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Interactions between *Yersinia enterocolitica* and Rabbit Ileal Mucus: Growth, Adhesion, Penetration, and Subsequent Changes in Surface Hydrophobicity and Ability To Adhere to Ileal Brush Border Membrane Vesicles

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Interactions between *Yersinia enterocolitica* and rabbit ileal mucus were examined. Strains carrying the *Yersinia* virulence plasmid, pYV, adhered to crude mucus but not to intestinal luminal contents that had been immobilized on polystyrene. Using an *Y. enterocolitica* O:9 mutant in which the *yadA* gene (formerly called *yopA*), encoding the high-molecular-weight outer membrane protein YadA (formerly called protein P1 or Yop1), had been inactivated and an *Escherichia coli* strain carrying the cloned *yadA* gene, we demonstrated that the ability to adhere to mucus correlated closely to expression of YadA. Thereafter, we evaluated possible consequences of binding between pYV-carrying *Y. enterocolitica* O:3 strains and constituents in the mucus layer. pYV-carrying strains were able to multiply at a high rate in mucus but not in luminal contents, and the ability to adhere to mucus could therefore facilitate bacterial colonization of the mucosa. However, we also showed in vitro that mucus acted as a barrier for a mucus-adherent, pYV-carrying *Y. enterocolitica* strain. Furthermore, penetration through, or preincubation with, mucus reduced subsequent adhesion of the pYV-carrying strain to brush border membrane vesicles without simultaneously causing bacterial aggregation. Preincubation with mucus also changed the bacterial surface of the same strain from hydrophobic to hydrophilic. Immunoglobulins present in mucus did not seem to be of importance for our observations. Interaction of *Y. enterocolitica* with intestinal mucus may thus reflect a host defense mechanism that reduces the pYV-mediated adhesion to the epithelial cell membrane, possibly by rendering the bacteria less hydrophobic.

Several mechanisms that promote adhesion of pathogenic yersiniae to eucaryotic cell membranes have been described previously (13, 17, 32, 37). In the intestinal tract, contact between an invading bacterium and the epithelial surface must be preceeded by penetration through the mucus layer. Interaction with mucus may thus take place before bacterial adherence to the epithelial cell membrane can be established. The existence of bacterium-mucus interactions has been documented in previous studies. Some microorganisms are able to adhere to intestinal mucus (8, 10, 20, 22, 27, 31, 33, 34, 39, 41, 46, 49). It has also been shown that microbial interaction with mucus may cause dramatic changes in the subsequent ability of the microbe to adhere to other surfaces. Increased (50) and decreased (6, 9, 27, 30) adhesion have been described previously.

A strain of *Yersinia enterocolitica* O:3 carrying the 70-kbp *Yersinia* virulence plasmid, pYV, has been reported to bind to immobilized intestinal mucus, and incubation of the same strain with mucus decreased subsequent adhesion to intestinal brush border membrane vesicles (BBVs) (27). In this study, we have further examined possible consequences of interaction between *Y. enterocolitica* and intestinal mucus. We demonstrate that mucus is an excellent nutrient medium for *Y. enterocolitica*, that the *Yersinia* outer membrane protein YadA promotes adhesion to immobilized mucus, that penetration through mucus is selectively delayed for strains carrying the pYV plasmid (pYV^+ strains), and that interaction with mucus decreases the surface hydrophobicity and subsequent adhesive abilities of pYV^+ strains without causing bacterial aggregation.

(In accordance with the nomenclature decided at the University of California at Los Angeles meeting on molecular biology of yersiniae, 22–27 February 1990, the *Yersinia* outer membrane protein that was formerly called protein P1 or Yop1 is designated YadA in this publication and the corresponding gene is designated *yadA* instead of, as formerly, *yopA*.)

MATERIALS AND METHODS

Bacteria and culture conditions. All strains used have been described previously. The 12 *Y. enterocolitica* strains $YeO301P^+$ to $YeO310P^+$ (serotype O:3), $NY8171^+$ (serotype O:8), and 3315^+ (serotype O:9) are all pYV^+, while strains $YeO301P^-$ to $YeO310P^-$, $NY8171^-$, and 3315^- are the corresponding pYV^- counterparts (35). Six other plasmid-lacking strains belonged to serotypes that are not commonly associated with disease: serotypes O:5a (strain 96874), O:6 (strain 96983), O:6 (strain 96999), O:6,30 (strain IP102), O:7 (strain IP107), and O:14 (strain 96222) (35). Four derivatives of *Y. enterocolitica* W22708 (serogroup O:9) were kindly donated by G. Cornelis (University Catholique de Louvain, Unité de Microbiologie, Brussells, Belgium): the wild-type strain W22708(pYVe227); the Tn*3* insertion derivative W22708(pYL4), which still expresses the *Yersinia* outer membrane proteins and is Ca^{2+} dependent; the insertion mutant W22708(pGB910), which does not produce YadA; and the plasmid-cured strain W22708 (4). *Escherichia coli* PM191 and PM191(pBR322), which contains the cloning

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vector, are negative controls for *E. coli* PM191(pYMS4), which contains the cloned *yadA* gene, encoding YadA, of *Y. enterocolitica* 6471/76 (serotype O:3) (44).

The strains were stored at −80°C in nutrient broth with 20% glycerol. For adhesion experiments, the bacteria were grown at 37°C for 20 h in Eagle minimal medium with Hanks balanced salts (EMM), (Flow Laboratories, Irvine, Scotland) and 10% fetal calf serum. These conditions promote the expression of YadA (19). Thymidine(methyl-^{3}H) (New England Nuclear, Boston, Mass.) (20 μCi/10 ml of medium) was added. After harvesting, the bacteria were washed twice in phosphate-buffered saline, pH 7.4 (PBS), and the concentration was adjusted spectrophotometrically at 540 nm with PBS to about 8×10^8 CFU/ml. Selective agents were kanamycin (100 μg/ml) and ampicillin (50 μg/ml). For experiments to study growth in mucus and luminal contents, *Y. enterocolitica* strains were grown on blood agar plates for 24 h at 20°C.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, strain YeO301P$^+$ was grown in a rich medium consisting of 1% tryptone, 0.5% yeast extract, and 0.2% glucose supplemented with the salts described by Higuchi et al. (14) and 20 mM sodium oxalate. The strain was inoculated into 100 ml of medium and grown for 16 h at 20°C in a shaking water bath. Thereafter, fresh medium was added to give a 20-times dilution and incubation was continued for 4 h at 37°C. The bacteria were harvested, washed twice, and kept in small aliquots at −20°C until immunoblotting was performed.

