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Adhesion of *Yersinia enterocolitica* to Purified Rabbit and Human Intestinal Mucin

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Interactions between *Yersinia enterocolitica* and purified intestinal mucins from rabbit and humans were investigated. Plasmid-bearing virulent organisms (but not plasmid-free nonvirulent bacteria) bound well to both mucins, suggesting that adherence was controlled by the virulence plasmid. Examination of binding to 14 different preparations of purified human intestinal mucin (8 preparations obtained from normal subjects and 6 samples from patients with cystic fibrosis) revealed no differences between normal and cystic fibrotic mucins in ability to serve as a binding substrate for virulent *Y. enterocolitica*. Analyses of binding curves suggested the presence of a single type of noninteracting receptor for *Y. enterocolitica* in both rabbit and human mucins with similar (but not necessarily identical) structures. Virulent bacteria bound to polystyrene through hydrophobic interactions that could be disrupted by treating the organisms with tetramethyl urea. In contrast, binding of plasmid-bearing *Y. enterocolitica* to intestinal mucin was not susceptible to tetramethyl urea and therefore does not appear to involve hydrophobic interactions. Prior incubation of organisms with mucin significantly inhibited binding to polystyrene, suggesting that mucin can mask hydrophobic adhesins on the bacterial surface. Hapten inhibition studies revealed that the monosaccharides galactose and *N*-acetylgalactosamine and the disaccharide lactose could markedly reduce (but not abolish) bacterial adherence to mucin but other monosaccharides and the RGD peptide had no effect on mucin binding. We conclude that virulent *Y. enterocolitica* is capable of interacting with the carbohydrate moiety of intestinal mucin. These interactions appear to be plasmid mediated and not hydrophobic.

Yersinia enterocolitica is an enteroinvasive bacterium that causes gastroenteritis (4, 40). The mechanisms enabling the organism to colonize the gut and produce disease have not yet been clearly defined, but all pathogenic strains (in contrast to nonpathogenic environmental isolates) contain a 42- to 50-MDa plasmid (6, 31), indicating that the plasmid is essential for virulence. A variety of proteins (16 to 20) are encoded by the plasmid, and their expression is regulated by both temperature and the availability of calcium (6, 31, 32, 43). Some of these proteins are secreted, and a number then associate with the outer membrane of the organism and are referred to as Yops (for yersinia outer membrane proteins) (22, 24, 25, 31, 32). In addition, the plasmid encodes for YadA, a fibrillar adhesin that is a true outer membrane protein (12). When expressed, these plasmid-encoded proteins change the surface charge and hydrophobicity of the bacterium, promote autoagglutination and mannose-resistant hemagglutination, enhance adherence to cell lines, collagen, and fibronectin, and confer on the organism the ability to resist phagocytosis by polymorphonuclear leukocytes and the bacteriocidal effects of serum (1, 9, 12–15, 22, 23, 29, 41, 46). Thus, plasmid proteins may participate in attachment to the intestinal wall and penetration of the mucosa and may facilitate survival and proliferation of the organism in the host tissue.

Following oral infection, *Y. enterocolitica* in the intestinal lumen must cross the mucus barrier covering the epithelial surface to invade the mucosa and produce clinical disease (4, 40). To investigate the early steps in the penetration process, we previously examined the ability of a plasmid-bearing strain of *Y. enterocolitica* (MCH700S, serotype O:3, biotype 4) to bind to small intestinal brush border membranes, mucus, and purified mucin (the gel-forming glycoprotein component of mucus) isolated from the rabbit. These studies showed that, when cultured at 37°C, the organism adhered strongly to intestinal cell membranes, mucus, and mucin (17). In contrast, the same organism cultured at 25°C (which suppresses plasmid expression) and its plasmid-cured isogenic strain (MCH700L) exhibited markedly diminished binding, indicating that bacterial adherence to intestinal cell membranes, mucus, and mucin was dependent on the production of plasmid-encoded proteins. The findings from these studies now have been independently confirmed (27, 28).

The objectives of the present study were to further pursue our earlier observations and to establish whether our strains of *Y. enterocolitica* bound to the same extent to a selection of human intestinal mucin preparations isolated from different individuals. In addition, we have explored the nature of the interactions between the organism and intestinal mucin, with particular focus on the role of bacterial cell surface hydrophobicity and the involvement of carbohydrate residues which constitute a large proportion (>80% by weight) of the mucin molecule.

MATERIALS AND METHODS

Preparation of mucin. Mucin was purified in the presence of proteolytic inhibitors from the small intestines (jejunum and ileum combined, from the ligament of Trietz to the ileo-cecal junction) of rabbits or humans as described previously (18–20). Approximately 12 New Zealand White rabbits were used for each mucin preparation. Human intestinal mucin, however, was isolated from individual subjects and preparations were not pooled. Following equilibrium density

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gradient centrifugation in CsCl (twice) and gel filtration on Sepharose 2B, highly purified polymeric mucin was harvested from the void-volume fractions of the column, dialyzed, lyophilized, and stored at −80°C. As in earlier studies, the purity of all mucin preparations was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (showing the absence of noncovalently bound contaminating protein), by DNA analysis (<2 μg/mg of mucin), and by gas-liquid chromatography (which failed to detect mannose, glucose, and uronic acid) (18–20). For binding assays, purified mucins were dissolved in phosphate-buffered saline (PBS; 0.1 M NaCl, 0.1 M Na_2HPO_4-NaH_2PO_4, 0.02% [wt/vol] NaN_3, pH 7.4) at a final concentration of 1 mg (dry weight)/ml.

