibit gonococcal adherence is unknown. However, we have shown in

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previous work that *L. jensenii* does not directly inhibit the growth of gonococci or coaggregate with gonococci either in medium or at the cell surface. Furthermore, the hydrogen peroxide produced by *L. jensenii* also does not affect gonococcal adherence to epithelial cells (38). In this study, we tested the hypothesis that *L. jensenii* utilizes a surface component to inhibit gonococcal adherence and examined the processes and components of the epithelial cells, gonococci, and lactobacilli that play a role in this inhibition.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. gonorrhoeae* MS11 (P^+ Tr) (34) and *N. gonorrhoeae* MS11-307 (Δ*pilE1*::*erm* Δ*pilE2* P^- Tr) (26) were grown at 37°C in a humidified 5% CO_2 environment on GC agar (Acumedia, Lansing, MI) with supplements (19) and the VCNT (vancomycin, colistin, nystatin, and trimethoprim) inhibitor (GCV) (Becton, Dickinson, and Company, Sparks, MD). *L. jensenii* ATCC 25258 ($H_2O_2^+$) was grown at 37°C in the presence of CO_2 on MRS agar (Becton, Dickinson). *Bacillus subtilis* strain RB 247 (42) was grown at 37°C on LB agar (Acumedia, Lansing, MI). *Escherichia coli* strains DH5α and BL21λDE3 were grown on LB agar at 37°C; 100 mg/liter kanamycin was added when pET24a and derivatives of this plasmid were used.

Cell culture. Human endometrial epithelial cell line Hec-1-B (ATCC HTB-113) was grown in Dulbecco's modified Eagle medium (with high glucose and L-glutamate) (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (FCS) (Invitrogen) at 37°C in the presence of CO_2. Cell culture assays were performed using 24-well cell culture plates with Hec-1-B cells grown to 50 to 90% confluence.

Glutaraldehyde fixation of Hec-1-B cells. Hec-1-B cells were incubated with 1 ml of glutaraldehyde (2.5%, vol/vol) for 30 min at 37°C in the presence of CO_2. The glutaraldehyde was then removed, and the fixed cells were washed five times with phosphate-buffered saline (PBS) immediately prior to use in adherence assays (37). Gonococcal adherence assays with glutaraldehyde-fixed cells were carried out as described below, except that fixed cells were lifted with saponin (1% [wt/vol] in GC broth) instead of PBS containing 5 mM EDTA.

Methanol and proteinase K treatment of *L. jensenii*. An *L. jensenii* inoculum was divided into three 2-ml aliquots containing 4×10^7 CFU/ml. Aliquot 1 (live lactobacilli) was kept at 37°C. Aliquots 2 and 3 were treated with an equal volume of ice-cold methanol (MeOH) for 10 min and then centrifuged at 14,000 × *g* for 3 min to pellet the bacteria. The supernatant was removed and replaced with 200 μl DMEM (aliquot 2, MeOH treated) or 160 μl DMEM and 40 μl proteinase K (ProK) (20 mg/ml) (aliquot 3, ProK treated). All three aliquots were then incubated for 2 h at 37°C. Aliquots 2 and 3 were boiled for 3 min and then centrifuged at 14,000 × *g* for 3 min to pellet the bacteria. The supernatants were removed, and the bacteria were resuspended in 2 ml fresh DMEM. Microscopic visualization of Gram-stained samples confirmed that MeOH treatment and ProK treatment left the lactobacilli intact.

Adherence assays. Adherence assays were performed as previously described (38). Briefly, wells containing 10^5 Hec-1-B cells were inoculated with *L. jensenii* at a multiplicity of infection (MOI) of 100 (1×10^7 CFU/ml), with *B. subtilis* at an MOI of 100 (1×10^7 CFU/ml), or with fresh DMEM supplemented with 5% FCS, 12.4 μM $Fe(NO_3)_3$ (GC supplement II), and 110 mM sodium pyruvate for mock infection. Following incubation at 37°C in the presence of CO_2 for 1 h, the epithelial cells were infected with *N. gonorrhoeae* at an MOI of 10 (1×10^6 CFU/ml). Following 3 h of incubation at 37°C in the presence of CO_2, the infected Hec-1-B cells were divided into two sets; the first set was used to determine the total number of bacteria in the well, and the second set was used to determine the number of cell-associated (adherent) bacteria. For the first set, the supernatant from each well (0.5 ml) was placed in a sterile tube. The Hec-1-B cells with the cell-associated bacteria were then lifted with 0.5 ml of PBS containing 5 mM EDTA (PBS/EDTA) and added to the supernatant. Serial dilutions were plated on appropriate selective media to determine the total number of CFU/well. For the second set, the supernatant was removed, and the epithelial cells were washed five times with sterile PBS. The epithelial cells with the cell-associated bacteria were then lifted with 1 ml PBS/EDTA and transferred to a sterile tube. Serial dilutions were plated on selective media to determine the number of cell-associated (adherent) CFU/well. The adherence frequency was calculated by dividing the number of cell-associated CFU/well by the total number of CFU/well. Where indicated, the adherence frequencies were normalized using the mock-infected preparations, which contained no lactobacilli.

Production of released surface components (RSC). A modification of the method of Reid et al. (31) was used to isolate released surface-associated proteins and biosurfactants from *L. jensenii*. Briefly, a 1-liter culture of *L. jensenii* in MRS broth was grown to an A_{600} of 1.6 to 1.8, and the bacteria were harvested by centrifugation (10,000 × *g* for 10 min at 7°C). The bacterial cell pellet was washed twice with sterile H_2O. The cell pellet was then resuspended in 134 ml PBS and gently stirred for 2 h at room temperature. The *Lactobacillus* suspension was then centrifuged for 20 min at 3,000 × *g*, and the supernatant was filtered (pore size, 0.22 μm) to remove the remaining bacteria. This cell-free preparation was concentrated to ~10 ml by Amicon ultraconcentration using a 10,000-molecular-weight-cutoff filter membrane. The protein concentration was determined using the Bradford method (Bio-Rad, Richmond, CA). The products obtained were then assayed to examine the inhibition of gonococcal adherence to Hec-1-B cells by pretreating the epithelial cells for 3 h prior to infection with *N. gonorrhoeae.*

Fibronectin blocking assay. To assess the effect of fibronectin (Fn) on the inhibitory activity of the RSC, 0.42 mg/ml RSC was incubated with 50 μl of soluble Fn (0 to 10 μM) for 1 h at room temperature. This mixture was used to treat Hec-1-B cell monolayers (10^5 cells/well) for 3 h. The suspension was then removed, and the cells were washed once with PBS. An adherence assay with *N. gonorrhoeae* was carried out using these cells as described above.