Isolation of crude mucus and luminal contents. Crude mucus was isolated from the ilea of four adult rabbits (stock SSc:CPH; Statens Seruminstitut, Copenhagen, Denmark) as described by Laux et al. (22). Simultaneously, ileal luminal contents were collected. Briefly, intestinal luminal contents were gently expressed from the excised ilea and transferred into 5 ml of ice-cold PBS. Thereafter, the ilea were opened and mucus was gently scraped off the walls with a rubber spatula into another 5 ml of PBS.

The mixtures were agitated on a vortex mixer for 10 min, and contaminating epithelial cells, membranes, and other debris were removed by centrifugation three times at 27,000 × *g* for 15 min. The protein concentrations were determined by the method of Lowry et al. (24). Crude mucus and luminal contents were kept in small aliquots at −20°C. The crude mucus was designated mucus(non-imm.).

Crude mucus was also isolated from two rabbits that 2 months previously each had received 10^{10} CFU of strain YeO301P$^+$ in 10 ml of 10% $NaHCO_3$ by intragastric inoculation. In the rabbit model, such an inoculum of *Y. enterocolitica* O:3 induced diarrhea and histopathological changes in the small intestine (38). The crude mucus thus isolated was designated mucus(imm.).

Adsorption of immunoglobulins from crude mucus. Immunoglobulins were adsorbed from mucus(non-imm.) and mucus(imm.) with agarose gel beads (Affi-Gel 10; Bio-Rad, Richmond, Calif.) coupled with swine antibody to rabbit immunoglobulins (Dakopatts, Copenhagen, Denmark) (3). Affi-Gel 10 (8 ml) was mixed with 14 ml of antibody (50 mg/ml in 0.1 M morpholinopropanesulfonic acid, pH 7.5 [Sigma, St. Louis, Mo.]), incubated for 4 h at 4°C with gentle agitation, and blocked with 0.8 ml of 1 M glycine, pH 8.0. After incubation for 1 h at 4°C, the gel was washed and divided into four equal portions. Mucus(non-imm.) (4 ml) and a similar volume of mucus(imm.) (both 15 mg of protein per ml) were each adsorbed twice with Affi-Gel 10-coupled anti-rabbit immunoglobulins. The first incubation was performed overnight at 4°C, and the second incubation was performed for 6 h at 4°C. Between the two adsorptions, the gel was removed by centrifugation and fresh gel was added. Mucus(non-imm.) and mucus(imm.) from which immunoglobulins had been adsorbed were designated mucus(non-imm./Ig-ads.) and mucus(imm./Ig-ads.), respectively. For control experiments, mucus samples were adsorbed with Affi-Gel 10 that had incubated with morpholinopropanesulfonic acid without immunoglobulins but otherwise had been treated as described above. The portions thus adsorbed were designated mucus(non-imm./c-ads.) and mucus(imm./c-ads.).

Isolation of BBVs. BBVs were prepared from the ilea of adult rabbits as previously described (35, 37). BBVs were suspended in PBS and kept in small aliquots at −20°C.

Adhesion assays. A previously described modification of the method described by Laux et al. (22) was used to quantitate adhesion of *Y. enterocolitica* (35, 37). In short, 96-well polystyrene microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with BBVs, different preparations of mucus, or luminal contents (0.2 mg in 0.1 ml). PBS was added to control wells. Residual binding sites were blocked with 0.15 ml of 5% bovine serum albumin (BSA). All experiments were performed in triplicate. Samples of radiolabeled *Y. enterocolitica* or *E. coli* (0.1 ml, 8×10^8 CFU/ml, if not stated otherwise) were added to each well. The plates were incubated for 1 h at 37°C and then washed three times with warm (37°C) PBS. Adherent bacteria were recovered with 0.15 ml of 5% SDS, and radioactivity was determined by liquid scintillation counting. Thereafter, the number of adherent bacteria per well was calculated (CFU/well = cpm/well × CFU/cpm). The variation of the method was determined as previously described (36). Within a single experiment, the coefficients of variation for adhesion to BBVs and crude mucus were 19 and 14%, respectively (95% upper confidence limits, 50 and 36%, respectively). Between experiments performed on different days, the coefficients of variation for adhesion to BBVs and crude mucus were 49 and 38%, respectively (95% upper confidence limits, 124 and 98%, respectively).

Periodate oxidation of immobilized mucus. Wells containing immobilized mucus(non-imm.) were incubated for 4 h at 4°C with 0.1 ml of 10 mM sodium metaperiodate in 0.2 M sodium acetate buffer (pH 4.5) (23). Control wells contained 10 mM sodium iodate in sodium acetate buffer or plain buffer. Adhesion assays with strain YeO301P$^+$ were performed after the wells were washed three times with PBS.

Addition of monosaccharides. L-Fucose, D-galactose, D-mannose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, or sialic acid was mixed with bacteria (strain YeO301P$^+$) in final concentrations of 10 mg/ml. Thereafter, the mixtures were added to wells with immobilized mucus(non-imm.) for adhesion assays as described above.

In vitro penetration through mucus and subsequent adhesion to BBVs. The mucus penetration assay was performed as described by others (29, 30, 34). BBVs immobilized in polystyrene wells were covered with a layer of one of the mucus preparations (0.1 ml, 5.0 mg of protein per ml) or BSA (similar volume and protein concentration) or PBS as a control. Samples of *Y. enterocolitica* (0.1 ml, 8×10^8 CFU/ml) were then carefully added. The bacteria were allowed to run down the sides of each well and across the upper surface of the mucus layers. The bacterial samples formed a distinct layer above the mucus and BSA layers but mixed almost instantaneously with the PBS. The plates were incubated for 4 h at 37°C. Shorter incubation failed to

promote significant adhesion in the control wells. One set of triplicate wells was aspirated to remove all bacteria except those which had penetrated all the way through the mucus layer. Another identical set of triplicate wells was also aspirated and thereafter washed three times with 37°C PBS, thus leaving only adherent bacteria. Radioactivity remaining in each set of triplicate wells was then determined as described above.

Incubation with mucus before adhesion to BBVs. Samples of *Y. enterocolitica* (8×10^8 CFU/ml) were incubated with similar volumes of the different mucus preparations or BSA (all 5 mg of protein per ml) or PBS for 30 min in a 37°C water bath. Thereafter, the mixtures were added (0.1 ml) to immobilized BBVs for adhesion assays or subjected (0.1 ml) to examinations of surface hydrophobicity.

