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Inhibition of Adhesion of *Escherichia coli* K88 to Piglet Ileal Mucus by *Lactobacillus* spp.

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Enteropathogenic *Escherichia coli* K88 colonizing the piglet ileum adhere to the mucosa by K88 fimbrial appendages. A recent study in our laboratory has implicated indigenous lactobacilli in the suppression of the colonization potential of enteropathogenic *E. coli* as measured by adhesion to ileal mucus. The aim of this study was to investigate the effect of *Lactobacillus* spp. of porcine origin on the adhesion of K88 fimbriae of *E. coli*. With an in vitro assay, the adhesion of *E. coli* K88ab strain G1108E and *E. coli* K88ac strain 1107 to 35-day-old piglet ileal mucus was studied in the presence of spent culture fluid of *Lactobacillus* spp. Detailed studies focused specifically on culture fluid of *Lactobacillus fermentum* 104R. Subsequently, the ileal mucus was exposed to the retentate of the spent culture fluid after dialysis and fractionation. Adhesion was confirmed to be attributable to K88 fimbriae when K88-specific monoclonal antibodies and isogenic mutants of *E. coli* K-12 with and without the plasmid containing the K88 gene were used. The active component was characterized by pretreatment of dialysis retentate with heat, periodate, pronase, and centrifugation, as well as by growth of the lactobacillus in various media and by assays at both 0 and 37°C. All three lactobacilli of porcine origin reduced adhesion of *E. coli* K88 by approximately 50%. Inhibition occurred when mucus was pretreated with either spent culture dialysis retentate or the void volume (fraction of >250,000 molecular weight) after gel filtration. The activity of the dialysis retentate was sensitive to pronase, but there was still activity at 0°C. It was concluded that *L. fermentum* 104R produces a proteinaceous component detectable in spent culture fluid during growth in both complex and defined media; this component inhibits the adhesion of K88ab and K88ac fimbriae to ileal mucus by interacting with mucus components.

Among the major causes of postweaning diarrhea in piglets are the enterotoxigenic *Escherichia coli* strains which bear the K88 fimbrial appendages. It has been shown that there is a correlation between the incidence of diarrhea and the presence of two plasmids, one encoding the genes for K88 fimbriae on the bacterial surface and a second plasmid encoding the genes for toxin production (21). Subsequently, it has been demonstrated that these K88 fimbriae mediate adhesion of the bacterial cell to the brush borders of the epithelial cells lining the small intestine (10). In that review (10), it is proposed that the adhesion step is a prerequisite for successful colonization of the tract by the pathogen. Consistent with this concept, it was shown that there exist resistant and sensitive pig phenotypes and that these either lack or carry, respectively, the epithelial cell surface receptor for the K88 fimbriae (20). More recently, K88-specific receptors have also been demonstrated in the mucus layer overlying piglet small intestinal epithelial cells (2, 3, 18, 23).

With the present awareness of the problems associated with the excessive use of antibiotics and the lack of success with K88-specific vaccines for the postweaned piglet, alternatives for preventing K88-induced diarrhea are required. Recently, there has been renewed interest in the age-old concept of using bacterial preparations with inhibitory activity against the enteric pathogens for protecting the piglet (14). This concept is often referred to as bacterial interference and has been applied to a range of ecosystems in addition to the gastrointestinal tract, e.g., the nasopharynx and the urinary tract (19, 22). Protection could occur because the lactic acid bacteria produce metabolites antagonistic to the growth of the pathogen. These metabolites can be low- or high-molecular-weight compounds, the latter being referred to as bacteriocins when they are proteinaceous and plasmid encoded. Several bacteriocins of lactic acid bacteria have been reported (9, 15). In addition to growth inhibition, the concept of competitive exclusion of pathogenic *E. coli* by lactobacilli has been studied for the intestine (5) and the urinary tract (4). In these in vitro studies, colonization of the epithelium with the lactobacilli sterically hindered the adhesion of *E. coli* to the surface.