Trypan blue exclusion assay. Wells containing 10^5 Hec-1-B cells were inoculated as described above for adherence assays with either sterile DMEM, *L. jensenii*, *N. gonorrhoeae*, RSC, or a combination of *N. gonorrhoeae* and *L. jensenii* or *N. gonorrhoeae* and RSC. Following 3 h of incubation at 37°C in the presence of CO_2, the cells were washed five times with PBS, and then trypan blue (0.2% in PBS) was added. After 3 min, the trypan blue was removed, and the cells were washed once with PBS. The numbers of live and dead cells for all conditions were counted visually, and the results were compared.

Collapsed drop analysis. To assess biosurfactant activity, the RSC was assayed by performing a collapsed drop analysis as described by Walenca et al. (47). Two microliters of paraffin oil was allowed to equilibrate overnight in the wells of a 96-well microtiter plate lid. Five microliters of each sample was dropped onto the oil-covered surface and observed for 1 h. A sample was considered positive if the drop flattened (had biosurfactant activity) and negative if the drop remained a bead on the surface of the oil (no biosurfactant activity). Water was used as a negative control.

Anion-exchange column chromatography. After dialysis against H_2O, Tris (final concentration, 50 mM; pH, 7.6) was added to the RSC, which was then separated by anion-exchange column chromatography (Econo-Pac Q; Bio-Rad) using a 0 to 1 M NaCl gradient in a buffer consisting of 50 mM Tris and 5 mM EDTA. The column flowthrough and a 25-ml wash fraction without NaCl were collected before the gradient was run. Twenty-five 2-ml fractions were collected with the gradient, and five 2-ml fractions were collected during a terminal wash with 1 M NaCl. The protein in fractions was quantified using the Bradford method, and samples were run on a 12% SDS-PAGE gel. The fractions were then dialyzed into PBS before they were assayed for inhibitory activity against gonococcal adherence to Hec-1-B cells. Inhibitory fractions were also assayed for biosurfactant activity using the collapsed drop analysis method described above.

Protein identification by tandem mass spectrometry (MS/MS). Proteins were separated on a 12% SDS-PAGE gel and stained with 1% Coomassie blue. The protein band of interest was excised from the gel and sent to the Michigan Proteome Consortium for analysis. Briefly, the sample was digested with trypsin, concentrated, and then spotted on a 192-well matrix-assisted laser desorption ionization (MALDI) target and dried at room temperature. The sample was analyzed with a 4800 proteomics analyzer (time of flight/time of flight; Applied Biosystems), and mass spectra were acquired in reflector positive ion mode for a peptide molecular mass range of 800 to 3,500 kDa. Mass spectra were summed from 2,000 laser shots from an Nd-YAG laser operating at 355 nm and 200 Hz. Internal calibration was performed using a minimum of three trypsin autolysis peaks. Database searches were performed using Applied Biosystems GPS Explorer v. 3.6 with Mascot v. 2.1. Spectra were subjected to seven-point Gaussian smoothing prior to peak picking. For the peptide mass fingerprinting search, a maximum of 65 peaks with a signal-to-noise ratio of 30 and a maximum peak density of 50 peaks per 200 Da were submitted. Data were searched against all *Lactobacillus* genome sequences in the NCBI database (www.ncbi.nlm.nih.gov/).

Cloning, expression, and purification of recombinant *L. jensenii* enolase. The sequences of the enolase (Eno) genes of *Lactobacillus gasseri* ATCC 33323, *Lactobacillus johnsonii* NC533, and all sequenced *L. jensenii* strains were compared using ClustalW2. Based on these sequences, primers were designed to amplify the putative enolase open reading frame (ORF) from *L. jensenii* ATCC 25258. The initial attempts to amplify the entire ORF were not successful. When conserved internal primers were used in combination with full-length primers, it

was found that while the 5′ end of the gene was highly conserved, the 3′ end was not highly conserved. To determine the sequence of the 3′ end, a primer specific for the gene located 3′ of the enolase gene in other lactobacilli (*secG*) was designed and used to PCR amplify the region. The DNA sequence of the product was determined and used to design a new primer for the 3′ end of the enolase gene, EnoRev, which included a NotI site. The 5′ primer, EnoFwd, contained an NdeI site which included the ATG start codon. The ORF was PCR amplified, purified using a PCR purification kit (Qiagen, Germantown, MD), and then digested with NotI and NdeI (New England Biolabs, Ipswich, MA). The insert was ligated into similarly digested pET24a (Novagen, EMD4 Biosciences, San Diego, CA) and transformed into *E. coli* DH5α. Transformants were identified, and the DNA sequence of the entire insert was determined. One isolate, pET24a-eno, was then transformed into *E. coli* BL21λDE3 for expression.

High-level production of His_6-tagged enolase (His_6-Eno) was performed using the method described by Studier (39). Briefly, 50 ml of an overnight culture of BL21λDE3(pET24a-eno) grown in LB medium containing 100 mg/liter kanamycin at 37°C was used to inoculate 2 liters of ZY-5052 autoinducing medium supplemented with 100 mg/liter kanamycin and incubated overnight at 30°C with shaking at 250 rpm. The bacteria were harvested by centrifugation and resuspended in 50 mM NaH_2PO_4 (pH 7.0), 300 mM NaCl. The cells were then lysed by shearing using an M-110P processor (Micro-fluidics Corp.) set at 20,000 lb/in^2. The insoluble material was removed by centrifugation, and the crude lysate was loaded onto an Ni-nitrilotriacetic acid (NTA) resin column (BD Biosciences) that was equilibrated with 50 mM NaH_2PO_4 (pH 7.0), 300 mM NaCl. The column was washed with 50 mM NaH_2PO_4 (pH 7.0), 300 mM NaCl, 10 mM imidazole, and then the bound protein was eluted using 50 mM NaH_2PO_4 (pH 7.0), 300 mM NaCl, 250 mM imidazole. The protein was purified further by anion-exchange chromatography as described above. Prior to use in cell culture assays, the protein was dialyzed against PBS, and the protein concentration was determined using the Bradford method. Purified protein was then applied to epithelial cells at various concentrations for 3 h before infection with *N. gonorrhoeae*.