Growth in mucus and luminal contents. Samples of mucus(non-imm.) (pH 7.35, 15.0 mg of protein per ml) and luminal contents (pH 7.35, 6.5 mg of protein per ml) were passed through a 0.45-μm-pore-size filter (Sartorius, Göttingen, Federal Republic of Germany). Thereafter, 0.45 ml of mucus(non-imm.) and 0.45 ml of luminal contents were each inoculated with 0.05 ml of PBS containing about 10^4 CFU of the strain to be tested. These preparations were incubated aerobically at 37°C. After 0, 5, and 20 h, 0.1-ml samples were removed, serially diluted, and plated out on blood agar medium.

SDS-PAGE. Whole-cell bacterial samples were dissolved in Laemmli sample buffer (21) by boiling for 3 min and thereafter were separated by SDS-PAGE (37). The samples were then electroblotted onto nitrocellulose paper (Schleicher & Schuell, Dassel, Federal Republic of Germany) as described by Shand et al. (42). After blocking of residual binding sites by incubation of the paper in 0.1% Tween in PBS for 1 h, the paper was cut into vertical strips and incubated for 2 h with samples of different mucus preparations diluted 1:20 in 0.1% Tween in PBS. All steps were performed at room temperature. After being washed three times, the nitrocellulose paper was incubated for 1 h with horseradish peroxidase-conjugated swine antibody to rabbit immunoglobulins (Dakopatts). After three washes, peroxidase activity was visualized with tetramethylbenzidine (Merck, Darmstadt, Federal Republic of Germany) (15).

Surface hydrophobicity. Surface hydrophobicity was evaluated as previously described (35, 37), using the two-phase dextran-polyethylene glycol separation system of Magnusson and Johansson (25).

Statistics. A change in adhesion of a specific strain was considered to be significant if it exceeded the 95% upper limit of the coefficients of variation. When test results from an experiment that was repeated six times were compared, a difference was considered significant if it was present in all six experiments, making the probability, P, of the difference being coincidental $\leq(1/2)^6$, i.e., 0.016. Differences in adhesion between two groups of strains were tested by means of the Wilcoxon rank sum test for paired data. The level of significance was preset to $P < 0.05$.

RESULTS

Adhesion to immobilized mucosal constituents. Adhesion of different *Y. enterocolitica* strains to mucus(non-imm.) immobilized in polystyrene wells is shown in Fig. 1A. Background binding to BSA is illustrated in Fig. 1B. pYV^+ strains adhered significantly better to mucus(non-imm.) than to BSA, while such a difference was found for neither pYV^- strains nor strains of nonpathogenic serotypes. Binding to immobilized luminal contents did not exceed background binding to BSA for any of the groups of strains examined. The number of CFU in the bacterial inocula did not change within the 60-min incubation period, as evaluated by serial dilution and plating on blood agar. Maximal binding of the pYV^+ strain YeO301P$^+$ to BBVs, mucus(non-imm.), and luminal contents is illustrated in Fig. 2. It is evident that the

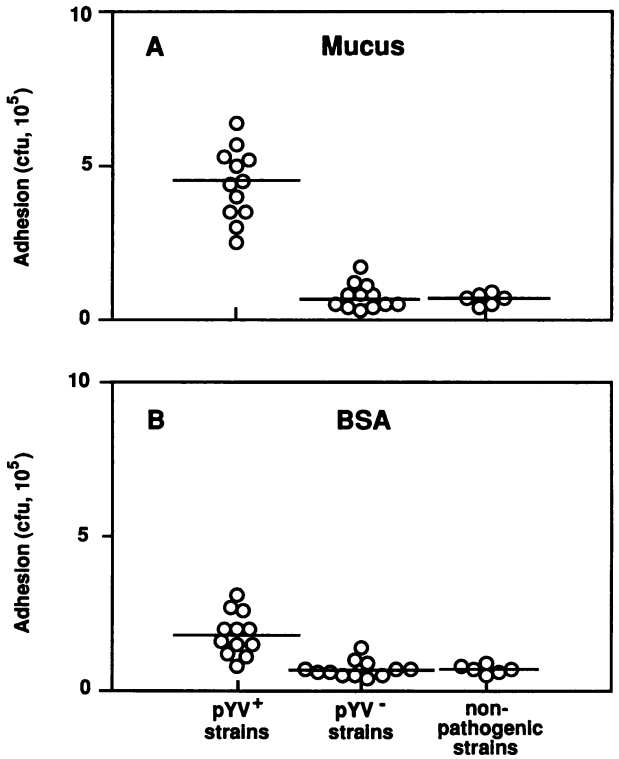

FIG. 1. Adhesion of isogenic pYV^+ and pYV^- and nonpathogenic strains of *Y. enterocolitica* to rabbit ileal mucus (A) and BSA (B) immobilized on polystyrene. Each point is the mean from triplicate samples. All determinations were performed within one experiment. Bars indicate median values.

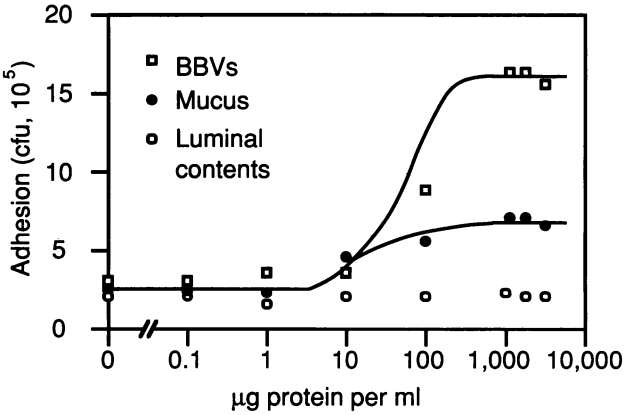

FIG. 2. Relation between concentrations of rabbit ileal BBVs, mucus, and intestinal luminal contents in coating solutions for polystyrene wells and adhesion of *Y. enterocolitica* YeO301P$^+$. Each point is the mean from triplicate samples. All determinations were performed within one experiment.