Bacteria and growth conditions. *Y. enterocolitica* MCH 700S and MCH700L were obtained from C. Pai. Strain MCH 700S was originally isolated from a patient with diarrhea and is known to cause clinical disease in rabbits (21, 29). Its isogenic strain MCH700L was cured of its plasmid as described by Pai and deStephano (29) and is known not to produce disease in rabbits (29). The presence or absence of the virulence plasmid was confirmed by agarose gel electrophoresis (16, 31). Samples from frozen stocks were cultured as described previously (17). After an overnight growth at 25°C in minimum essential medium without methionine (Dulbecco's modified Eagle medium, no. 320-1970; GIBCO Laboratories, Grand Island, N.Y.), bacteria were diluted (1:10) into fresh medium and incubated at 37°C for a total of 3 h. After 30 min, 100 μCi of ^{35}S-methionine (specific activity, >1,000 Ci/mmol; Amersham Canada, Oakville, Ontario, Canada) was added to the culture medium; 1 h later, 5 mg of cold methionine was added. At the end of incubation, bacteria were pelleted by centrifugation, washed twice with sterile PBS, and finally suspended in the same buffer. The optical density of the bacterial suspension at 540 nm was adjusted with PBS to ~10^{10} organisms per ml (confirmed by dilution and colony counting). To determine the radioactivity incorporated per bacterium, a count was made on a 50-μl aliquot of the final suspension by scintillation spectrometry.

Binding assay. The binding of *Y. enterocolitica* to purified intestinal mucins from rabbit and humans was assessed by using polystyrene microtiter plates as described earlier (17). In these experiments, we used three separate rabbit mucin preparations, eight individual preparations of human intestinal mucin isolated from normal subjects without gastrointestinal disease, and six preparations obtained from patients with cystic fibrosis (CF). The biochemical characteristics of these mucins have been described previously (19, 20). For the binding assay, microtiter wells (4) were coated with 50 μl of mucin solution overnight at 4°C. Residual binding sites were blocked by addition of skim milk (350 μl; 20 mg/ml in PBS) and incubation for 2 h at 25°C. After thorough washing of the plate, bacteria (50 μl) were added to the wells, and the plates were incubated for 2 h at 25°C. After another thorough washing, wells were filled with 350 μl of sodium dodecyl sulfate (1% [wt/vol] solution), and the plates were incubated overnight at 37°C. The solution was then removed, and radioactivity counts were done by scintillation spectrometry. Experiments showed that these desorption conditions were sufficient to remove >90% of the radioactivity (and hence, adherent bacteria) from the wells. The number of *Y. enterocolitica* bound to each well was calculated on the basis of the specific activity of the bacterial preparation used for that particular experiment. Assays were performed at least three times. Means ± standard errors were calculated, and means were compared by one-way analysis of variance and Student's *t* test.

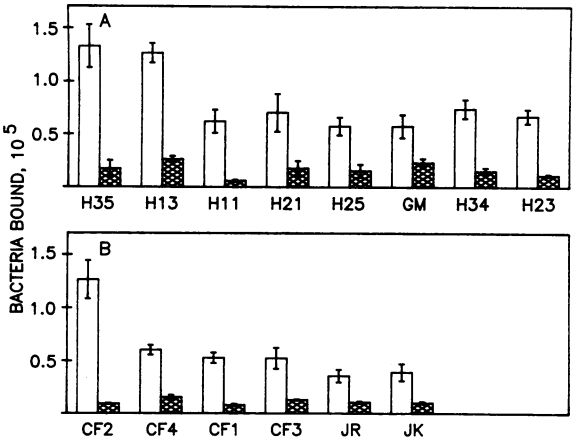

FIG. 1. Adherence of *Y. enterocolitica* to different preparations of purified human intestinal mucin. Mucins (50 μl of 1 mg/ml) were immobilized on polystyrene microtiter plate wells, and the remaining binding sites in the wells were blocked with skim milk (350 μl of 20 mg/ml) before addition of bacteria (50 μl of ~2 × 10^9 organisms per ml). (A) Mucins obtained from normal subjects with no history of gastrointestinal disease. (B) Mucins isolated from patients with CF. Open boxes denote binding of plasmid-bearing (virulent) bacteria; crosshatched boxes show binding of plasmid-cured (nonvirulent) organisms. In all cases, adherence of plasmid-cured bacteria was significantly lower than that of plasmid-bearing organisms ($P < 0.02$).

Inhibition assays. Initially, bacteria were suspended in PBS and added to uncoated polystyrene wells, a known hydrophobic surface. The remainder of the binding assay was performed as described above. To inhibit hydrophobic interactions, bacterial suspensions were first incubated for 1 h at 25°C in tetramethyl urea (TMU) at final concentrations of 0.06 to 1.0 M before addition to the untreated polystyrene wells. These experiments were then repeated with wells coated with purified rabbit intestinal mucin (50 μg) and skim milk.