Statistical analysis. All data were analyzed by using unpaired Student's *t* test. A *P* value of <0.05 was considered statistically significant.

RESULTS

***Lactobacillus* inhibition of gonococcal adherence to fixed epithelial cells.** In our experimental system, there are three types of cells that could be involved in the *Lactobacillus* inhibition of gonococcal adherence to Hec-1-B cells: the epithelial cells, the lactobacilli, and the gonococci. Since intestinal *Lactobacillus* strains have been shown to induce epithelial cells to become more resistant to pathogen adherence (15, 20, 23, 29), we utilized glutaraldehyde-fixed Hec-1-B cells to determine the role of the epithelial cells in the inhibitory interaction. *L. jensenii* (MOI, 100) was used to precolonize both glutaraldehyde-fixed and live epithelial cells for 1 h prior to gonococcal infection as described in Materials and Methods. The cells were then lifted with saponin and plated onto selective media (MRS medium for lactobacilli and GCV for gonococci). When *L. jensenii* was present, 8.9% ± 3.9% of the total gonococci present adhered to live epithelial cells, compared to 20.2% ± 8.0% of the gonococci in the absence of lactobacilli. This was a significant reduction in the adherence of gonococci ($P < 0.001$) (Fig. 1A). The inhibition was specific to lactobacilli, as pretreatment of the epithelial cells with *B. subtilis* (MOI, 100) had no effect on the frequency of adherence of gonococci compared to the control (90.7% ± 13.5% of the control) ($P = 0.117$).

Gonococci adhered at a 2-fold-lower frequency to fixed epithelial cells than to live epithelial cells. Nonetheless, the presence of *L. jensenii* reduced the level of gonococcal adherence on fixed cells from 11.2% ± 2.8% to 4.4% ± 2.0% ($P = 0.036$) (Fig. 1B). The level of inhibition of gonococcal adherence to fixed epithelial cells was not significantly different from the level of inhibition observed for live epithelial cells ($P = 0.433$), suggesting that *Lactobacillus*-mediated inhibition of gonococcal adherence to epithelial cells does not require the epithelial cells to be metabolically active.

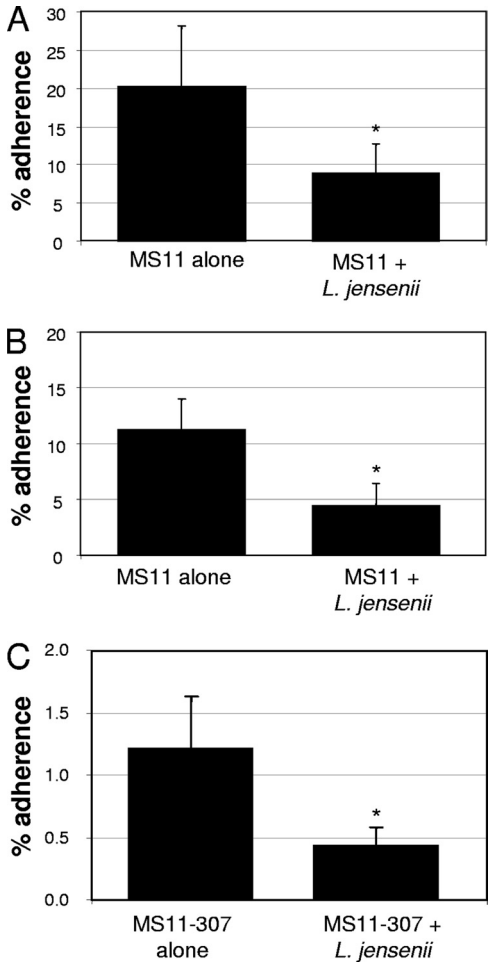

FIG. 1. Effect of *L. jensenii* on gonococcal adherence. (A) Adherence of *N. gonorrhoeae* to live Hec-1-B cells in the presence or absence of *L. jensenii*. The data are averages of the results of 20 independent experiments performed in triplicate. The error bars indicate standard errors. $P < 0.001$, as determined by Student's *t* test. (B) Adherence of *N. gonorrhoeae* to fixed Hec-1-B cells in the presence or absence of *L. jensenii*. The data are averages of the results of four independent experiments performed in triplicate. The error bars indicate standard errors. $P = 0.036$, as determined by Student's *t* test. (C) Adherence of nonpiliated (MS11-307) gonococci in the presence and absence of *L. jensenii*. The data are averages of the results of six independent experiments performed in triplicate. The error bars indicate standard errors. $P = 0.008$, as determined by Student's *t* test. An asterisk indicates that the *P* value is <0.05.

Pilus-mediated gonococcal adherence is not targeted by *L. jensenii*. The type IV pilus is the primary adhesin for gonococci and has been shown to be necessary for infection (18, 41). We hypothesized that lactobacilli inhibit the adherence of *N. gonorrhoeae* by specifically targeting pilus-mediated adherence. The effect of *L. jensenii* on the nonpiliated gonococcal strain MS11-307 was examined to test this hypothesis. Nonpiliated gonococci adhered to Hec-1-B cells at a frequency of 1.2% ± 0.4% (Fig. 1C), which is significantly lower than the value for the P^+

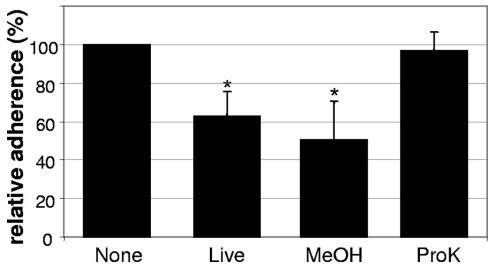

FIG. 2. Adherence of *N. gonorrhoeae* in the presence of live, MeOH-fixed, and ProK-treated *L. jensenii*. The relative adherence was determined by dividing the percentage of adherent gonococci in the presence of lactobacilli by the percentage of adherent gonococci in the corresponding control. None, no lactobacilli; Live, untreated *L. jensenii*; MeOH, methanol-treated *L. jensenii*; ProK, proteinase K-treated *L. jensenii.* The data are averages of the results of three or more independent experiments performed in triplicate. The error bars indicate standard errors. The *P* values determined by Student's *t* test were <0.001 (Live), 0.001 (MeOH), and 0.533 (ProK). An asterisk indicates that the *P* value is <0.05.