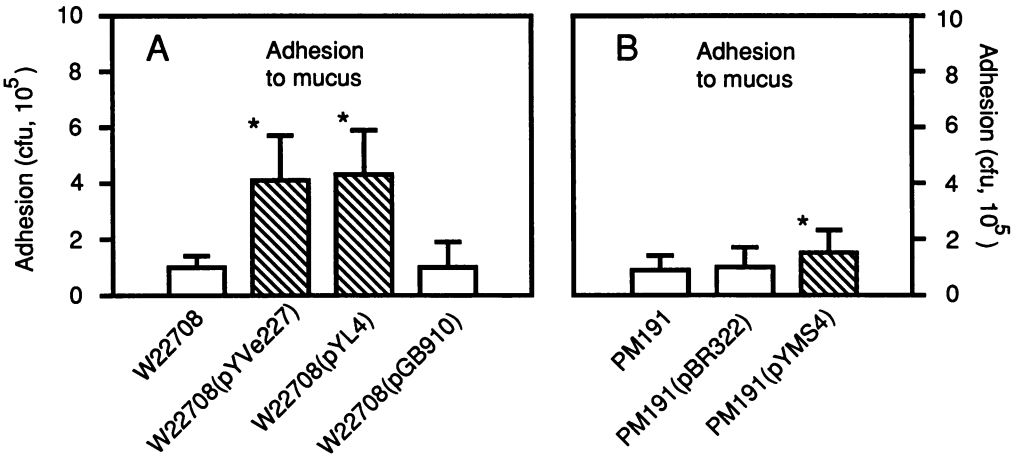

FIG. 3. Adhesion of *Y. enterocolitica* O:9 strains (A) and *E. coli* PM191 strains (B) to immobilized rabbit ileal mucus. Mean values and standard deviations from six experiments (each performed in triplicate) are given. Hatched columns indicate YadA-producing strains. Asterisks indicate significant differences between a YadA-producing strain and each of the controls that do not produce YadA.

binding potential to BBVs was greater than that to mucus.

Characteristic of the binding of pYV$^+$ *Y. enterocolitica* to mucus. Adhesion of the pYV$^+$ strains to mucus(non-imm./Ig-ads.) and mucus(non-imm./c-ads.) was very similar. Medians (ranges) were 3.3 (1.8 to 7.6) $\times$ 10^5 and 3.4 (1.6 to 9.3) $\times$ 10^5 CFU/ml, respectively. Addition of sugars present in mucin, the glycoprotein responsible for the viscoelastic character of mucus (1), did not cause inhibition of binding of strain YeO301P$^+$ to mucus(non-imm.) (range of adhesion, 82 to 111% of PBS control adhesion). Oxidative treatment of potential receptor molecules present in immobilized mucus(non-imm.) with sodium metaperiodate was also found not to decrease binding (adhesion, 111% of adhesion of control with sodium iodate and 95% of that of control with buffer).

Inactivation of the *yadA* gene (the gene encoding YadA) in *Y. enterocolitica* W22708(pYL4) yielded the insertion mutant W22708(pGB910) (4). Adhesion to mucus(non-imm.) in strain W22708 was significantly reduced to a level comparable with that of the plasmid-cured strain W22708 (Fig. 3A). When the *yadA* gene was introduced into *E. coli* PM191, adhesion to mucus increased significantly compared with that in controls (Fig. 3B). The increase was small but present in all experiments performed.

Incubation with, and penetration through, mucus and the subsequent adhesion to BBVs. Strain YeO301P$^+$, but not strain YeO301P$^-$, was significantly retarded in its travel through mucus(non-imm.) compared with its travel through PBS and BSA (Fig. 4A). After penetration through PBS and BSA, adhesion to BBVs by strain YeO301P$^+$ was two to three times greater than that by strain YeO301P$^-$. However, penetration through mucus(non-imm.) significantly reduced the adherence of strain YeO301P$^+$ to a level comparable with that of strain YeO301P$^-$ (Fig. 4B). Preincubation with mucus(non-imm.) resulted in a similar reduction in subsequent adhesion of strain YeO301P$^+$ to BBVs (Fig. 5A). Surface hydrophobicity of strain YeO301P$^+$, as evaluated in the two-phase partitioning system, was unaffected by preincubation with PBS and BSA. However, preincubation with mucus(non-imm.) conferred on the bacteria a significantly more hydrophilic surface (Fig. 5B).

To study whether secretory immunoglobulins in the mucus layer contributed to the reduction in binding of strain YeO301P$^+$ to BBVs, crude mucus from normal nonimmunized rabbits and perorally infected rabbits were subjected to adsorption of immunoglobulins. The efficacy of the adsorption was documented by means of immunoblotting (Fig. 6). Because mucus from normal rabbits did not contain detectable antibodies against *Y. enterocolitica* antigens, we included mucus from immunized animals in these experiments. Penetration of YeO301P$^+$ through the different mucus preparations was not altered, and neither was the

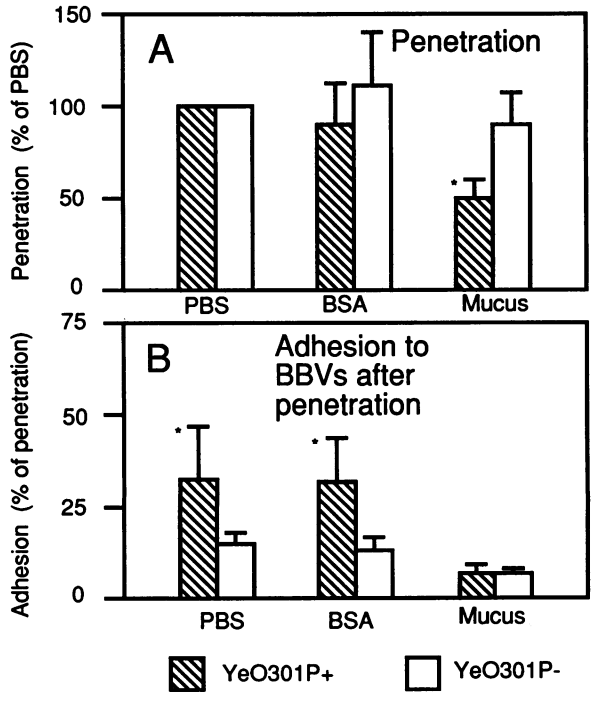

FIG. 4. Penetration of *Y. enterocolitica* YeO301P$^+$ and YeO301P$^-$ through rabbit ileal mucus or controls (A) and subsequent adhesion to BBVs (B). Mean values and standard deviations from six experiments (each performed in triplicate) are given. Asterisks indicate significant differences between the two strains tested.