Recently, we showed a correlation between the presence of indigenous lactobacilli on the surface of the squamous gastric epithelium in vivo and the extent to which *E. coli* K88 could adhere in vitro to ileal mucus from the pig (2). *E. coli* K88 adhered more poorly to ileal mucus collected from piglets in which the gastric tissue was densely colonized by lactobacilli compared with mucus from piglets with sparsely colonized gastric tissue. The question was therefore raised as to whether the presence of lactobacillus metabolites influenced the adhesion of *E. coli* K88 in the gastrointestinal ecosystem. The aim of the work presented here was to establish in vitro whether porcine lactobacillus-produced metabolites will interfere with the mechanism of adhesion of K88 fimbria-bearing cells.

MATERIALS AND METHODS

Bacteria and cultural conditions. *Lactobacillus crispatus* 152 (2) and *Lactobacillus fermentum* 104R and 104S (12) were isolated from porcine gastric squamous epithelium; *Lactobacillus murinus* C39 (11) was isolated from piglet feces. *L. fermentum* 737 was isolated from rodent gastric squamous epithelium (6). Primary cultures (from stocks

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stored at −70°C in 20% glycerol) and subcultures were grown overnight in broth (Mann, Rogosa and Sharpe; Oxoid), using a 10% inoculum, 37°C, and a candle jar. Cultures were similarly grown in brain heart infusion broth (BHI; Oxoid), lactobacillus defined medium (LDM) prepared by the Conway and Kjelleberg (7) modification of the medium published by Kotarski and Savage (16), or lactobacillus basal medium (LBM: yeast extract, 20 g; tryptone, 0.5 g; sodium acetate, 1.0 g; $Na_2HPO_4 \cdot 2H_2O$, 0.2 g; K_2HPO_4, 0.7 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $MnSO_4 \cdot H_2O$, 0.06 g; $CaCl_2$, 0.05 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; $CaCl_2 \cdot 2H_2O$, 0.06 g; glucose, 30 g; Milli-Q water, 1 liter, purified by Milli-Q plus [Millipore, Corp.]). Spent culture fluid was collected by centrifuging overnight cultures at 8,000 × *g* for 15 min and dialyzing (maximum, 50-ml aliquots) three times at 4°C against 5 liters of Milli-Q water for approximately 6 h. When specified, the dialysis was carried out with phosphate-buffered saline (PBS; 0.01 M, pH 7.2). Spent culture fluids and retentates were stored at −70 or −20°C prior to use. As controls, uninoculated media were treated as for the spent culture fluid.

Primary cultures of *E. coli* K88ac strain 1107 (kindly supplied by O. Söderlind; as described in references 2 and 3), *E. coli* K88ab strain G1108E (kindly supplied by C. Deneke, Tufts University, Boston, Mass), and *E. coli* K-12 and K-12(K88ab) (kindly supplied by P. Cohen, University of Rhode Island, Kingston) were grown overnight in tryptone soy broth (Oxoid) at 37°C (1% inoculum) from stocks stored at −70°C in 20% glycerol. *E. coli* K-12 and *E. coli* K-12 (K88) were isogenic strains, with the latter carrying the plasmid containing the gene encoding for K88 fimbriae as described in reference 8. By using a 5% inoculum from these primary cultures, *E. coli* cells were radioactively labelled by growing in 10 ml of tryptone soy broth containing 5 mCi of [*methyl*-1,2-^{3}H]thymidine (117 Ci · mmol^{-1}; Amersham International) · ml^{-1} at 37°C to an A_{600} of 0.5 (approximately 2 h). Cells were harvested by centrifugation (approximately 3,000 × *g*), washed in PBS, and resuspended in PBS to the original volume prior to use in the adhesion assay. In addition, washed cells were also resuspended to the original volume in spent culture fluid dialysis retentate instead of PBS. These cells were used for studying the effect on adhesion of the presence of, rather than mucus pretreatment with, this retentate.