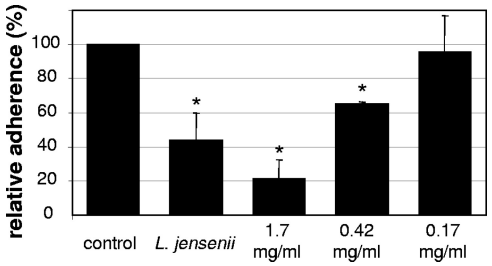

FIG. 3. RSC inhibition of gonococcal adherence is dose dependent. Hec-1-B cells were incubated with either 1.7 mg/ml, 0.42 mg/ml, or 0.17 mg/ml RSC for 3 h before the addition of gonococci. The levels of gonococcal adherence to epithelial cells were 43.9% ± 21.4% (*L. jensenii*) ($P = 0.01$), 21.5% ± 14.4% (1.7 mg/ml RSC) ($P = 0.001$), 65.1% ± 1.7% (0.42 mg/ml RSC) ($P = <0.001$), and 95.6% ± 27.9% (0.17 mg/ml RSC) ($P = 0.81$) of the untreated control value. The data are averages of the results of three independent experiments performed in triplicate. The error bars indicate standard errors. An asterisk indicates that the *P* value is <0.05.

gonococcal strain (20.2% ± 8.0%) (Fig. 1A). Again, when the epithelial cells were precolonized with *L. jensenii*, the frequency of adherence of P^- gonococci was reduced significantly, to 0.43% ± 0.13% ($P = 0.008$) (Fig. 1C). After the data were normalized using the appropriate controls, the frequencies of adherence of the *L. jensenii*-treated nonpiliated and piliated gonococcal strains were compared. No statistically significant difference was found ($P = 0.334$), which suggests that *L. jensenii* does not inhibit gonococcal adherence by specifically targeting type IV pilus-mediated adherence to epithelial cells.

Surface components of *L. jensenii* inhibit gonococcal adherence. Since we determined that the epithelial cells do not need to be metabolically active for *L. jensenii* to inhibit gonococcal adherence to host cells (Fig. 1B), we next focused on determining the components of lactobacilli that are involved in this inhibition by comparing the effects of treatment of the lactobacilli with MeOH and/or ProK prior to infection with gonococci. As shown in Fig. 2, MeOH-treated lactobacilli inhibited gonococcal adherence to epithelial cells to 50.2% ± 25.9% of the control ($P = 0.001$), which was similar to the level of inhibition observed with live lactobacilli (63.0% ± 14.9%) ($P < 0.001$). This result suggests that the inhibitory factor is a surface component of *L. jensenii*. Additionally, ProK treatment eliminated *Lactobacillus*-mediated inhibition of gonococcal adherence (96.9% ± 12.7%) ($P = 0.533$). Together with the MeOH results, this suggests that a constitutive protein on the exposed surface of *L. jensenii* is involved in the inhibition of gonococcal adherence to epithelial cells.

Released surface components (RSC) from *L. jensenii* inhibit gonococcal adherence to epithelial cells in a dose-dependent manner. *Lactobacillus* species have been shown to produce several compounds that can inhibit pathogen adherence, including hydrogen peroxide, bacteriocins, and biosurfactants (5, 27, 30, 47). A probiotic *Lactobacillus* species, *Lactobacillus helveticus*, has been shown to utilize constitutive surface layer proteins to inhibit *E. coli* O157:H7 adherence to epithelial cells (16). Since we previously reported that *L. jensenii* inhibits gonococcal adherence by a mechanism that does not involve secretion of inhibitory substances (38) and we have shown that surface proteins are involved in the inhibition of gonococcal adherence, we next determined if surface components released from *L. jensenii* could inhibit gonococcal adherence to epithelial cells. Using a method described by Reid et al. (31) to isolate biosurfactants from lactobacilli, we isolated surface components released from lactobacilli, and the biosurfactant activity of each preparation was tested by collapsed drop analysis. Every drop of the RSC collapsed on the oily surface, demonstrating that the RSC has biosurfactant activity, while the water controls remained beaded on the oily surface.

The RSC was then tested for inhibition of gonococcal adherence to epithelial cells by treating Hec-1-B cells for 3 h with various concentrations of RSC prior to infection with gonococci in an adherence assay. The results of this experiment showed that gonococcal adherence was reduced nearly 5-fold when the epithelial cells were treated with 1.7 mg/ml RSC (Fig. 3). When the epithelial cells were treated with 0.42 mg/ml RSC, the gonococcal adherence was inhibited less than 2-fold, and 0.17 mg/ml RSC had no effect on gonococcal adherence. The adherence of gonococci to the treated epithelial cells decreased as the amount of RSC increased, indicating that gonococcal adherence to epithelial cells is inhibited in a dose-dependent manner.

To rule out the possibility that the apparent reduction in gonococcal adherence to epithelial cells was caused by cell death or detachment caused by treatment with either RSC or bacteria, cell number and viability were assessed by trypan blue exclusion. There was no significant difference in the total number of nonviable cells (the cells that took up the trypan blue dye) after each treatment compared to Hec-1-B cells for fresh medium (85.7 ± 22.2 blue cells per well), RSC (78.0 ± 11.3 blue cells per well) ($P = 0.704$), lactobacilli (99.0 ± 16.0 blue cells per well) ($P = 0.552$), gonococci (118.3 ± 35.1 blue cells per well) ($P = 0.368$), lactobacilli plus gonococci (73.7 ± 6.9 blue cells per well) ($P = 0.535$), or RSC plus gonococci (89.3 ± 12.2 blue cells per well) ($P = 0.857$). Therefore, the effect on gonococcal adherence was not due cell death. When the numbers of live cells present in the cell monolayer (the cells that excluded the trypan blue dye) were compared after the treat-