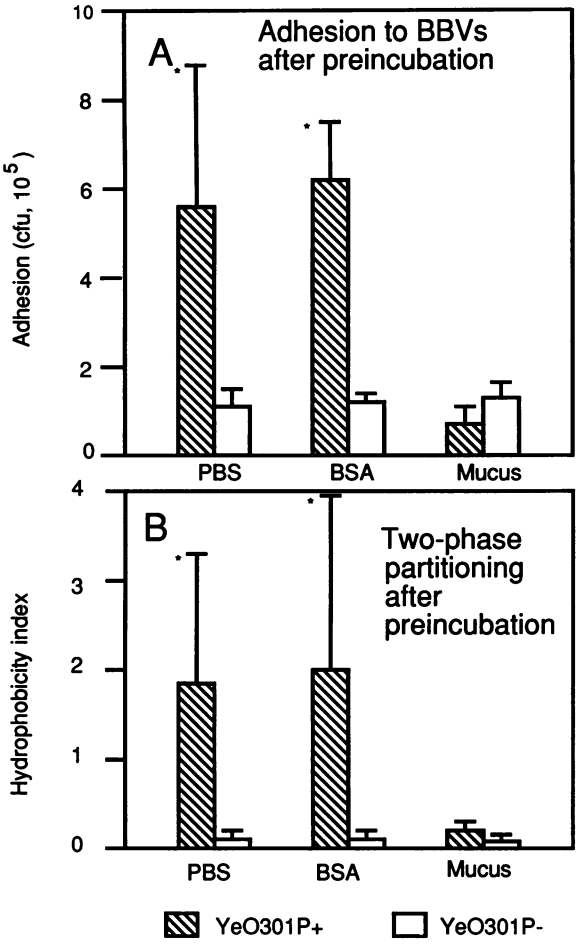

FIG. 5. Adhesion (A) and two-phase partitioning (B) of *Y. enterocolitica* YeO301P$^+$ and YeO301P$^-$ to rabbit ileal BBVs after preincubation with mucus or controls. Mean values and standard deviations from six experiments (each performed in triplicate) are given. Asterisks indicate significant differences between the two strains tested.

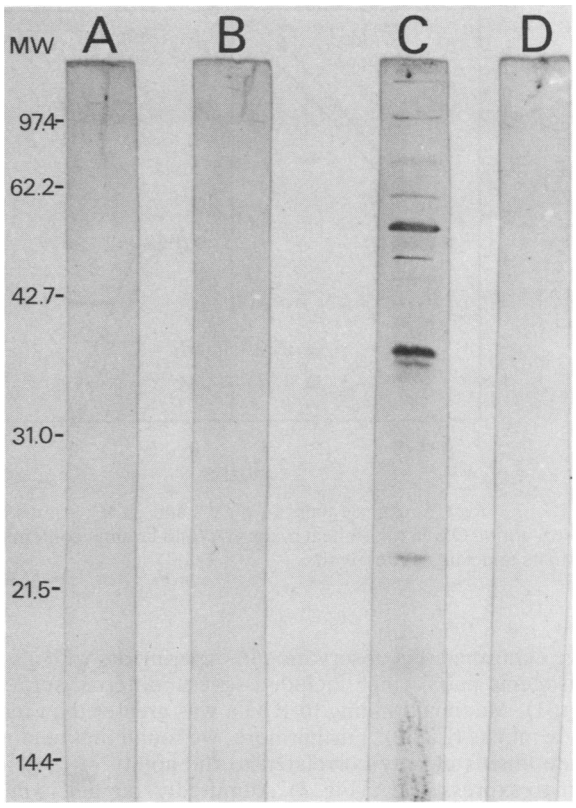

FIG. 6. Immunoblot analysis of mucus(non-imm./c-ads.) (lane A), mucus(non-imm./Ig-ads.) (lane B), mucus(imm./c-ads.) (lane C), and mucus(imm./Ig-ads.) (lane D) against whole-cell bacterial samples of *Y. enterocolitica* YeO301P$^+$. The sizes of molecular weight markers (MW) are given in kilodaltons.

subsequent adhesion to BBVs (Table 1). Correspondingly, incubation of YeO301P$^+$ with each of the mucus preparations prior to adhesion assays reduced binding to BBVs to similar degrees (Table 1). Incubation with mucus did not cause aggregation of the bacteria, as evaluated by means of phase-contrast microscopy performed on wet mounts.

Growth in mucus and luminal contents. Three pYV$^+$ strains and the three corresponding pYV$^-$ strains were inoculated into separate samples of mucus(non-imm.) and luminal contents. All strains grew well in mucus(non-imm.) but not in luminal contents (Fig. 7).

DISCUSSION

The composition of intestinal mucus is complex. Its major components are mucins, high-molecular-weight glycoproteins that are able to bind large quantities of water, thus conferring on mucus the characteristic viscoelastic, gel-forming properties (1). Other constituents in mucus include nonmucin glycoproteins, secretory immunoglobulin A, fragments of sloughed epithelial cells, and plasma proteins. It is often claimed that the mucus layer acts as a mechanical barrier for microorganisms present in the intestinal lumen (12), thus protecting the epithelial surface from being colonized by enteropathogenic organisms. The experiments described in this communication were performed in order to examine the possible consequences of interaction between *Y. enterocolitica* and the intestinal mucus layer.

Mantle et al. (27) found that a pYV$^+$ strain of *Y. enterocolitica* O:3 adhered better to immobilized rabbit intestinal crude mucus than did its isogenic pYV$^-$ counterpart. We

TABLE 1. Influence of interaction of *Y. enterocolitica* YeO301P$^+$ with different mucus preparations[a]

Prepn	% Penetration through PBS	% Adhesion to BBVs after:	
		Penetration[b]	Preincubation[c]
PBS	100	29 ± 15	100
BSA	81 ± 14	35 ± 10	107 ± 24
Mucus(non-imm./Ig-ads.)	45 ± 7.1	6.2 ± 3.2	12 ± 4.7
Mucus(non-imm./c-ads.)	54 ± 10.0	5.5 ± 3.0	10 ± 3.1
Mucus(imm./Ig-ads.)	49 ± 27.0	3.0 ± 1.7	10 ± 2.2
Mucus(imm./c-ads.)	54 ± 4.0	2.8 ± 1.3	9.2 ± 6.0

[a] For a detailed description of experimental conditions, see text. All experiments were performed in triplicate and repeated six times. Mean values ± standard deviations are given.