In vitro adhesion assay. The adhesion of radioactively labelled *E. coli* cells to ileal mucus from a 35-day-old pig was studied by using a modification of the method of Laux et al. (17) as outlined in Conway et al. (8). Basically, mucus was collected in HEPES (*N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid)-Hanks buffer from the ileum of a 35-day-old piglet and stored at −20°C. It was immobilized (0.5 mg of protein · ml^{-1}) on polystyrene microtiter wells (Nunc) by overnight incubation at 4°C. Excess mucus was removed by washing twice with HEPES-Hanks buffer. Spent culture fluid dialysis retentates or fractions (0.25 ml per well) were added to the immobilized mucus, and the microtiter trays were then incubated at 37°C for 30 min. Wells similarly treated with uninoculated media served as controls. The mucus-coated wells were then washed twice with HEPES-Hanks buffer, and the adhesion of the radioactively labelled cells was studied as described previously (8). Adhesion was quantified as the amount of radioactivity remaining in the well, and the amount of inhibition was assessed as the percentage in the test relative to the uninoculated medium control. All assays were performed in triplicate, and the number of separate experiments are detailed in Results. The amount of adhesion attributable specifically to K88 fimbriae was determined by using the isogenic mutants of *E. coli* K-12 with and without the K88 plasmid containing the gene encoding for the K88 fimbriae. In addition, adhesion was studied in the presence of a monoclonal antibody (PAB 10) confirmed previously by immunoblotting to be specific for K88 fimbriae (2).

Fractionation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Only spent culture fluid from the growth of *L. fermentum* 104R in LDM and BHI was fractionated. Spent culture fluid retentates, after dialysis against Milli-Q water, were fractionated by membrane filtration at 4°C, using membranes (Millipore) with molecular weights cutoffs of 10,000 and 30,000 and thus yielding fractions with molecular weights of <10,000, 10,000 to 30,000, and >30,000. Fractions were stored at −70°C.

Spent culture fluid of strain 104R after growth in LDM broth was also fractionated by gel filtration. Initially, 50 ml of spent culture fluid was concentrated 25 times by pressure dialysis, using a 10K-cutoff membrane (Millipore). This concentrate was applied to an XK 26/40 column packed with Sephadex 200 SF (Pharmacia) and eluted with PBS at a flow rate of 1.1 ml cm^{-2} h^{-1}. The fractionation was carried out at 5°C, and the eluate was collected as 2-ml fractions. The optical density (280 nm) of the fractions was recorded prior to storage at −20°C. Fractions 28, 34, and 42, which corresponded to relative molecular weights (M_r) of >250,000, 250,000, and 150,000, respectively, were analyzed by SDS-PAGE after being concentrated five times by centrifugal ultrafiltration at 5,000 × *g* for 1 h at 4°C, using tubes with a 10K-cutoff membrane (Filtron). The concentrated material was solubilized and separated by SDS-PAGE (1).

Characterization of the adhesion-inhibiting component. Before the spent culture fluid or control medium was added to the immobilized mucus to test for the presence of the adhesion-inhibiting component, spent culture fluid from the growth of strain 104R in LDM broth and uninoculated LDM broth were pretreated with pronase, heat, or periodate. The pronase treatment involved the addition of 100 μg of pronase (Calbiochem) ml^{-1} and then incubation at 40°C for 15 min, after which the material was transferred to ice. The adhesion inhibition assay was then carried out on ice instead of at 37°C (referred to as 0°C). Spent culture fluid and media were also heat treated at 80°C for 15 min and then centrifuged at about 13,000 × *g* for 5 min prior to being tested in the adhesion assay. For periodate treatment, $NaIO_4$ or $NaIO_3$ as the control (both at 10 mg ml^{-1}) was added to the supernatants, which were then incubated at room temperature for 30 min, after which 20 μl of ethylene glycol ml^{-1} was added. The mixture was dialyzed overnight and then tested in the adhesion inhibition assay.

Control experiments. To ensure that the spent culture fluid was not releasing mucus components from the surface, mucus was radioactively labelled and the amount of label was quantified before and after treatment with the spent culture fluid. The mucus was labelled by reductive methylation, using a modification of the method of Jentoft and Dearborn (13). This involved suspending mucus in 3 ml of labelling solution (2 mM ^{3}H-formaldehyde, 20 mM NaC NBH_3, and 100 mM HEPES-Hanks buffer) to yield a protein concentration of 1 mg ml^{-1} and incubating at 22°C for 2 h. The labelling was stopped by centrifugal ultrafiltration as outlined above. The retentate was washed three times in HEPES-Hanks buffer (3 ml) by repeated ultrafiltration. Labelled mucus was stored at −20°C and then used in the adhesion assay as described above.