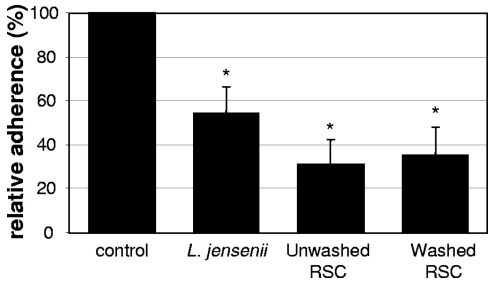

FIG. 4. Washing of RSC-treated cells prior to inoculation with gonococci does not remove the inhibitory activity. RSC was incubated with Hec-1-B cells for 3 h prior to infection with gonococci. The levels of gonococcal adherence to epithelial cells were 53.3% ± 17.2% (*L. jensenii*) ($P = 0.01$), 31.2% ± 15.0% (unwashed RSC) ($P = 0.001$), and 34.9% ± 18.1% (washed RSC) ($P = 0.003$) of the control value. The data are averages of the results of three independent experiments performed in triplicate. The error bars indicate standard errors. The P value was 0.729 when the washed RSC sample was compared to the unwashed sample, as determined by Student's t test. An asterisk indicates that the P value is <0.05.

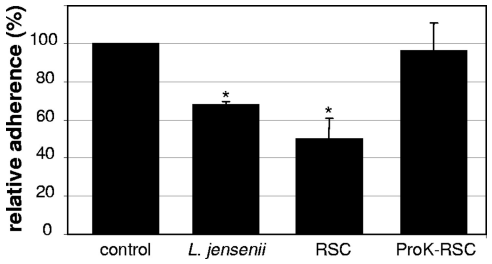

FIG. 5. Proteinase K treatment of RSC eliminates the inhibitory effect on *N. gonorrhoeae* adherence to Hec-1-B cells. RSC was incubated for 2 h at 37°C with proteinase K at 20 mg/ml. The levels of gonococcal adherence to epithelial cells were 68.2% ± 2.0% (*L. jensenii*) ($P < 0.001$), 50.1% ± 14.6% (RSC) ($P = 0.004$), and 96.3% ± 18.9% (ProK-RSC) ($P = 0.755$) of the control value. The data are averages of the results of three independent experiments performed in triplicate. The error bars indicate standard errors. An asterisk indicates that the P value is <0.05.

ments, there was again no significant difference in cell counts compared to Hec-1-B cells for fresh medium ($7.83 \times 10^4 \pm 2.89 \times 10^4$ cells/well), *N. gonorrhoeae* ($9.0 \times 10^4 \pm 4.0 \times 10^4$ cells/well) ($P = 0.779$), *L. jensenii* ($8.67 \times 10^4 \pm 1.89 \times 10^4$ cells/well) ($P = 0.786$), RSC ($9.67 \times 10^4 \pm 1.44 \times 10^4$ cells/well) ($P = 0.533$), lactobacilli plus gonococci ($8.67 \times 10^4 \pm 1.89 \times 10^4$ cells/well) ($P = 0.786$), or RSC plus gonococci ($9.33 \times 10^4 \pm 8.89 \times 10^4$ cells/well) ($P = 0.589$). This suggests that any effects on the gonococcal adherence observed was due to an effect on gonococcal adherence to the epithelial cells, not to an effect on the adherence of the epithelial cells to the cell culture plate.

Inhibition of gonococcal adherence to epithelial cells remains after removal of RSC. The RSC contains biosurfactant activity, which by definition indicates that surface-active molecules are present (32). Therefore, we hypothesized that a surface component of *L. jensenii* in the RSC inhibits gonococcal adherence by interacting with the surface of the epithelium. To test whether a component of the RSC interacts with the epithelial cell surface to inhibit gonococcal adherence, RSC at a concentration of 1.7 mg/ml was incubated with Hec-1-B cells and then either washed away before infection with gonococci or left in the well during infection. The relative adherence frequencies were then compared. There was no significant difference in the inhibition of gonococcal adherence when the RSC was removed (Fig. 4) ($P = 0.729$). This suggests that the inhibitory component(s) of the RSC remained associated with the epithelial cells.

Characterization of the inhibitory components of the RSC. To determine if the inhibitory component(s) of the RSC was a protein, an aliquot of RSC (1.7 mg/ml) was treated with ProK and incubated at 37°C for 2 h. A second aliquot was incubated at 37°C for 2 h as a negative control. Both aliquots were heat treated to inactivate the protease and centrifuged to remove any debris. The resulting samples were then used to pretreat Hec-1-B cells for 3 h before infection with gonococci (MOI, 10). At 3 h after gonococcal infection, the cells were lifted and plated to quantify the adherence. Compared to the control, the gonococcal adherence to RSC-treated cells was reduced 2-fold ($P = 0.004$) (Fig. 5). However, when the epithelial cells were pretreated with ProK-treated RSC the gonococcal adherence frequency was similar to the adherence frequency of the untreated control ($P = 0.755$). These results suggest that the inhibitory activity of the RSC is due to the presence of a protein(s).

Purification of the RSC proteins. We next fractionated the inhibitory protein components of the RSC by anion-exchange chromatography. A column was eluted with a 0 to 1 M NaCl gradient, and the proteins were detected by using A_{260}. A representative elution profile is shown in Fig. 6A. Peak 1 (P1) eluted at 20 mM NaCl, and peak 2 (P2) eluted at 1 M NaCl. SDS-PAGE analysis of samples from P1 and P2 showed there were several proteins in each pool (Fig. 6B). The most abundant protein in P1 and the most abundant protein in P2, indicated in Fig. 6B, were excised and analyzed by tandem mass spectrometry. The protein from P1 had two peptides with observed molecular masses of >1,000 kDa that exhibited 100% sequence identity to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *L. johnsonii* NCC 533. The protein from P2 had eight peptides with observed molecular masses of >1,000 kDa that exhibited 100% sequence identity to enolase from *L. johnsonii* NCC 533 and *L. gasseri* ATCC 33323. P1 and P2 were dialyzed into PBS and then assayed for inhibitory activity. P1 (0.42 mg/ml) inhibited gonococcal adherence to epithelial cells 2-fold. Similarly, P2 (0.42 mg/ml) inhibited gonococcal adherence 2-fold (Fig. 6C). This suggests that there are at least two separable inhibitory components in the RSC.