[b] Results are given as adherent radioactivity in percentages of penetrating radioactivity.

[c] Results are given as percent adhesion after preincubation with PBS.

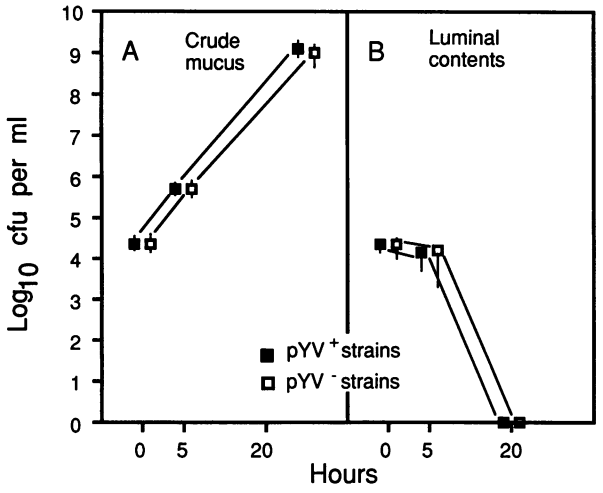

FIG. 7. Growth of three pairs of pYV$^+$ and pYV$^-$ strains of *Y. enterocolitica* O:3 in rabbit ileal mucus (A) and luminal contents (B). Medians and ranges are given.

have confirmed this observation in experiments with a series of isogenic pairs which included several different serotypes (Fig. 1). Maximal binding to BBVs was greater than that to crude mucus (Fig. 2). Furthermore, we found that binding to crude mucus closely correlated to the ability of the bacterium to express YadA (Fig. 3). Admittedly, binding to mucus by *E. coli* PM191(pYMS4) did not reach the same level as that by *Y. enterocolitica* O:9 W22708(pYVe227), though both of these strains expressed YadA. This apparent discrepancy might be due to a low expression of YadA by the *yadA* gene carried in the pYMS4 plasmid, or the YadA molecules might dislodge more easily from the surface of *E. coli* PM191(pYMS4) during washing than from *Y. enterocolitica* W22708(pYVe227) (37). We have previously demonstrated that expression of YadA is associated with increased adherence to ileal BBVs and increased surface hydrophobicity (37), the latter being a recognized promoter of bacterial adhesion to eucaryotic cell surfaces (5). Strong hydrophobic interactions between an *E. coli* strain and mucin have also been reported elsewhere (41). YadA-producing strains also demonstrated increased binding to punched-out mucus-including disks of intestinal tissue (37) in a model that measured pYV-encoded adhesion (36).

The ability to bind to mucus constituents might be advantageous for the bacterium and facilitate the colonization of the mucus layer. In order for such colonization to take place, the mucus-adherent bacteria must multiply at a rate exceeding the rate at which they are expelled into the gut lumen together with sloughed mucus. That *Y. enterocolitica* is indeed able to multiply at a high rate in intestinal mucus is illustrated in Fig. 7. In contrast, this organism did not grow in luminal contents. Other studies have shown that a normal human fecal *E. coli* isolate and an avirulent *Salmonella typhimurium* strain also had the ability to grow in intestinal mucus but not in luminal contents (29, 45). Certain bacteria are able to degrade mucin and use it as a nutrient (16). However, we do not know whether *Y. enterocolitica* utilizes mucin or other constituents present in crude mucus as energy sources.

On the other hand, binding to mucus could also act as a host defense mechanism against invading pathogens. Such binding might prolong the time of mucus penetration. Correspondingly, we found in vitro that the pYV$^+$ strain YeO301P$^+$ penetrated a mucus layer less efficiently than its pYV$^-$ counterpart (Fig. 4A). It has previously been shown that a lipopolysaccharide-deficient, and thus more hydrophobic, *S. typhimurium* mutant was retarded in its passage through mucus compared with the wild-type parent (29).

Binding of pYV$^+$ strains of *Y. enterocolitica* to mucus constituents or vice versa could also lead to changes in surface characteristics of the bacterium and thus in adhesive abilities. We found that adhesion of YeO301P$^+$ to BBVs was significantly reduced when the bacteria were allowed to interact with mucus prior to the adhesion assay, either by means of penetration (Fig. 4B) or by simple mixing and incubation (Fig. 5A). Gastrointestinal mucus has previously been reported to inhibit subsequent adhesion of organisms such as *Entamoeba histolytica*, *Shigella flexneri*, *Y. enterocolitica*, and *Campylobacter jejuni* (6, 9, 27, 30). A similar inhibitory effect of saliva on adhesion of oral streptococci has been recognized for many years (47, 48). Adhesion of single cultured epithelial cells to a monolayer was also inhibited by gastric mucus (2).

The inhibitory effect of mucus or saliva on adhesion is often reported to be mediated by bacterial aggregation, caused by either secretory immunoglobulin A (47) or mucin (48). However, inhibition of adhesion has also been reported to occur without simultaneous aggregation of the microorganisms (9). In the case of *Entamoeba histolytica*, inhibition is due to specific binding of human colonic mucin to the adherence lectin of the parasite (6). In this study, interaction of *Y. enterocolitia* with mucus did not promote bacterial aggregation but it changed the bacterial surface from hydrophobic to hydrophilic (Fig. 5B). This is an effect of mucus-microbe interaction that, to the best of our knowledge, has not previously been reported.

It has been observed that colostrum secretory immunoglobulin A may render bacteria more hydrophilic (26). Therefore, in order to evaluate the role of immunoglobulins in our observations, the immunoglobulins from crude mucus preparations (obtained from normal rabbits and from rabbits immunized orally with live *Y. enterocolitica*) were adsorbed. Such adsorption did not change the adhesion of strain YeO301P$^+$ to mucus, nor did it change the inhibitory effect of mucus on subsequent binding of strain YeO301P$^+$ to BBVs (Table 1). Mantle et al. demonstrated that mucin glycoprotein accounted for 35 to 45% of the binding of *Y. enterocolitica* to rabbit intestinal mucus (27). Furthermore, both intestinal crude mucus and its purified mucin component were inhibitors of the binding of *Y. enterocolitica* to BBVs. We believe that factors other than secretory immunoglobulin A, probably mucin, are the main mediators of the effects caused by mucus on bacterial adhesion in our study. *Y. enterocolitica* was able to bind to collagen types I and IV (11). However, though intestinal mucus contains sloughed enterocyte fragments and transudated serum proteins (7), there are no reports of collagen being detected in mucus. Therefore, we doubt that collagen binding plays a major role in adhesion of *Y. enterocolitica* to mucus.