TABLE 1. Effect of spent culture dialysis retentate of *L. murinus* C39 and *L. fermentum* 104R and 104S grown in LDM broth on adhesion of *E. coli* strains to piglet ileal mucus after pretreatment of the immobilized mucus with retentate

E. coli strain	Fimbriae	Adhesion index (%)[a]		
		104R	104S	C39
1107	K88ac	53 ± 7 (7)	52 ± 5 (3)	61 ± 14 (3)
G1108E	K88ab	35 ± 8 (2)	NT[b]	NT
K-12 (K88)	K88ab	45 ± 6 (2)	NT	NT

[a] Results are expressed as the mean ± standard deviation. The number in parentheses is the number of experiments, each of which was performed in triplicate. The adhesion index is expressed as the adhesion of *E. coli* to the test mucus relative to the control mucus.

[b] NT, not tested.

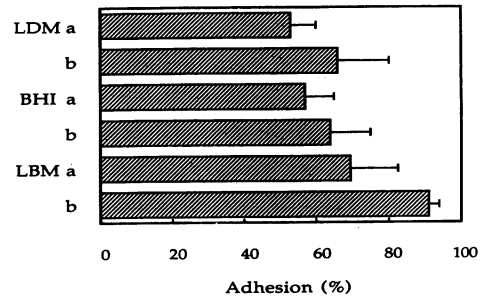

FIG. 1. Adhesion of *E. coli* K88ac strain 1107 to piglet ileal mucus pretreated with spent culture dialysis retentate after growth of *L. fermentum* 104R in LDM, BHI, or LBM broth. Samples were dialyzed against (a) deionized and filtered water or (b) PBS. Results are expressed as the percent adhesion relative to the uninoculated medium control (mean ± standard deviation; n = 7 for LDM medium and n = 2 for BHI and LBM). Each experiment was done in triplicate.

Spent culture fluid from the growth of strain 104R in LDM was centrifuged at 27,000 × g for 1.5 h, and the resultant supernatant was tested for adhesion inhibitory activity to determine whether the active component contained cell wall fragments.

To establish whether the adhesion of K88 was decreased by a nonspecific protein rather than a specific lactobacillus growth component, bovine serum albumin (0.5 μg ml^{-1}) instead of spent culture fluid was added to the immobilized mucus prior to the adhesion assay.

RESULTS

Effect of spent culture fluid dialysis retentate on adhesion of *E. coli* K88 strains. The adhesion of *E. coli* K88 strain G1108E was reduced to 36 and 48% when, prior to the adhesion assay, *E. coli* K88 cells were resuspended in BHI spent culture fluid dialysis retentate of *L. murinus* C39 and *L. crispatus* 152, respectively. Conversely, BHI spent culture fluid dialysis retentate of *L. fermentum* 737 of rodent origin had no effect on adhesion of strain G1108E. Reduction of adhesion of *E. coli* K88 strain 1107 also occurred when the immobilized mucus was pretreated with the dialysis retentate of LDM-grown cells relative to uninoculated LDM medium and then washed before the adhesion assay. This inhibitory effect on adhesion was demonstrated for retentate from *L. murinus* C39 and *L. fermentum* 104R and 104S (Table 1) (strain 152 not tested). Because other aspects of strain 104R were being extensively studied in our laboratory, all subsequent studies of the reduction of K88 adhesion were carried out with spent culture fluid of strain 104R. The adhesion of *E. coli* K88ab strain G1108E, K88ac strain 1107, and K-12(K88ab) was inhibited by dialysis retentate of LDM-grown cells of strain 104R (Table 1). As reported previously with monoclonal antibody PAB10 and 35-day-old piglet ileal mucus, >90% of the adhesion of K88ac strain 1107 in these studies was mediated by K88 fimbriae. In addition, adhesion of *E. coli* K-12 without the plasmid containing the K88 gene was <10% of that observed for the isogenic mutant K-12(K88) containing the K88ab gene-bearing plasmid.