To further separate the proteins in P2, the pool of proteins was dialyzed against 50 mM Tris (pH 7.6), concentrated by ultrafiltration, and then separated by gel filtration size exclusion chromatography (Sephacryl S-200HR). Samples from fractions containing proteins were visualized by SDS-PAGE and assayed for biosurfactant activity. Fraction 49 was the only fraction that contained biosurfactant activity, and when visualized by SDS-PAGE, a sample produced a single protein band. However, when it was assayed for inhibition of gonococcal adherence, this sample did not contain inhibitory activity. This suggests that the biosurfactant activity of the RSC is distinct and can be separated from the inhibitory activity.

Fraction 36, another fraction that produced a single protein

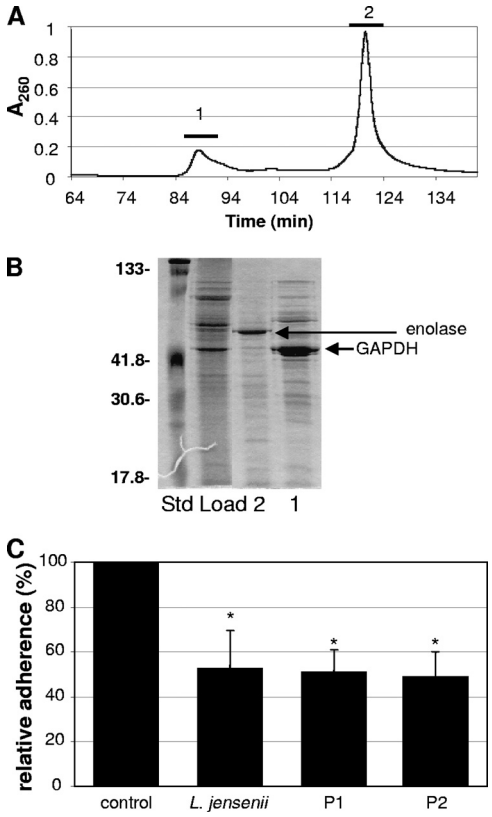

FIG. 6. Fractionation of RSC by anion-exchange chromatography. (A) Elution profile of samples detected by UV absorbance (λ = 260 nm) as the protein eluted from the column. Peaks 1 and 2 are labeled. (B) SDS-PAGE analysis of P1(lane 1) and P2 (lane 2). The long arrow indicates the band with similarity to the enzyme enolase, and the short arrow indicates the band with similarity to the enzyme GAPDH, as determined by MS/MS. Lane Std contained a molecular mass standard. (C) Gonococcal adherence to epithelial cells pretreated with P1 and P2. Treatment of epithelial cells inhibited gonococcal adherence, and the levels of adherence were 52.9% ± 23.9% (*L. jensenii*) ($P = 0.027$), 51.2% ± 14.0% (P1) ($P = 0.004$), and 48.8% ± 14.8% (P2) ($P = 0.004$) of the control value. The data are the averages of the results of three independent experiments performed in duplicate. The error bars indicate standard errors. An asterisk indicates that the P value is <0.05.

band, was tested for inhibitory activity. This fraction also did not inhibit gonococcal adherence to epithelial cells. Two other fractions that were assayed for inhibition of gonococcal adherence were fractions 24 and 29. These fractions were chosen because when they were visualized by SDS-PAGE, they both appeared to contain the putative enolase shown in Fig. 6B. Fraction 24 contained the putative enolase along with an additional 65.8-kDa protein, and it reduced the gonococcal adherence to epithelial cells to 26.5% of the value for the no-RSC control. Fraction 29 contained the putative enolase, the 65.8-kDa protein of fraction 24, and an additional 30.6-kDa protein. This fraction reduced the gonococcal adherence to 40.4% of the value for the no-RSC control. The level of inhibition appeared to correlate with the amount of the putative enolase in the fraction as determined by SDS-PAGE, and fraction 24 contained more of the putative enolase than fraction 29 contained. The band correlated with the putative enolase was excised from an SDS-PAGE gel and was verified by tandem

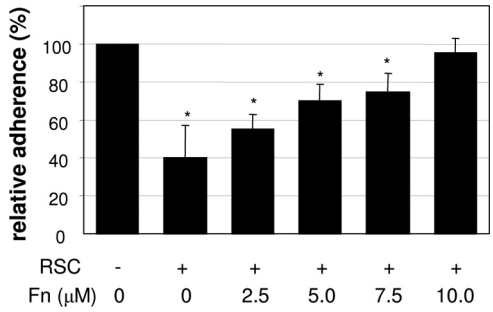

FIG. 7. Gonococcal adherence to cells treated with RSC that had been pretreated with different amounts of soluble fibronectin. Gonococcal adherence frequencies are expressed as values relative to the value for a no-RSC–no-Fn control, and Student's t tests were used to compare each Fn treatment with the no-Fn treatment. The levels of adherence to epithelial cells were 40.4% ± 17.0% (0.42 mg/ml RSC), 54.9% ± 8.0% (RSC and 2.5 μM Fn) ($P = 0.374$), 70.1% ± 8.7% (RSC and 5.0 μM Fn) ($P = 0.121$), 74.6% ± 9.9% (RSC and 7.5 μM Fn) ($P = 0.091$), and 95.3% ± 7.5% (RSC and 10 μM Fn) ($P = 0.019$) of the control value. The data are averages of the results of three independent experiments performed in duplicate. The error bars indicate standard errors. An asterisk indicates that the P value is <0.05.

mass spectrometry to have homology to enolase (seven peptides with m/z of >1,000 and 100% sequence identity to an enolase from *L. gasseri* ATCC 33323).

