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Binding of *Yersinia enterocolitica* to Purified, Native Small Intestinal Mucins from Rabbits and Humans Involves Interactions with the Mucin Carbohydrate Moiety

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Plasmid-bearing (but not plasmid-cured) *Yersinia enterocolitica* is known to bind to purified small intestinal mucins from rabbits and humans. This study examined which region(s) of the mucin molecule is important for bacterial adherence. Pronase digestion of mucin and removal of nonglycosylated or poorly glycosylated peptide regions had no effect on bacterial binding, suggesting that plasmid-bearing *Y. enterocolitica* interacts with mucin carbohydrate. Periodate oxidation also did not alter bacterial adherence, indicating that vicinal hydroxyl groups in the mucin sugars are not important for binding. Boiling of mucin, depolymerization by reduction of disulfide bonds, or removal of noncovalently associated lipid actually enhanced bacterial adherence, suggesting that plasmid-bearing *Y. enterocolitica* can interact with additional domains in the mucin molecule revealed by these treatments. These domains were destroyed by pronase digestion. In delipidated mucin (but not in reduced or boiled mucin), binding to these domains appeared to be hydrophobic since it could be prevented by treatment of bacteria with tetramethyl urea. Oligosaccharides obtained from both human and rabbit small intestinal mucins were capable of inhibiting attachment of plasmid-bearing (but not plasmid-cured) *Y. enterocolitica* to mucin. After removal of terminal and backbone sugar residues by treatment of mucin with trifluoromethanesulfonic acid, binding of plasmid-bearing bacteria increased significantly when *N*-acetylgalactosamine, either alone or with galactose attached, was revealed, indicating that core regions of the sugar side chains are involved in bacterial binding. Adherence of plasmid-cured organisms was unaffected by trifluoromethanesulfonic acid treatment of mucin. We concluded that virulent *Y. enterocolitica* interacts with the carbohydrate moiety of native small intestinal mucin through a plasmid-mediated process. When mucin becomes denatured, binding of the organism can increase through hydrophobic and nonhydrophobic interactions with (most likely) the mucin protein.

Yersinia enterocolitica, a gram-negative, facultatively anaerobic bacterium, is recognized as an important cause of infectious enteritis (5, 43). All pathogenic strains of the organism (in contrast to nonpathogenic environmental isolates) contain a 42- to 50-MDa plasmid (8, 37), indicating that the plasmid is essential for virulence. The plasmid encodes a fibrillar adhesin known as YadA, which is a true outer membrane protein (17), and a variety of other proteins, including those referred to as yersinia outer membrane proteins because they are secreted and then adhere to the outer surface of the organism (30, 37, 38). Expression of the plasmid is regulated by both temperature and calcium availability (8, 37, 38, 47). Although the functions of only a few of these plasmid-encoded proteins have been elucidated, their production changes the surface charge and hydrophobicity of the bacterium; increases bacterial binding to cell lines, epithelial cell membranes, collagen, and fibronectin; and allows the organism to resist phagocytosis and the bacteriocidal effects of serum (1, 12, 17, 18, 21, 22, 28, 29, 34, 44, 50). Thus, plasmid proteins may be involved in attachment to and penetration of intestinal tissue and may help the organism to survive and proliferate in the mucosa.

Following infection, *Y. enterocolitica* must cross the mucus barrier that covers the intestinal epithelium before invading the mucosa and causing tissue damage and clinical disease (5, 43). The mammalian intestine is lined with a layer of mucus gel produced by goblet cells. The major component of mucus that is responsible for its gel-forming properties is mucin, a large (>2 × 10^6 Da), polymeric molecule composed of highly glycosylated (~80% [by weight] carbohydrate) glycoprotein monomers and a smaller, less glycosylated component of 118 kDa that may be the cleaved C-terminal region of one type of glycoprotein monomer (2, 7, 23, 27, 54). The entire mucin polymer is held together by disulfide bonds (2, 7, 23, 27). The polymeric structure of mucin is critical for gel formation: reduction of disulfide bridges or proteolytic digestion of nonglycosylated mucin peptide releases glycoprotein monomers that do not gel under normal physiologic conditions (2, 45). The mucus layer protects the intestine by forming a barrier that invading bacteria (like *Y. enterocolitica*) must interact with and penetrate to gain entry into the mucosa.