For hapten inhibition studies, bacteria were incubated for 1 h at 25°C with potential haptens (all purchased from Sigma Chemical Co., St. Louis, Mo.), and then the mixtures were added to wells coated with purified rabbit intestinal mucin (50 μg) and skim milk. Antibody inhibition studies were performed by adding 50 μl of undiluted antisera to wells coated with purified rabbit intestinal mucin (50 μg) and skim milk. After incubation for 1 h at 25°C, wells were washed and bacteria (2 × 10^9 CFU in 50 μl) were added. The antibodies used for these experiments were specific for either rabbit intestinal mucin or its disulfide-bound, 118-kDa glycoprotein component (19, 20).

RESULTS

Binding of *Y. enterocolitica* to different human intestinal mucin preparations. Figure 1 illustrates the binding of virulent and plasmid-cured strains of *Y. enterocolitica* to different samples of purified human intestinal mucin isolated from normal subjects (eight) and patients with CF (six). Binding of plasmid-bearing bacteria to 11 mucin preparations was comparable, but 3 preparations (H35, H13, and CF2) showed higher levels of adherence. At present, we do not know why bacteria bound so well to these three mucins which were

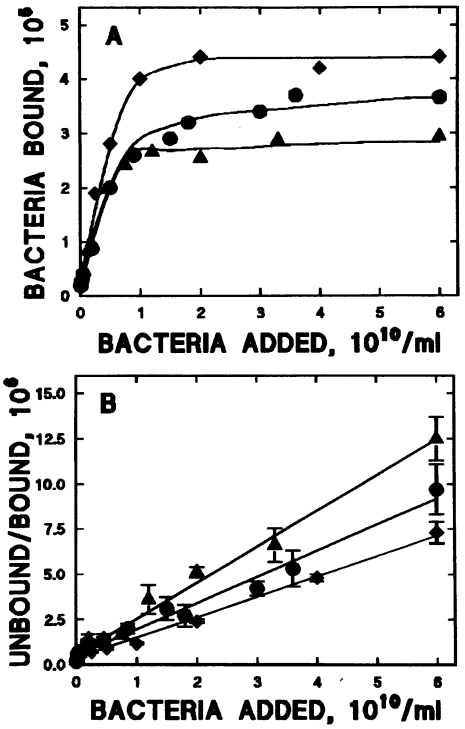

FIG. 2. Binding of plasmid-bearing *Y. enterocolitica* to purified intestinal mucins. Bacteria (50-μl aliquots) were added to microtiter plate wells coated with mucin and skim milk as described in the legend to Fig. 1. (A) Binding to rabbit intestinal mucin (◆) and to human intestinal mucins (▲ and ●; H35 and CF2 preparations obtained from a normal subject and a patient with CF, respectively). Standard error of the mean bars have been omitted for clarity. (B) Langmuir isotherm plots derived from the data in panel A. Correlation coefficients (r) were 0.99 or greater for all three mucins.

prepared and purified in exactly the same manner as all of the other mucins tested and did not differ in their composition and biochemical properties (19). Since our experimental findings likely reflect subtle differences among individual mucins in terms of the number or type of receptors present, elucidation of the binding site for virulent *Y. enterocolitica* could also improve our understanding of mucin structure. Notwithstanding the variability in binding, adherence of virulent *Y. enterocolitica* was always significantly higher than that of plasmid-cured organisms to all of the mucins tested. Overall, no differences were detected between normal and CF mucins in terms of ability to serve as a binding substrate for virulent (or plasmid-cured) bacteria.

Using two human intestinal mucins with the greatest bacterial binding (H35 and CF2) and rabbit intestinal mucin, we explored in more detail the characteristics of adherence. The binding curves of virulent *Y. enterocolitica* to all three mucins were very similar, showing dependence on bacterial concentration and saturation at concentrations of 1×10^{10} to 2×10^{10} CFU/ml (Fig. 2A). Figure 2B shows the derived Langmuir isotherms from the data in Fig. 2A. Linear regression analyses gave straight lines for all three isotherms, suggesting a single type of noninteracting receptor in each mucin. Calculations of N (saturation of mucin sites expressed as CFU per microgram of mucin) and K (the association constant expressed as milliliters per microgram of mucin) were similar for all three mucins (Table 1), suggesting common (but not necessarily identical) mucin

TABLE 1. Values of N and K for *Y. enterocolitica* binding to intestinal mucins[a]

Mucin	N (CFU/μg of mucin)	K (ml/μg of mucin)
Rabbit	8.9×10^3	2.9×10^{-10}
H35	5.0×10^3	3.5×10^{-10}
CF2	6.9×10^3	2.8×10^{-10}

[a] Langmuir isotherms were constructed from the results of binding experiments of virulent *Y. enterocolitica* to purified intestinal mucins (Fig. 2). H35 and CF2 mucins were obtained from a normal subject and a CF patient, respectively. The average number of binding sites on the mucin, N, and the association constant, K, were derived from the slope ($1/N$) and the intercept ($1/KN$), respectively, of each isotherm plot.

receptors. Values for K were in the same range as those reported previously for *Pseudomonas cepacia* (36) and *Pseudomonas aeruginosa* (38) binding to intestinal mucin. However, N values indicated that the number of binding sites in mucin for *Y. enterocolitica* is somewhat lower than those for *P. cepacia* and *P. aeruginosa* (36, 38), suggesting that the receptor for *Y. enterocolitica* may represent a more restricted, less common structural component of mucin.