Soluble fibronectin blocks inhibitory activity of the RSC. Gonococci are known to bind to fibronectin (Fn) during invasion of epithelial cells (43). Since enolases of other *Lactobacillus* species have been shown to bind Fn (3, 10, 17) and a putative enolase is the main component of one of the inhibitory fractions of the RSC, we hypothesized that the RSC might reduce gonococcal adherence to epithelial cells by blocking gonococcal binding to extracellular matrix components, such as fibronectin. To test this hypothesis, RSC was treated with different amounts of soluble Fn prior to addition to epithelial cells. After 3 h, the RSC-Fn solution was removed, and the cells were infected with *N. gonorrhoeae*. When no Fn was added, 0.42 mg/ml RSC reduced the level of gonococcal adherence to 40.4% ± 17.0% of the control. When the RSC was treated with 2.5, 5, and 7.5 μM Fn, the level of gonococcal adherence was reduced, but as the amount of Fn increased, the inhibitory activity of the RSC was reduced (54.9% ± 8.0%, 70.1% ± 8.7%, and 74.6% ± 9.9% of the control, respectively) (Fig. 7). When the RSC was treated with 10 μM soluble Fn, the level of gonococcal adherence was 95.3% ± 7.5% of the control level, which was essentially no different than the level of gonococcal adherence in the absence of RSC. This is consistent with the hypothesis that the mechanism of RSC inhibition of gonococcal adherence is inhibition of gonococcal interactions with Fn on the cells.

Effect of His_6-Eno on gonococcal adherence to Hec-1-B cells. One of the two proteins of the RSC that copurified with the inhibitory activity had significant similarity to an enolase from *L. gasseri.* To determine if this protein is the inhibitory component, the enolase gene from *L. jensenii* ATCC 25258 was recombinantly expressed as a His_6-tagged protein in *E. coli* and purified by chromatography (Fig. 8A). The purified protein was then used at various concentrations to treat epithelial cells prior to gonococcal infection. His_6-Eno inhibited gonococcal

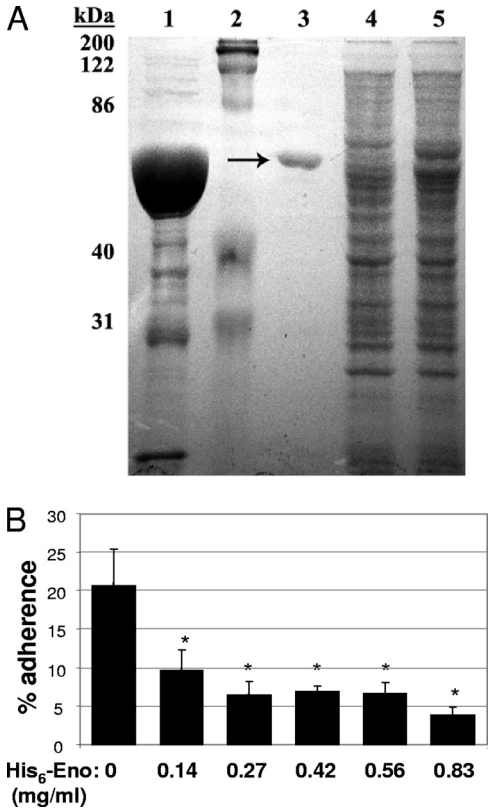

FIG. 8. Purification of His_6-Eno and effect of His_6-Eno on gonococcal adherence to Hec-1-B cells. (A) SDS-PAGE gel analysis of His_6-Eno. Lane 1, 18 μg purified His_6-Eno; lane 2, molecular mass standards; lane 3, 0.72 μg purified His_6-Eno; lane 4, uninduced *E. coli* BL21λDE3(pET24a-eno); lane 5, induced *E. coli* BL21λDE3(pET24a-eno). The arrow indicates the position of His_6-Eno. (B) Gonococcal adherence to cells pretreated with His_6-Eno. The levels of gonococcal adherence were as follows: in the absence of His_6-Eno, 20.1% ± 4.0%; in the presence of 0.14 mg/ml His_6-Eno, 10.4% ± 2.8% ($P = 0.028$); in the presence of 0.27 mg/ml His_6-Eno, 6.5% ± 1.8 ($P = 0.013$); in the presence of 0.42 mg/ml His_6-Eno, 6.9% ± 0.7% ($P = 0.004$); in the presence of 0.56 mg/ml His_6-Eno, 6.7% ± 1.4% ($P = 0.013$); and in the presence of 0.83 mg/ml His_6-Eno, 3.8% ± 1.0% ($P = 0.006$). The data are averages of the results of three independent experiments performed in duplicate. The error bars indicate standard errors. An asterisk indicates that the P value is <0.05.

adherence to epithelial cells at all concentrations tested by 50 to 80% (Fig. 8B). As the concentration of His_6-Eno increased, the adherence of gonococci to epithelial cells decreased, again indicating that there was a dose-dependent response.

DISCUSSION

Adherence is a critical first step in the infection of a new host for many pathogens, including *N. gonorrhoeae.* As incoming pathogens, gonococci have to overcome the host's defenses and compete with the indigenous microbiota to effectively colonize the endocervical epithelia. *L. jensenii*, one of the bacterial species most commonly isolated from the healthy human vaginal tract, has been shown to inhibit gonococcal adherence to and invasion of epithelial cells using a coculture model of infection (38). The inhibition of gonococcal adherence was not caused by any secreted factor, such as hydrogen peroxide, by coaggregation of the gonococci and the lactobacilli, or by competition for receptors. The ability to inhibit gonococcal adherence without direct killing of the pathogen might be specific to *Lactobacillus* species, as we found no inhibition of adherence when cells were pretreated with another Gram-positive bacterium, *B. subtilis*. This finding correlates with research which showed that vaginal *Lactobacillus* isolates inhibit gonococcal adherence to the ME180 cell line better than intestinal isolates (46), suggesting that the inhibitory mechanism may have developed due to competition in the vaginal ecosystem. In this work, we examined the components of lactobacilli, gonococci, and the epithelial cells involved in an effort to elucidate the mechanism(s) of this inhibition.

In our *in vitro* model of gonococcal infection, precolonization of glutaraldehyde-fixed epithelial cells with *L. jensenii* reduced gonococcal adherence 2-fold (Fig. 1B), suggesting that *L. jensenii* inhibits gonococcal adherence by a mechanism that does not require the epithelial cells to be metabolically active. Since lactobacilli inhibit gonococcal adherence to epithelial cells by a mechanism that does not require a response from the epithelial cells, this suggests that a component of *L. jensenii* is important for the interaction. Our results show that methanol-fixed *L. jensenii* and live lactobacilli inhibited gonococcal adherence to epithelial cells to similar extents (Fig. 2). Therefore, it is likely that the components of lactobacilli necessary to inhibit gonococcal adherence to epithelial cells are surface associated. Treatment of methanol-fixed *L. jensenii* with proteinase K eliminated the inhibition, indicating that the inhibitory factor is a protein or has a protein component.