Effect of growth media and dialysis conditions on adhesion inhibitory activity. As illustrated in Fig. 1, there is a tendency for the effect on *E. coli* K88ac adhesion of spent culture fluid dialysis retentate of strain 104R to be greater when the dialysis is carried out with Milli-Q water instead of PBS for all growth media tested. The pH of the various retentates varied depending on whether water or PBS was used. The pHs of the dialysis retentate were approximately 3.45 and 7.00 after dialysis against water or PBS, respectively, for all media tested. Uninoculated and undialyzed medium controls had a pH value of 4.29, 5.44, or 3.78 for LDM, BHI, or LBM when dialyzed against water and a pH of 7.00 when PBS was used. Furthermore, the effect was slightly less pronounced when LBM medium was used. This effect of PBS and LBM was also observed when strain C39 instead of 104R was used (data not shown). Consequently, all subsequent studies were carried out with LDM as the growth medium for 104R, and the dialysis retentates were produced with water rather than PBS.

Fractionation and SDS-PAGE of spent culture fluid dialysis retentate of strain 104R. Fractionation of 104R LDM spent culture fluid dialysis retentate by membrane filtration revealed that the component which affected adhesion of both K88ac (strain 1107) and K88ab (strain G1108E) had a molecular weight of >30,000 (Fig. 2). Similarly, fractionation of retentate from dialysis of spent culture fluid of C39 BHI-grown cells also resulted in >30K fraction containing the active components. When the retentate of LDM-grown 104R cultures was fractionated by gel filtration, the adhesion inhibitory activity was mainly detected in the void volume which represented components with molecular weights of >250,000 (Fig. 3). While this could indicate that cell wall fragments of the lactobacilli could be the active agents, centrifugation at 27,000 × g for 1.5 h failed to remove the activity of either K88ab (strain K12K88) or K88ac (strain 1107) from the retentate (Fig. 2).

SDS-PAGE analysis of fractions with molecular weights of >250,000, 250,000, and 150,000 after gel filtration of 104R LDM spent culture dialysis retentate is presented in Fig. 4. One band corresponding to a very high molecular weight (marked with an arrow) was only detectable in one fraction. This fraction corresponded to an M_r of >250,000 and inhibited K88 adhesion by about 50%.

Chemical characterization of the adhesion inhibitory activity from LDM cultures of strain 104R. Pretreatment of the spent culture fluid dialysis retentate with pronase completely removed the adhesion inhibitory effect of the material (Fig. 5). As a control for pronase treatment, the assay was carried out at both 0 and 37°C, and no significant difference in the effect of the retentate on the adhesion inhibitory activity was noted (data not presented). The activity was stable to heat treatment (data not presented). It was not possible to estab-

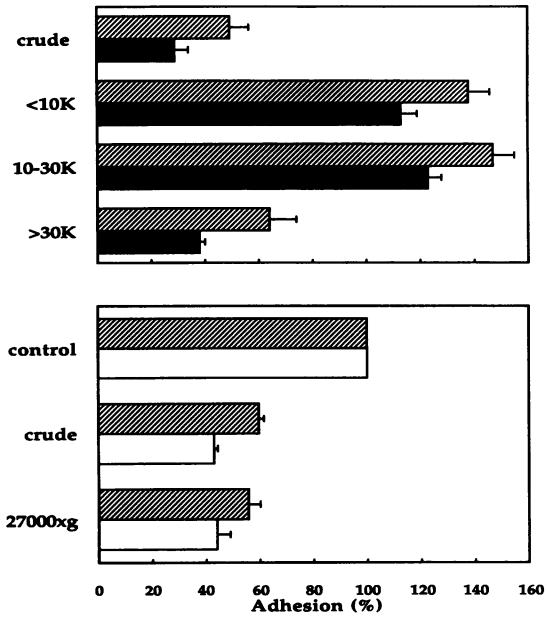

FIG. 2. Effect on adhesion in K88ac strain 1107 (▨), K88ab strain G1108E (■), and K-12 (K88ab) (□) in spent culture dialysis retentate of LDM-grown 104R cells after fractionation by membrane filtration into molecular sizes <10K, 10K to 30K, and >30K or not fractionated (crude) (top); or uninoculated LDM (control), unfractionated retentate (crude), and centrifugation at 27,000 × *g* for 1.5 h (centrifuged) (bottom). Results are expressed as given in the legend to Fig. 1. Each experiment (n = 2) was assayed in triplicate.

lish the effect of periodate oxidation on activity because activity was lost after treatment with both periodate and the iodate control of both Milli-Q water- and PBS-dialyzed retentate of LDM cultures of 104R.