The product of the chromosomal *inv* gene is probably a main promoter of adhesion to (and penetration through) the intestinal epithelial surface. This product, invasin, is efficiently expressed at temperatures below 37°C and therefore is already present in the bacterial outer membrane before the microbe is ingested (18). However, the introduction of a lesion in the *inv* gene of a *Yersinia pseudotuberculosis* strain only delayed the onset of death after oral inoculation in a mouse model, indicating that other factors contribute to

adhesion and/or penetration (40). YadA confers on pYV$^+$ strains of *Y. enterocolitica* and *Y. pseudotuberculosis* an increased ability to bind to the enterocyte membrane (37). Being expressed already in the gut lumen (43), YadA could be expected to be important for adhesion to the enterocyte barrier. However, binding of the bacterium to one or several mucus constituents seems to act as a host defense mechanism that renders the bacterium less hydrophobic and decreases the ability to penetrate the mucus layer and subsequently adhere to the brush border. In accordance with this, infection with *Y. enterocolitica* led to an increase in intestinal mucus secretion in the rabbit model (28).

We do not presently know at which stage of the infection the YadA-promoted adhesion plays the most important role in vivo. After penetration of the enterocyte barrier, the bacterium might benefit from the ability of the YadA to bind to collagen fibers (11). The product of the chromosomally located *ail* gene is an adhesin that, like YadA, is also optimally expressed at body temperature. YadA might operate in concert with the *ail* gene product for adhesion to migrating cells of the immune system. This could promote further spread through the host organism (18). The relative importance of the YadA in the different phases of the pathogenesis remains to be determined.

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REFERENCES

1. **Allan, A., W. J. Cunliffe, J. P. Pearson, L. A. Sellers, and R. Ward.** 1984. Studies on gastrointestinal mucus. Scand. J. Gastroenterol. **93**:101–113.
2. **Allan, A., and S. M. Minnikin.** 1975. The binding of the mucoprotein from gastric mucus to cells in tissue culture and the inhibition of cell adhesion. J. Cell Sci. **17**:617–631.
3. **Andersson, B., O. Porras, L. Å. Hanson, T. Lagergård, and C. Svanborg-Edén.** 1986. Inhibition of attachment of *Streptococcus pneumoniae* and *Haemophilus influenzae* by human milk and receptor oligosaccharides. J. Infect. Dis. **153**:232–237.
4. **Balligand, G., Y. Laroche, and G. Cornelis.** 1985. Genetic analysis of virulence plasmid from a serogroup 9 *Yersinia enterocolitica* strain: role of outer membrane protein P1 in resistance to human serum and autoagglutination. Infect. Immun. **48**:782–786.
5. **Beachey, E. H.** 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. **143**:325–345.
6. **Chadee, K., W. A. Petri, Jr., D. J. Innes, and J. I. Ravdin.** 1987. Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. J. Clin. Invest. **80**:1245–1254.
7. **Clamp, J. R., and M. Creeth.** 1984. Some non-mucin components of mucus and their possible biological roles, p. 121–136. *In* Mucus and mucosa (Ciba Foundation symposium 109). Pitman, London.
8. **Cohen, P. S., J. C. Arruda, T. J. Williams, and D. C. Laux.** 1984. Adhesion of a human fecal *Escherichia coli* strain to mouse colonic mucus. Infect. Immun. **48**:139–145.
9. **Dinari, G., T. L. Hale, O. Washington, and S. B. Formal.** 1986. Effect of guinea pig or monkey colonic mucus on *Shigella* aggregation and invasion of HeLa cells by *Shigella flexneri* 1b and 2a. Infect. Immun. **51**:975–978.
10. **Drumm, B., A. M. Roberton, and P. M. Sherman.** 1988. Inhibition of attachment of *Escherichia coli* RDEC-1 to intestinal microvillus membranes by rabbit ileal mucus and mucin in vitro. Infect. Immun. **56**:2437–2442.
11. **Emödy, L., J. Heesemann, H. Wolf-Watz, M. Skurnik, G. Kapperud, P. O'Toole, and T. Wadström.** 1989. Binding to collagen by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*: evidence for *yopA*-mediated and chromosomally encoded mechanisms. J. Bacteriol. **171**:6674–6679.
12. **Florey, H. W.** 1933. Observations on the functions of mucus and the early stages of bacterial invasion of the intestinal mucosa. J. Pathol. Bacteriol. **37**:283–289.
13. **Heesemann, J., and L. Grüter.** 1987. Genetic evidence that the outer membrane protein YOP1 of *Yersinia enterocolitica* mediates adherence and phagocytosis resistance to human epithelial cells. FEMS Microbiol. Lett. **40**:37–41.
14. **Higuchi, K., L. L. Kupferberg, and J. L. Smith.** 1959. Studies on the nutrition and physiology of *Pasteurella pestis*. J. Bacteriol. **77**:317–321.
15. **Hindersson, P., J. D. Knudsen, and N. H. Axelsen.** 1987. Cloning and expression of *Treponema pallidum* common antigen (Tp-4) in *Escherichia coli* K12. J. Gen. Microbiol. **133**:587–596.
16. **Hoskins, L. C., M. Agustines, W. B. McKee, E. T. Boulding, M. Kriaris, and G. Niedermeyer.** 1985. Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood groups antigens and oligosaccharides from mucin glycoproteins. J. Clin. Invest. **75**:944–953.
17. **Isberg, R. R.** 1989. Determinants for thermoinducible cell binding and plasmid-encoded cellular penetration detected in the absence of the *Yersinia pseudotuberculosis* invasin protein. Infect. Immun. **57**:1998–2005.
18. **Isberg, R. R.** 1990. Pathways for the penetration of enteroinvasive *Yersinia* into mammalian cells. Mol. Biol. Med. **7**:73–82.
19. **Kapperud, G., E. Namork, M. Skurnik, and T. Nesbakken.** 1987. Plasmid-mediated surface fibrillae of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*: relationship to outer membrane protein YOP1 and possible importance for pathogenesis. Infect. Immun. **55**:2247–2254.
20. **Kennedy, M. J., P. A. Volz, C. A. Edwards, and R. J. Yancey.** 1987. Mechanisms of association of *Candida albicans* with intestinal mucosa. J. Med. Microbiol. **24**:333–341.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Laux, D. C., E. F. McSweegan, and P. S. Cohen.** 1984. Adhesion of enterotoxigenic *Escherichia coli* to immobilized intestinal mucosal preparations: a model for adhesion to mucosal surface components. J. Microbiol. Methods **2**:27–39.
23. **Laux, D. C., E. F. McSweegan, T. J. Williams, E. A. Wadolkowski, and P. S. Cohen.** 1986. Identification and characterization of mouse small intestine mucosal receptors for *Escherichia coli* K-12(K88ab). Infect. Immun. **52**:18–25.
24. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
25. **Magnusson, K.-E., and G. Johansson.** 1977. Probing the surface of *Salmonella typhimurium* and *Salmonella minnesota* SR and R bacteria by aqueous biphasic partitioning in systems containing hydrophobic and charged polymers. FEMS Microbiol. Lett. **2**:225–228.
26. **Magnusson, K.-E., and I. Stjernström.** 1982. Mucosal barrier mechanisms. Interplay between secretory IgA (SIgA), IgG and mucins on the surface properties and association of salmonellae with intestine and granulocytes. Immunology **45**:239–248.
27. **Mantle, M., L. Basaraba, S. C. Peacock, and D. G. Gall.** 1989. Binding of *Yersinia enterocolitica* to rabbit intestinal brush border membranes, mucus, and mucin. Infect. Immun. **57**:3292–3299.
28. **Mantle, M., E. Thakore, J. Hardin, and D. G. Gall.** 1989. Effect of *Yersinia enterocolitica* on intestinal mucin secretion. Am. J. Physiol. **256**:G319–G327.
29. **McCormick, B. A., B. A. D. Stocker, D. C. Laux, and P. S. Cohen.** 1988. Roles of motility, chemotaxis, and penetration