Nature of the interaction between *Y. enterocolitica* and intestinal mucin. Cell surface hydrophobicity is a recognized promoter of bacterial adhesion. To investigate whether the binding properties of *Y. enterocolitica* depended on hydrophobicity, we first examined adherence of virulent and plasmid-cured strains to polystyrene, a known hydrophobic surface (Fig. 3A). Virulent organisms bound to this solid matrix extremely well and to a greater extent than nonvirulent bacteria. Incubation with TMU, a potent disrupting agent of hydrophobic associations, significantly inhibited, in a dose-dependent manner, the binding of virulent organisms to polystyrene at concentrations >0.25 M (Fig. 3B). TMU

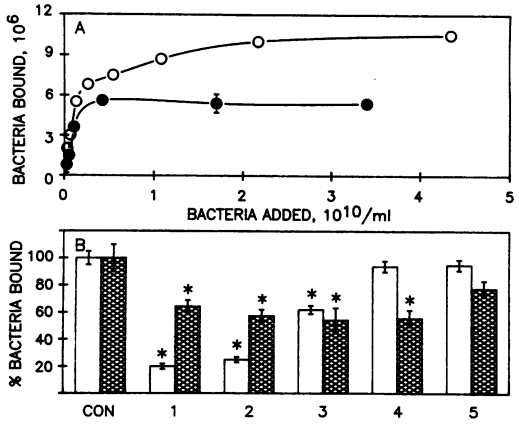

FIG. 3. Binding of *Y. enterocolitica* to polystyrene. (A) Bacteria (50-μl aliquots) were added to polystyrene microtiter plate wells, and the plates were incubated at 25°C for 2 h. After washing, bound organisms were removed and the number bound was determined. The graph shows the binding of plasmid-bearing (virulent) bacteria (○) and plasmid-cured (nonvirulent) organisms (●). (B) Bacteria (50 μl of ~10^{10} organisms per ml) were incubated in TMU at final concentrations of 0.06 to 1.0 M for 1 h at 25°C before addition to polystyrene wells. Data are expressed relative to controls incubated with PBS (no TMU). CON, control, untreated bacteria; 1, 1 M TMU; 2, 0.5 M TMU; 3, 0.25 M TMU; 4, 0.125 M TMU; 5, 0.06 M TMU. Open boxes denote binding of virulent bacteria; cross-hatched boxes show binding of nonvirulent organisms. *, significantly different from respective control ($P < 0.01$).

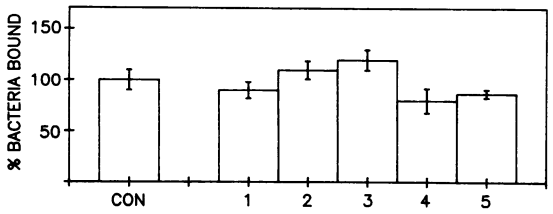

FIG. 4. Effects of TMU on binding of *Y. enterocolitica* to mucin. Purified rabbit intestinal mucin (50 μl of 1 mg/ml) was immobilized on polystyrene microtiter plate wells, and the remaining binding sites in the wells were blocked with skim milk (350 μl of 20 mg/ml). Plasmid-bearing bacteria (50 μl of ~2×10^{10} organisms per ml) were incubated in TMU at final concentrations of 0.06 to 1.0 M for 1 h at 25°C before addition to mucin-coated wells. Data are expressed relative to controls incubated with PBS (no TMU). CON, control, untreated bacteria; 1, 1 M TMU; 2, 0.5 M TMU; 3, 0.25 M TMU; 4, 0.125 M TMU; 5, 0.06 M TMU. TMU treatment had no significant effects on bacterial adherence to mucin.

also inhibited the adherence of plasmid-cured bacteria to polystyrene by 30 to 40% at concentrations over 0.125 M (Fig. 3B). However, in this case, inhibition of binding was not dose dependent. These findings suggest that even plasmid-cured organisms have some hydrophobic surface properties that are involved with adherence to polystyrene, but the surface hydrophobicity of virulent bacteria is greater and accounts for a larger proportion of their polystyrene binding. Treatment of *Y. enterocolitica* with TMU up to concentrations of 1 M did not affect bacterial viability. In addition, the effects of TMU were irreversible, since washing of organisms with PBS (up to three times) did not alter the TMU-dependent reduction in bacterial adherence to polystyrene.

We then examined whether hydrophobicity was involved in the binding of *Y. enterocolitica* to mucin. These studies were performed on rabbit intestinal mucin because it is more readily available than human mucin but shows similar characteristics of bacterial binding. Comparing adherence of plasmid-bearing organisms to mucin (shown in Fig. 2A) with that to polystyrene (Fig. 3A), it is evident that binding to mucin is an order of magnitude lower than that to the nonbiological substrate. As illustrated in Fig. 4, treatment with TMU (even at concentrations as high as 1 M) had no effect on adherence of virulent *Y. enterocolitica* to mucin, indicating that binding does not occur through hydrophobic interactions. Since binding of plasmid-cured bacteria to mucin is so poor, we did not assess the effects of TMU on these organisms, thinking that changes (particularly reductions) in adherence could not be accurately measured.