In earlier studies on the first steps of the penetration process, we showed that a virulent, plasmid-bearing strain of *Y. enterocolitica* (MCH700S, serotype O:3) bound to rabbit small intestinal and colonic mucus and purified mucin (22). Binding occurred only when bacteria were cultured at 37°C, not after culturing at 25°C, which suppressed plasmid expression. In addition, a plasmid-cured isogenic strain (MCH700L) exhibited markedly diminished binding, suggesting that bacterial adherence to small intestinal and colonic mucus and mucin involves plasmid-encoded proteins. The findings from these studies have recently been independently confirmed (32, 33). Subsequently, we demonstrated that bacterial binding to purified rabbit small intestinal mucin, although dependent on

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plasmid expression, was not related to the surface hydrophobicity of the organism but may involve interactions with sugars present in the mucin oligosaccharide side chains (25). Hapten inhibition studies revealed that certain sugars (particularly galactose and *N*-acetylgalactosamine) were capable of significantly decreasing (by approximately 40%) bacterial adherence to mucin but not completely abolishing it, suggesting that the binding site for virulent *Y. enterocolitica* in mucin comprises more than a single sugar residue (25).

The objectives of the present study were to pursue these earlier observations further and to explore in more detail which region(s) of small intestinal mucin is important for bacterial binding. This was accomplished by investigating whether adherence of *Y. enterocolitica* is altered by treatment of the mucin in ways that affect its protein component, carbohydrate moiety, or macromolecular structure and/or conformation.

MATERIALS AND METHODS

Preparation of mucin. Mucin was purified, in the presence of proteolytic inhibitors, from the small intestines of New Zealand rabbits (the jejunum and ileum combined, from the ligament of Trietz to the ileocecal junction) as described previously (23, 27). Following equilibrium density 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 compositional analyses for amino acid and sugar contents (23, 27). 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. Purified rabbit small intestinal mucin was used for most of our studies because it is readily accessible and resembles small intestinal mucins from other species in terms of its composition and structure and its binding capacity for *Y. enterocolitica* (7, 22, 23, 25, 27). However, for some experiments, human and rat small intestinal mucins were required. These were isolated and purified exactly as described above for rabbit mucin (27).

Bacteria and growth conditions. Two isogenic strains of *Y. enterocolitica* (MCH700S [plasmid bearing] and MCH700L [plasmid cured], originally obtained from C. Pai, Asian Medical Centre, Seoul, Korea) were used for the present studies. Plasmid-bearing organisms were initially isolated from a patient with enteritis and are known to cause clinical disease in rabbits (34, 35). Isogenic strain MCH700L was cured of its plasmid as described by Pai and DeStephano (34) and is known not to produce disease in rabbits (20, 34). The presence or absence of the virulence plasmid was confirmed by agarose gel electrophoresis (37). The binding properties of these two organisms to rabbit, human, and rat small intestinal mucins have been described previously (22, 25). Samples from frozen stocks of both strains were grown as in earlier studies (22, 23). After overnight growth at 25°C in modified Eagle medium (no. 320-1965; GIBCO Laboratories, Grand Island, N.Y.) without methionine, bacteria were diluted (1 in 10) into fresh medium and incubated at 37°C for 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, followed 1 h later by 5 mg of cold methionine. 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 $\sim 10^{10}$ organisms per ml (confirmed by dilution and colony counting). To determine the radioactivity incorporated per bacterium, a 50-μl aliquot of the final suspension was counted by scintillation spectrometry.

Binding assay. The binding of *Y. enterocolitica* to purified small intestinal mucin was assessed by using polystyrene microtiter plates as described earlier (22, 25). Briefly, four or six wells were coated with 50 μg of mucin in 50 μl of PBS 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 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 determined by scintillation spectrometry. Experiments showed that these desorption conditions were sufficient to remove >95% of the radioactivity (and hence bacteria) from the wells. The number of *Y. enterocolitica* bacteria 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.