Control experiments. The reductive methylation of mucus did not alter the K88 receptors in the mucus because adhesion of *E. coli* K-12 (K88ab) to radioactively labelled mucus was consistent with that obtained for unlabelled mucus. With the radioactively labelled mucus, no significant

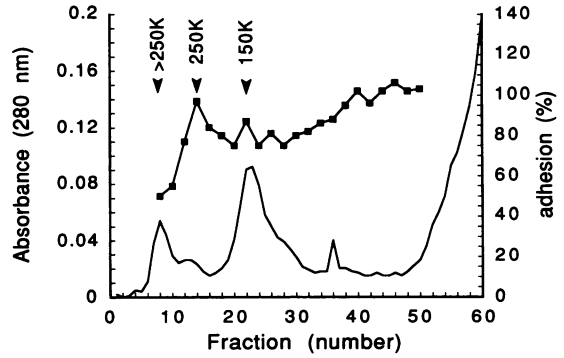

FIG. 3. Adhesion of *E. coli* K88ac strain 1107 to immobilized mucus pretreated with fractions of spent culture dialysis retentate of 104R cells grown in LDM. Fractions were collected after elution from a Sephadex 200SF gel filtration column. A_{280} is plotted as a continuous line. Relative molecular weights (M_rs) of the fractions are as marked. Adhesion is expressed as a percentage as in the legend to Fig. 1. Experiments (n = 3) were done in triplicate.

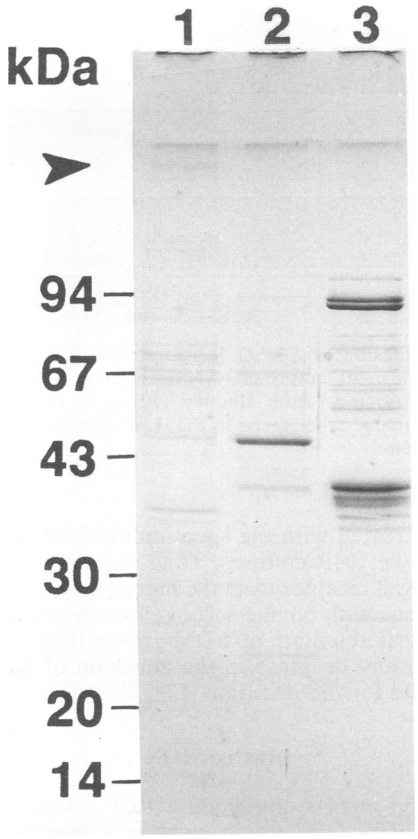

FIG. 4. SDS-PAGE analysis of the fractions from gel filtration corresponding to an M_r of >250,000 (lane 1), 250,000 (lane 2), or 150,000 (lane 3). Only the >250K fraction had a significant affect on adhesion of K88. The band marked with an arrowhead appears specific for this fraction.

lost of radioactivity was noted after the mucus was pretreated with either LDM medium or LDM spent culture dialysis retentate. Relative to the LDM medium control, 91% ± 12% of the radioactivity was detectable after the

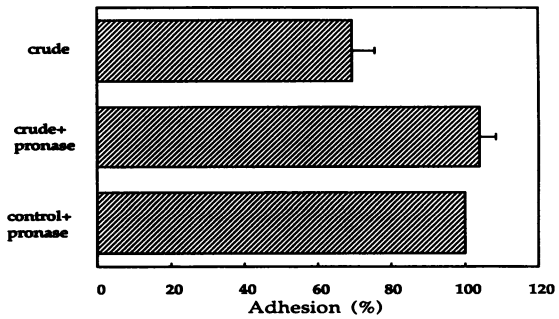

FIG. 5. Effect of pronase (100 μg · ml^{-1}) pretreatment of culture fluid dialysis retentates of LDM-grown 104R cultures on adhesion of *E. coli* K88ac strain 1107 to mucus: after pretreatment with pronase, the entire assay was carried out on ice with dialysis retentate without pronase addition (crude), dialysis retentate with pronase addition (crude + pronase), and uninoculated medium with pronase (control + pronase). Results are typical of three individual experiments and are expressed as given in the legend to Fig. 1.