To investigate whether mucin could inhibit bacterial binding to polystyrene, virulent *Y. enterocolitica* organisms (~2×10^9) were preincubated for 1 h at 37°C with 100 μg of rabbit intestinal mucin, and then the mixture was added to polystyrene wells. Adherence to the polystyrene was reduced to 19.5% ± 2.0% of control levels, suggesting that mucin can mask hydrophobic adhesins on the bacteria.

A large variety of bacteria have been reported to interact with mucin carbohydrate, including *Streptococcus sanguis* and *Streptococcus mutans* (2, 7), *P. aeruginosa* (33, 35) and *P. cepacia* (36), species of *Staphylococcus* (39), strains of *E. coli* (3, 37, 48), and *Helicobacter pylori* (47). We therefore examined whether sugars could interfere with the binding of virulent *Y. enterocolitica* to rabbit intestinal mucin in hapten inhibition studies (Table 2). Galactose (Gal) and *N*-acetylgalactosamine (GalNAc) significantly reduced bacterial adherence to mucin at concentrations of 0.1 M, but binding could not be abolished even with concentrations of 0.5 M. Lactose [Gal (β1-4)glucose] decreased adherence to the same extent as Gal and GalNAc. Other monosaccharides tested had no inhibitory effect on the binding of *Y. enterocolitica* to mucin. Similarly, adherence was not diminished by the RGD peptide, suggesting that the bacterial adhesin for mucin is unlikely to involve invasin, which recognizes this integrin-binding domain and appears to be important for attachment and invasion of cell lines by *Y. enterocolitica* (10, 11). Finally, treatment of mucin-coated wells with antisera specifically against purified rabbit intestinal mucin or the disulfide-bound, 118-kDa glycoprotein component of mucin did not prevent bacterial adherence, suggesting that the antigenic sites of the mucin molecule are not the same as the binding site(s) for *Y. enterocolitica*.

TABLE 2. Hapten inhibition of *Y. enterocolitica* binding to rabbit intestinal mucin[a]

Hapten	Binding (% of control)
Control	100 ± 15
Galactose	66 ± 4*
N-Acetylgalactosamine	62 ± 5*
Glucose	75 ± 8
N-Acetylglucosamine	82 ± 7
Mannose	84 ± 8
Sialic acid	112 ± 13
Fucose	111 ± 15
Lactose	60 ± 5*
RGD peptide	107 ± 10

[a] Polystyrene microtiter plate wells were coated with purified rabbit intestinal mucin (50 μl of 1 mg/ml), and the remaining binding sites in the wells were blocked with skim milk (350 μl of 20 mg/ml). Plasmid-bearing bacteria (~2×10^{10} organisms per ml) were incubated with monosaccharide haptens (0.125 to 0.5 M final concentrations), lactose (0.06 to 0.25 M final concentration), or RGD peptide (1.25- to 5-mg/ml final concentration) for 1 h at 25°C, and then 50-μl aliquots of the suspension were added to mucin-coated wells. Data are expressed relative to controls in which bacteria were incubated with PBS only. *, significantly different from control ($P < 0.01$).

DISCUSSION

In previous studies, we showed that a virulent strain of *Y. enterocolitica* was capable of adhering to purified rabbit intestinal mucin while its isogenic, plasmid-cured derivative (which is not virulent [29]) exhibited markedly reduced binding (17). These findings suggested that the ability to adhere to mucin may constitute a virulence factor in *Y. enterocolitica*. On examining the attachment of *Y. enterocolitica* to 14 different preparations of human intestinal mucin (eight from normal subjects and six from patients with CF), we found that virulent bacteria bound to all mucins to a greater extent than plasmid-cured organisms. These findings reinforce the plasmid dependency of adhesion and also suggest that all of the mucins shared a common (if not identical) receptor. On comparing normal with CF mucins, no overall differences were observed in the ability to serve as a binding substrate for virulent (or plasmid-cured) *Y. enterocolitica*, despite the fact that CF intestinal mucin differs from normal mucin by having longer, more branched oligosaccharide chains, a higher sulfate content (49), and alterations in its nonglycosylated peptide moiety and macromolecular structure (19, 42). These findings imply that peripheral carbohydrate residues and naked peptide regions (which include the disulfide-bound, 118-kDa glycoprotein compo-

nent) of intestinal mucin may not contain the binding ligand for *Y. enterocolitica* and that the additional sulfate present in CF mucin may not impair bacterial attachment, as has been noted for *P. aeruginosa* (34).