through and growth in intestinal mucus in the ability of an avirulent strain of *Salmonella typhimurium* to colonize the large intestine of streptomycin-treated mice. Infect. Immun. **56**:2209–2217.

30. **McSweegan, E., D. H. Burr, and R. I. Walker.** 1987. Intestinal mucus gel and secretory antibody are barriers to *Campylobacter jejuni* adherence to INT 407 cells. Infect. Immun. **55**:1431–1435.
31. **McSweegan, E., and R. I. Walker.** 1986. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect. Immun. **53**:141–148.
32. **Miller, V. L., J. B. Bliska, and S. Falkow.** 1990. Nucleotide sequence of the *Yersinia enterocolitica ail* gene and characterization of the Ail protein product. J. Bacteriol. **172**:1062–1069.
33. **Mouricout, M. A., and R. A. Julien.** 1987. Pilus-mediated binding of bovine enterotoxigenic *Escherichia coli* to calf small intestinal mucins. Infect. Immun. **55**:1216–1223.
34. **Nevola, J. J., D. C. Laux, and P. S. Cohen.** 1987. In vivo colonization of the mouse large intestine and in vitro penetration of intestinal mucus by an avirulent smooth strain of *Salmonella typhimurium* and its lipopolysaccharide-deficient mutant. Infect. Immun. **55**:2884–2890.
35. **Pærregaard, A., F. Espersen, and N. Baker.** Acta Pathol. Microbiol. Immunol. Scand., in press.
36. **Pærregaard, A., F. Espersen, J. Hannover Larsen, and N. Høiby.** 1990. Adhesion of *Yersinia enterocolitica* to human epithelial cell lines and to rabbit and human small intestinal tissue. Acta Pathol. Microbiol. Immunol. Scand. **98**:53–60.
37. **Pærregaard, A., F. Espersen, and M. Skurnik.** Acta. Pathol. Microbiol. Immunol. Scand., in press.
38. **Pai, C. H., V. Mors, and T. A. Seemayer.** 1980. Experimental *Yersinia enterocolitica* enteritis in rabbits. Infect. Immun. **28**: 238–244.
39. **Ramphal, R., C. Guay, and G. B. Pier.** 1987. *Pseudomonas aeruginosa* adhesins for tracheobronchial mucin. Infect. Immun. **55**:600–603.
40. **Rosqvist, R., M. Skurnik, and H. Wolf-Watz.** 1988. Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. Nature (London) **334**:522–525.
41. **Sajjan, S. U., and J. F. Forstner.** 1990. Characteristics of binding of *Escherichia coli* serotype O157:H7 strain CL-49 to purified intestinal mucin. Infect. Immun. **58**:860–867.
42. **Shand, G. H., S. S. Pedersen, R. Tilling, M. R. W. Brown, and N. Høiby.** 1988. Use of immunoblot detection of serum antibodies in the diagnosis of chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis. J. Med. Microbiol. **27**:169–177.
43. **Skurnik, M., and K. Poikonen.** 1986. Experimental intestinal infection of rats by *Yersinia enterocolitica* O:3. Scand. J. Infect. Dis. **18**:355–364.
44. **Skurnik, M., and H. Wolf-Watz.** 1989. Analysis of the *yopA* gene encoding the Yop1 virulence determinants of *Yersinia* spp. Mol. Microbiol. **3**:517–529.
45. **Wadolkowski, E. A., D. C. Luax, and P. S. Cohen.** 1988. Colonization of the streptomycin-treated mouse large intestine by a human fecal *Escherichia coli* strain: role of growth in mucus. Infect. Immun. **56**:1030–1035.
46. **Wanke, C. A., S. Cronan, C. Goss, K. Chadee, and R. L. Guerrant.** 1990. Characterization of binding of *Escherichia coli* strains which are enteropathogens to small-bowel mucin. Infect. Immun. **58**:794–800.
47. **Williams, R. C., and R. J. Gibbons.** 1972. Inhibition of bacterial adherence by secretory IgA: a mechanism of antigen disposal. Science **177**:697–699.
48. **Williams, R. C., and R. J. Gibbons.** 1975. Inhibition of streptococcal attachment to receptors on human buccal epithelial cells by antigenically similar salivary glycoproteins. Infect. Immun. **11**:711–718.
49. **Yamamoto, T., and T. Yokota.** 1988. Electron microscopic study of *Vibrio cholerae* O1 adherence to the mucus coat and villus surface in the human small intestine. Infect. Immun. **56**:2753–2759.
50. **Zenian, A., and F. D. Gillin.** 1985. Interactions of *Giardia lamblia* with human intestinal mucus: enhancement of trophozoite attachment to glass. J. Protozool. **32**:664–668.