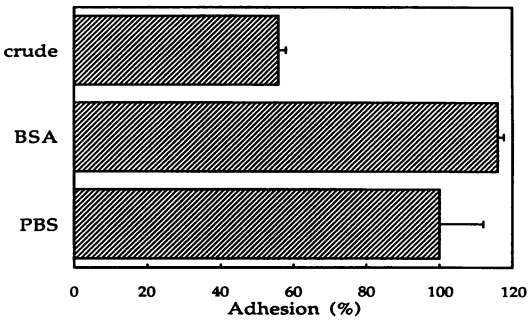

FIG. 6. Adhesion of *E. coli* K88ac strain 1107 to ileal mucus pretreated with spent culture dialysis retentate of LDM-grown 104R cells (crude), bovine serum albumin (BSA) or PBS. Results are expressed as given in the legend to Fig. 1. Experiments ($n = 2$) were done in triplicate.

mucus was treated with the spent culture dialysis retentate of LDM-grown 104R cultures. Thus, the retentate does not release the K88 receptor from the mucus layer. The pretreatment of mucus with bovine serum albumin instead of spent culture dialysis retentate of LDM-grown 104R cultures did not result in any decrease in the adhesion of K88ac strain 1107, as it did for the retentate (Fig. 6).

DISCUSSION

This study reports the finding that several strains of lactobacilli of porcine origin produce components in the spent culture fluid which reduce, or interfere with, the adhesion of *E. coli* K88-bearing cells to porcine ileal mucus. While there are many published reports that lactobacillus strains produce spent culture fluid with bacteriocidal activity against enteropathogenic bacteria, few studies direct attention to interference with the adhesion of the pathogen to the host epithelial mucosa. Adhesion of pathogenic *E. coli* to ileal epithelial cells can be inhibited by pretreating the ileal cells with whole lactobacillus cells (5). Similarly, Chan et al. (4) competitively excluded the adhesion of uropathogens from human uroepithelial cells by lactobacillus whole cells and cell wall fragments. They therefore proposed that the lactobacillus whole cells and cell wall fragments sterically hindered the adhesion of the pathogen rather than adhering to or directly interfering with the receptor used by the pathogen.

The pretreatment of the immobilized mucus with the spent culture dialysis retentate of strains C39, 104R, and 104S grown in LDM broth resulted in decreased adhesion of the K88 cells to the mucus. Even though the adhesion was decreased relative to the uninoculated media and one could conclude that the active component was of bacterial origin, it cannot be ignored that the bacterial growth may simply modify a constituent of the medium. However, the interference with adhesion of the K88 cells must be attributable to a component(s) of lactobacillus origin because LDM is a defined medium and contains no proteins or other constituents which would be retained in the dialysis retentate. Because the activity was not removed by centrifuging at 27,000 × *g* for 1.5 h (Fig. 2), it can be proposed that the active component is not a cell wall fragment as reported by Chan et al. (4). Furthermore, the adhesion was inhibited to the same degree when the spent culture dialysis retentate was either present in the adhesion assay or used to pretreat the immobilized mucus and removed prior to the adhesion assay. The site of action must therefore be the mucus components. The lactobacillus component(s) could bind to the K88 receptor or to other mucus constituents such that the K88 receptor is not accessible or not recognized by the K88 fimbriae. Additional studies are required to clarify this point, and these could include use of purified K88 receptors.