Hydrophobic interactions have been shown to play a role in the binding of several bacteria to mucin (5, 37, 38). Expression of the virulence plasmid of *Y. enterocolitica* is known to render the organism more hydrophobic (13, 14, 22) and certainly increases its binding to polystyrene. That adherence to this nonbiological matrix was mostly dependent on hydrophobic interactions was confirmed by our experiments showing that binding could be inhibited by TMU. In contrast, however, adherence of plasmid-bearing *Y. enterocolitica* to mucin clearly does not involve hydrophobic interactions since it is not reduced even by high concentrations (1 M) of TMU. Studies of a variety of mucins have demonstrated that the nonglycosylated regions of their protein cores are rich in hydrophobic domains (8, 42, 44, 45). This would suggest that the binding site(s) for *Y. enterocolitica* in mucin is unlikely to be located in the exposed peptide moiety. Interestingly, preincubation of plasmid-bearing *Y. enterocolitica* with intestinal mucin significantly reduced subsequent binding of the organism to polystyrene, suggesting that mucin may mask hydrophobic adhesins on the bacterial surface and make the organism more hydrophilic. Using a two-phase partitioning system, Paerregaard et al. (28) found that incubation of *Y. enterocolitica* with crude intestinal mucus preparations also rendered the bacteria significantly more hydrophilic. Since it has been postulated that *Y. enterocolitica* may adhere to intestinal cell membranes through hydrophobic interactions (27, 28), it seems possible that mucus and mucin may protect the host by coating the bacterial surface, decreasing its hydrophobicity, and thereby interfering with attachment to epithelial cells.

Hapten inhibition studies suggested that *Y. enterocolitica* may interact with specific carbohydrate residues in the oligosaccharide chains of intestinal mucin, notably, Gal and GalNAc. However, while both of these monosaccharides (individually) were capable of significantly reducing the binding of *Y. enterocolitica* to mucin by ~40%, neither was capable of abolishing adherence, even at high (0.5 M) concentrations. It therefore appears that binding of *Y. enterocolitica* to mucin involves carbohydrate but the actual receptor must be made up of more than one sugar residue. Because Gal and GalNAc can occur in both the core (internal) and the peripheral (external) regions of mucin oligosaccharides, it is not yet possible to speculate which segment of the sugar chain carries the receptor for *Y. enterocolitica*.

In recent studies, Paerregaard et al. (28) could not demonstrate the involvement of carbohydrate in the binding of *Y. enterocolitica* to rabbit ileal mucus and concluded that the organism attached by hydrophobic interactions. However, these conclusions were based on the fact that individual monosaccharides did not inhibit bacterial adherence to crude mucus preparations, and experiments to confirm the involvement of hydrophobic interactions were not performed. Crude mucus is a complex secretion, containing not only mucin but also proteins, DNA, lipid, and probably membrane fragments from shed epithelial cells. *Y. enterocolitica* may interact with a variety of these components by different mechanisms. In support of this hypothesis, we previously showed that binding to mucin actually only accounted for 35 to 45% of the total binding to crude mucus preparations (17). Thus, overall binding to mucus may reflect the sum of a number of adhesive bonds, and inhibition of one type of interaction (with individual monosaccharides) while others remain intact may not cause a detectable change in gross binding. The differences between our findings and those of Paerregaard et al. (28) are therefore likely due to the use of purified mucin and not crude, unfractionated mucus as the binding substrate for *Y. enterocolitica*.

The mechanism by which *Y. enterocolitica* adheres to mucin is not yet known. Both chromosomally encoded (the *inv* gene product invasin and the *ail* gene product) and plasmid-mediated (YadA) surface proteins are known to promote bacterial attachment to and invasion of several cell lines (9–12, 26). Since invasin and the *ail* gene product are only synthesized by pathogenic strains of *Y. enterocolitica* (26, 30), they may be adhesins for mucin. However, reduced expression of invasin at 37°C (10), the fact that plasmid-bearing organisms cultured at 25°C (when invasin is produced) do not bind to mucin (17), and the fact that the RGD peptide (the integrin-binding domain for invasin) does not prevent adherence of *Y. enterocolitica* to mucin all suggest that invasin is not the bacterial adhesin for this substrate. Although the *ail* gene is expressed at 37°C, its receptor has not yet been defined. YadA is thought to be responsible for many of the plasmid-mediated changes in the surface properties of *Y. enterocolitica* (1, 12, 23) and has been implicated in the ability of the organism to bind to HEp-2 cells (9), fibronectin (46), various types of collagen (41), and intestinal brush border membranes (17, 27, 28). Since no specific receptors for YadA have been identified, it is generally assumed that its adhesive properties result from hydrophobic interactions with different substrates. If this assumption is correct, it would argue against YadA having an important role in the binding of *Y. enterocolitica* to intestinal mucin which is evidently not due to hydrophobic interactions. However, it remains to be determined whether attachments to mucin carbohydrate involve YadA, the *ail* gene product, or some other (as yet unidentified) adhesin.

In summary, we have shown that plasmid-bearing (but not plasmid-cured) *Y. enterocolitica* organisms are capable of binding well to purified intestinal mucins from humans and rabbits. Bacterial attachment appears to involve mucin carbohydrate (Gal and GalNAc residues) and is not hydrophobic in nature. Although interactions between bacteria and intestinal and/or colonic mucin may protect the host by preventing the organism from attaching to the underlying epithelium, they may also benefit the pathogen. If bacteria are able to bind strongly to components of the intestinal mucus layer, clearance of the organism by the motile and abrasive forces of digestion may be delayed and colonization of the intestinal tract may be favored. Moreover, if the rate of bacterial growth in and penetration of the mucus barrier exceeds the rate at which this layer naturally turns over and is eliminated from the gut, then clearance will be further delayed, again favoring colonization of the gut, invasion of the mucosa, and subsequent disease. Thus, the ability of *Y. enterocolitica* to interact with mucin may be an important early step in the pathogenic process. Future studies will attempt to define more precisely the structure of the receptor and region of the mucin molecule that are required for adherence of *Y. enterocolitica*.