N. gonorrhoeae utilizes a type IV pilus for adherence to epithelial cells in culture (24, 40), and this adhesin has been shown to be essential for gonococci to colonize human volunteers (18, 41). Therefore, pilus-mediated adherence is a likely target for the observed *Lactobacillus* inhibition. However, when the effect of *L. jensenii* precolonization of epithelial cells on the adherence of piliated gonococci and nonpiliated gonococci was examined, piliated gonococci and nonpiliated gonococci were inhibited to similar extents, suggesting that the mechanism of inhibition is not specific to the main gonococcal adhesin, type IV pili, but is nonspecific.

One mechanism that could explain this global inhibition of gonococcal adherence is the production of a biosurfactant by the lactobacilli. Biosurfactants are amphipathic molecules produced by microorganisms that have a variety of purposes, including adsorption to surfaces (32). It is possible that *L. jensenii* produces a biosurfactant that adheres to the epithelial cell surface, which is left behind when the bacterium desorbs. The molecule left on the epithelial cell surface could change the nature of the surface such that it is less able to support gonococcal adherence. Over 15 species of lactobacilli have been shown to produce biosurfactants (31). One of the best-characterized *Lactobacillus*-produced biosurfactants is produced by *Lactobacillus reuteri* RC-14, and this biosurfactant was found to be effective for preventing the adherence of *Enterococcus faecalis* to hydrophilic glass and silicone rubber surfaces (14, 44, 45). The biosurfactant from *L. reuteri* RC-14 was also found to inhibit abscess formation by *Staphylococcus aureus* in a surgical implant model in rats, demonstrating that biosurfactants can inhibit pathogen adherence to biotic surfaces as well as abiotic surfaces (13). Furthermore, a surface-associated protein was

purified from this biosurfactant and identified as the inhibitory component (14). This protein was identified as a collagen-binding protein; however, it was not determined if this protein alone had biosurfactant activity.

Using a method to isolate biosurfactants, *L. jensenii* was induced to release surface proteins. The released surface component (RSC) mixture had surfactant activity, as demonstrated by collapsed drop analysis, and it also inhibited gonococcal adherence in a dose-dependent manner (Fig. 3). However, when the RSC was fractionated by column chromatography, the inhibitory activity was separated from the biosurfactant activity. A cell-free protein extract from *L. helveticus*, which did not have biosurfactant activity, was also found to inhibit pathogen adherence to epithelial cells (16). This preparation was an extract of surface layer proteins. Surface layer proteins form a paracrystalline layer associated with the outer surface of some bacteria, and they play a role in adhesion to surfaces (16). However, we do not consider surface layer proteins a potential inhibitory factor, since *L. jensenii* does not produce surface layer proteins (28).

In the RSC there are at least two inhibitory components, since two distinct inhibitory fractions were isolated from the RSC following anion-exchange chromatography. The first fraction contains several proteins, as shown by SDS-PAGE (Fig. 6B), and the major band was identified by MS/MS as a putative glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The second fraction was analyzed further, and an inhibitory protein was identified as a putative enolase by MS/MS. We subsequently cloned the enolase gene from *L. jensenii*, purified the recombinant His_6-tagged protein, and tested it to determine its ability to inhibit gonococcal adherence to epithelial cells. Since His_6-Eno was able to inhibit the adherence of *N. gonorrhoeae* (Fig. 8B), we concluded that the putative enolase was one of the inhibitory factors present in the RSC.

While both GAPDH and enolase are normally thought of as cytosolic enzymes involved in glycolysis, both of these enzymes have been found to be surface associated in several *Lactobacillus* species (4, 10, 21). Furthermore, lactobacillus enolase has been shown to bind to extracellular matrix components, such as fibronectin, collagen, and laminin (3, 17, 33). Our data show that preincubation of the RSC with soluble fibronectin before use in gonococcal adherence assays eliminated the inhibitory effect of the RSC in a dose-dependent manner (Fig. 7), which is consistent with the hypothesis that *L. jensenii* inhibits gonococcal interactions with epithelial cells by occluding fibronectin binding sites with its enolase. In the vaginal tract, fibronectin is secreted and coats the mucosal surface (12), indicating that the hypothesis that a surface component of *L. jensenii* binds to fibronectin to prevent gonococcal adherence is biologically relevant. Additional experiments are necessary to determine if the putative enolase is in fact the fibronectin binding protein and whether the enzymatic activity is necessary for the inhibition.

While a putative GAPDH was identified as the most abundant protein in P1 from the RSC (Fig. 6C), it still has not been identified as the inhibitory component in this fraction. Since GAPDH from other *Lactobacillus* species is known to bind colonic mucin and fibronectin (33), this protein is a candidate for an inhibitor of gonococcal adherence to epithelial cells; however, this hypothesis remains to be tested.

In summary, we showed that *L. jensenii* inhibits gonococcal adherence to epithelial cells independent of an epithelial cell response, utilizing at least one surface-associated protein. When removed from the surface of lactobacilli, the protein(s) can still inhibit gonococcal adherence to epithelial cells; however, inhibition is eliminated by treatment with fibronectin, suggesting that the inhibitory protein(s) blocks gonococcal binding to this extracellular matrix component. Now, with gonococci having achieved "superbug" status, new methods of treatment and prevention that do not rely solely on antibiotics must be developed. The potential use of *Lactobacillus* products for prevention of gonorrhea is an exciting possibility that needs to be explored further.

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AUTHOR'S CORRECTION

Lactobacillus gasseri Surface-Associated Proteins Inhibit *Neisseria gonorrhoeae* Adherence to Epithelial Cells

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In the course of recent studies we have determined that what our laboratory has reported as *Lactobacillus jensenii* 25258 is in fact *L. gasseri* 33323. This was determined by sequencing the 16S rDNA from all of the *Lactobacillus* strains in our collection, including all derivatives of the original strain reported in this manuscript. The fact that our strain is actually *L. gasseri* 33323 does not change any of the conclusions in the paper. While the experiments were done with what we thought was *L. jensenii* 25258, no reports of similar studies have been done with *L. gasseri*, at least not to our knowledge. Future experiments by our group will refer to this organism as *L. gasseri* 33323. We sincerely apologize to all our colleagues for any difficulties or confusion arising from this unfortunate error.

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