The spent culture dialysis retentate of LDM cultures of strain 104R reduced adhesion of wild-type strains of *E. coli* bearing both K88ab (strain G1108E) and K88ac (strain 1107) fimbriae (Table 1). Willemsen and deGraaf (23) have shown that, while the ab and ac serotypes of K88 fimbriae have an affinity for the same three mucus proteins, the ab serotype binds to an additional protein. Here we show comparable levels of interference by the 104R component in adhesion of K88ab and -ac strains. Consequently, the site of action of the metabolite may be a receptor common for both the ab and ac serotypes. Both wild-type *E. coli* K88 strains tested here also produce Type 1 fimbriae, and because adhesion was inhibited by about 50%, the question can be raised as to which fimbriae are affected. By using monoclonal antibodies, it has previously been established that >90% of K-12(K88) (2) and K88 strain 1107 (8) adhesion was attributable to the K88 fimbriae in our system. The specificity of the antibody was confirmed by immunoblotting of cellular homogenates from a wild-type *E. coli* K88 strain, *E. coli* K-12 carrying the plasmid containing the gene for K88, and the isogenic K-12 strain lacking this K88-encoding plasmid (8). Furthermore, this monoclonal antibody with affinity for K88ab, K88ac, and K88ad fimbriae inhibited adhesion of ^{3}H-labelled *E. coli* K88 strain 1107 to 35-day-old piglet ileal mucus by >90% ($P < 0.001$) (8). In the latter study, it was also confirmed that adhesion of *E. coli* K88 strain 1107 was reduced by about 75% by pretreating mucus with purified K88 fimbriae bound to Sepharose. It is therefore reasonable to propose that the 104R dialysis retentate interferes with the adhesion of the K88 fimbriae. The extent of reduction of adhesion was generally about 50% (see Table 1).

Growth and dialysis conditions influenced the extent of inhibitory activity detected in the retentates (Fig. 1). The greatest activity was always detected in dialysis retentates obtained by dialyzing against water rather than PBS. This suggests that the active component(s) may be more stable at an acid pH because the pH after dialysis against water was considerably lower than that obtained after PBS was used (Table 2); however, dialysis with the same buffer at the various pH values would be required to confirm that ionic strength does not influence the activity of the inhibiting component. One can eliminate the fact that the inhibitory effect is attributable to pH because the pH of retentate dialyzed against PBS was neutral, and yet still inhibitory, while the uninoculated medium controls at the same pH had no effect. Membrane filtration data confirm this point because the organic acids produced by the lactobacilli would separate into the fractions with an M_r of <30,000, and these had no effect on the adhesion (Fig. 2). Furthermore, only the fraction with an M_r of >250,000 decreased adhesion relative to the smaller fractions and the controls when PBS was used as the mobile phase.

Fractionation by membrane filtration and gel filtration confirmed that the active component had a molecular weight of >30,000 and, in fact, >250,000 (Fig. 2 and 3). That *L. fermentum* 104R releases proteins into the culture supernatant is consistent with other studies showing the presence in lactobacillus culture supernatant of components of bacterial origin such as bacteriocins (reviewed in reference 9) and

adhesive proteins (7). In the latter study, the same defined LDM broth was utilized.

The inhibitory component(s) of 104R was most likely a proteinaceous compound, which is stable at 80°C because the activity was destroyed by pronase but not by heating (Fig. 5). Because the iodate control destroyed the activity of the component, it is not possible, using metaperiodate, to establish whether the active protein also contains carbohydrate. From the control experiments, it was established that the inhibitory activity was not attributable simply to binding to the mucus of nonspecific proteins because the adhesion of K88-bearing cells was not affected by bovine serum albumin (Fig. 6). It is possible that the active component in the dialysis retentate directly affects the K88 receptor, either chemically or by steric hindrance when interacting with a neighboring moiety. Furthermore, because inhibition also occurred when 0°C was used instead of 37°C, the activity cannot be attributed to proteases of the 104R-destroying mucus proteins.

It can therefore be concluded that *L. fermentum* 104R releases a proteinaceous component(s) into the culture supernatant which affects mucus such that the adhesion of the K88ab- and K88ac-bearing cells is reduced. Studies to purify the active component and then to ascertain the mechanism of inhibition and whether it is active in vivo are under way. Such a component could be effective in protecting the piglet from infection by *E. coli* K88 strains.

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