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REFERENCES

1. **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.
2. **Bergey, E. J., M. J. Levine, M. S. Reddy, S. D. Bradway, and I. Al-Hashimi.** 1986. Use of the photoaffinity cross-linking agent *N*-hydroxysuccinimidyl-4-azidosalicylic acid to characterize salivary-glycoprotein-bacterial interactions. Biochem. J. **234:** 43–48.
3. **Cohen, P. S., J. C. Arruda, T. J. Williams, and D. C. Laux.** 1985. Adhesion of a human fecal *Escherichia coli* strain to mouse colonic mucus. Infect. Immun. **48:**139–145.
4. **Cover, T. L., and R. C. Aber.** 1989. *Yersinia enterocolitica*. N. Engl. J. Med. **321:**16–24.
5. **Drumm, B., A. W. Neumann, Z. Policova, and P. M. Sherman.** 1989. Bacterial cell surface hydrophobicity properties in the mediation of in vitro adhesion by the rabbit enteric pathogen *Escherichia coli* strain RDEC-1. J. Clin. Invest. **84:**1588–1594.
6. **Gemski, P., J. R. Lazere, and T. Casey.** 1980. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. **27:**682–685.
7. **Gibbons, R. J., and J. V. Qureshi.** 1978. Selective binding of blood group-reactive salivary mucins by *Streptococcus mutans* and other oral organisms. Infect. Immun. **22:**665–671.
8. **Gwozdzinski, K., A. Slomiany, H. Nishikawa, K. Okazaki, and B. L. Slomiany.** 1988. Gastric mucin hydrophobicity: effects of associated and covalently bound lipids, proteolysis, and reduction. Biochem. Int. **17:**907–917.
9. **Heesemann, J., and L. Gruter.** 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.
10. **Isberg, R. R., A. Swain, and S. Falkow.** 1988. Analysis of expression and thermoregulation of the *Yersinia pseudotuberculosis inv* gene with hybrid proteins. Infect. Immun. **56:**2133–2138.
11. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.
12. **Kapperud, G., E. Namork, M. Skurnik, and T. Nesbakken.** 1987. Plasmid-mediated surface fibrillae of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*: relationship to the outer membrane protein YOP1 and possible importance for pathogenesis. Infect. Immun. **55:**2247–2254.
13. **Lachica, R. V., and D. L. Zink.** 1984. Plasmid-associated cell surface charge and hydrophobicity of *Yersinia enterocolitica*. Infect. Immun. **44:**540–543.
14. **Laird, W. J., and D. C. Cavanaugh.** 1980. Correlation of autoagglutination and virulence of *Yersiniae*. J. Clin. Microbiol. **11:**430–432.
15. **Lian, C.-J., W. S. Hwang, and C. H. Pai.** 1987. Plasmid-mediated resistance to phagocytosis in *Yersinia enterocolitica*. Infect. Immun. **55:**1176–1183.
16. **Lian, C.-J., and C. H. Pai.** 1985. Inhibition of human neutrophil chemiluminescence by plasmid-mediated outer membrane proteins of *Yersinia enterocolitica*. Infect. Immun. **49:**145–151.
17. **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.
18. **Mantle, M., G. G. Forstner, and J. F. Forstner.** 1984. Biochemical characterization of the component parts of intestinal mucin from patients with cystic fibrosis. Biochem. J. **224:**345–354.
19. **Mantle, M., and Stewart, G.** 1989. Intestinal mucins from normal subjects and patients with cystic fibrosis: variable contents of the disulphide-bound 118kDa glycoprotein and different reactivities with an anti-(118kDa glycoprotein) antibody. Biochem. J. **259:**243–253.
20. **Mantle, M., and E. Thakore.** 1988. Rabbit intestinal and colonic mucins: isolation, partial characterization, and measurement of secretion using an enzyme-linked immunoassay. Biochem. Cell Biol. **66:**1045–1054.
21. **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.
22. **Martinez, R. J.** 1983. Plasmid-mediated and temperature-regulated surface properties of *Yersinia enterocolitica*. Infect. Immun. **41:**921–930.
23. **Martinez, R. J.** 1989. Thermoregulation-dependent expression of *Yersinia enterocolitica* protein 1 imparts serum resistance to *Escherichia coli* K-12. J. Bacteriol. **171:**3732–3739.
24. **Michiels, T., and G. R. Cornelis.** 1990. Secretion of Yop proteins of *Yersinia*. Infect. Immun. **58:**2840–2849.
25. **Michiels, T., and G. R. Cornelis.** 1991. Secretion of hybrid proteins by the *Yersinia* Yop export system. J. Bacteriol. **173:**1677–1685.
26. **Miller, V. L., J. J. Farmer III, W. E. Hill, and S. Falkow.** 1989. The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. Infect. Immun. **57:** 121–131.
27. **Paerregaard, A., F. Espersen, and N. Baker.** 1990. The influence of properties encoded by the *Yersinia* virulence plasmid on adhesion of *Yersinia enterocolitica* to ileal brush border membrane vesicles. Acta Pathol. Microbiol. Immunol. Scand. **98:** 927–932.
28. **Paerregaard, A., F. Espersen, O. M. Jensen, and M. Skurnik.** 1991. 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. Infect. Immun. **59:**253–260.
29. **Pai, C. H., and L. deStephano.** 1982. Serum resistance associated with virulence in *Yersinia enterocolitica*. Infect. Immun. **35:**605–611.
30. **Pierson, D. E., and S. Falkow.** 1990. Nonpathogenic isolates of *Yersinia enterocolitica* do not contain functional *inv*-homologous sequences. Infect. Immun. **58:**1059–1064.
31. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31:**775–782.
32. **Portnoy, D. A., H. Wolf-Watz, I. Bolin, A. B. Beeder, and S. Falkow.** 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43:**108–114.
33. **Ramphal, R., C. Carnoy, S. Fievre, J.-C. Michalski, N. Houdret, G. Lamblin, G. Strecker, and P. Roussel.** 1991. *Pseudomonas aeruginosa* recognizes carbohydrate chains containing type 1 (Galβ1-3GlcNAc) or type 2 (Galβ1-4GlcNAc) disaccharide units. Infect. Immun. **59:**700–704.
34. **Ramphal, R., N. Houdret, L. Koo, G. Lamblin, and P. Roussel.** 1989. Differences in adhesion of *Pseudomonas aeruginosa* to mucin glycopeptides from sputa of patients with cystic fibrosis and chronic bronchitis. Infect. Immun. **57:**3066–3071.
35. **Ramphal, R., and M. Pyle.** 1983. Evidence for mucins and sialic acid as receptors for *Pseudomonas aeruginosa* in the lower respiratory tract. Infect. Immun. **41:**339–344.
36. **Sajjan, S. U., M. Corey, M. A. Karmali, and J. F. Forstner.** 1992. Binding of *Pseudomonas cepacia* to normal human intestinal mucin and respiratory mucin from patients with cystic fibrosis. J. Clin. Invest. **89:**648–656.
37. **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.
38. **Sajjan, S. U., J. Reisman, P. Doig, R. T. Irvin, and J. F. Forstner.** 1992. Binding of nonmucoid *Pseudomonas aeruginosa* to normal human intestinal mucin and respiratory mucin from patients with cystic fibrosis. J. Clin. Invest. **89:**657–665.
39. **Sanford, B. A., V. L. Thomas, and M. A. Ramsay.** 1989. Binding of staphylococci to mucus in vivo and in vitro. Infect. Immun. **57:**3735–3742.
40. **Schiemann, D. A.** 1989. *Yersinia enterocolitica* and *Yersinia*

pseudotuberculosis, p. 601–672. *In* M. P. Doyle (ed.), Foodborne bacterial pathogens—1989. Marcel Dekker, Inc., New York.

41. **Schulze-Koops, H., H. Burkhardt, J. Heesemann, K. von der Mark, and F. Emmrich.** 1992. Plasmid-encoded outer membrane protein YadA mediates specific binding of enteropathogenic yersiniae to various types of collagen. Infect. Immun. **60:**2153–2159.
42. **Shankar, V., B. Naziruddin, S. R. de la Rocha, and G. P. Sachdev.** 1990. Evidence of hydrophobic domains in human respiratory mucins. Effect of sodium chloride on hydrophobic binding properties. Biochemistry **29:**5856–5864.
43. **Skurnik, M.** 1985. Expression of antigens encoded by the virulence plasmid of *Yersinia enterocolitica* under different growth conditions. Infect. Immun. **47:**183–190.
44. **Slomiany, B. L., V. L. N. Murty, J. Sarosiek, J. Piotrowski, and A. Slomiany.** 1988. Role of associated and covalently bound lipids in salivary mucin hydrophobicity: effect of proteolysis and disulfide bridge reduction. Biochem. Biophys. Res. Commun. **151:**1046–1053.
45. **Smith, B. F., and J. T. LaMont.** 1984. Hydrophobic binding properties of bovine gallbladder mucin. J. Biol. Chem. **259:**12170–12177.
46. **Tertti, R., M. Skurnik, T. Vartio, and P. Kuusela.** 1992. Adhesion protein YadA of *Yersinia* species mediates binding of bacteria to fibronectin. Infect. Immun. **60:**3021–3024.
47. **Tzouvelekis, L. S., A. F. Mentis, A. M. Makris, C. Spiliadis, C. Blackwell, and D. M. Weir.** 1991. In vitro binding of *Helicobacter pylori* to human gastric mucin. Infect. Immun. **59:**4252–4254.
48. **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.
49. **Wesley, A., J. Forstner, R. Qureshi, M. Mantle, and G. Forstner.** 1983. Human intestinal mucin in cystic fibrosis. Pediatr. Res. **17:**65–69.