Treatments of the mucin substrate. The mucin substrate was treated as follows: (i) Purified rabbit small intestinal mucin was exhaustively digested with pronase (type XIV from *Streptomyces griseus*; Sigma Chemical Co., St. Louis, Mo.; final enzyme-to-substrate ratio, 1:3) for 72 h at 37°C, as described previously (23, 27), and then dialyzed against PBS (molecular mass cutoff, 1,000 Da). (ii) Mucin (2 mg) was digested with protease-free endo-β-*N*-acetylglucosaminidase F (endo F; 4 U) in PBS containing 50 mM disodium EDTA (pH 7.6) for 24 h at 37°C (as described by the manufacturer [Boehringer GmbH, Mannheim, Federal Republic of Germany]). Digested mucin was dialyzed overnight against PBS to remove free oligosaccharides. (iii) Mucin (2 mg/ml in H_2O) was oxidized with periodic acid (final concentration, 0.01 M in 0.2 M acetate buffer, pH 4.0) for 2 h at 37°C and then dialyzed against PBS overnight. (iv) Mucin was delipidated by extraction (three times) with chloroform-methanol (2:1) (24). Dried, delipidated mucin samples were weighed and reconstituted in PBS. Chloroform-methanol extracts were dried under nitrogen, reconstituted in 200 μl of chloroform, and applied to thin-layer chromatography plates (silica gel G; Analtech Inc., Newark, Del.). Plates were then sprayed with 0.6% potassium dichromate in 50% H_2SO_4 and developed for 10 min at 80°C. A brown stain at the application point of the first chloroform-methanol extract only indicated the presence of lipids. (v) Mucin was reduced with 0.2 M 2-mercaptoethanol at 100°C for 5 min, alkylated with iodoacetamide (0.5 M) at 4°C overnight (23, 27), and then dialyzed against PBS. (vi) Mucin was heated at 100°C for 10 min and then cooled. The final solutions obtained following all of the above-described procedures were adjusted to a volume that gave a concentration equivalent to 1 mg of original mucin per ml with PBS. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (19), and gels were stained with Bio-Rad silver stain (bulletin 1089, Bio-Rad Laboratories, Richmond, Calif.). Treated mucins were then used to coat the wells in the binding assay described above, and the ability of *Y. enterocolitica* to adhere to these modified preparations was assessed.

In separate experiments, we verified that treatment of the mucin did not alter its binding to the microtiter plate wells during the first step of the assay procedure. The protein moiety of purified mucin was ^{14}C labelled by reductive methylation (16), and then aliquots were either boiled for 10 min, reduced and alkylated, or delipidated (as described above). Microtiter plate wells were subsequently coated with 50 μl of untreated, boiled, reduced and alkylated, or delipidated, radiolabelled mucin exactly as performed for the bacterial binding assay. After overnight incubation, wells were washed to remove unbound mucin, filled with 350 μl of sodium dodecyl sulfate (1% [wt/vol] solution), and incubated overnight at 37°C. The solution was then removed, and radioactivity counts were determined by scintillation spectrometry. These conditions removed >95% of the radioactivity (and hence mucin) from the wells.

To investigate whether hydrophobic interactions were involved in the binding of plasmid-bearing *Y. enterocolitica* to treated mucin preparations, bacterial suspensions were first incubated for 1 h at 25°C in tetramethyl urea (TMU; a strong inhibitor of hydrophobic interactions) at a final concentration of 0.5 M (25). Bacteria were harvested by centrifugation, washed to remove excess TMU, and then added to wells coated with either native or treated rabbit small intestinal mucin (50 μg) and skim milk. As in previous studies (25), experimental findings were identical regardless of whether or not TMU-treated bacteria were washed before addition to the mucin-coated wells.

Removal of mucin oligosaccharides. Purified rabbit and human small intestinal mucins (20 mg of each) were subjected to alkaline borohydride reduction (β elimination of oligosaccharides) by treatment with 0.1 M KOH and 1 M KBH_4 at 45°C for 26 h (11). After titration to pH 4.5 with 4 M acetic acid, the reaction mixture was passed through an AG50WX8 ion-exchange column (Bio-Rad Laboratories). Oligosaccharides were eluted with water, taken to pH 4.5 with triethylamine, and evaporated to dryness. After removal of borate by extraction (six times) with methanol, oligosaccharides were dissolved in PBS at a concentration of 3 mg (dry weight)/ml. The protein contents of these preparations were established by amino acid analyses, and the sugar compositions were determined by gas-liquid chromatography (4).

Oligosaccharides recovered from the above-described experiments were then used as haptens in inhibition studies. Bacteria were incubated for 1 h at 25°C with oligosaccharide preparations (50 μl), and then the mixtures were added to wells coated with purified rabbit small intestinal mucin (50 μg) and skim milk. In control incubations, oligosaccharides were substituted with PBS.

Purified rabbit, human, and rat small intestinal mucins (3 mg of each) were treated with trifluoromethanesulfonic acid (TFMS) for 2 h at 25°C to remove terminal and backbone sugar residues (6, 26). Partially deglycosylated preparations were then dialyzed against PBS overnight. Loss of sugars was confirmed by gas-liquid chromatography (4), and recovery of protein was determined by amino acid analyses as described previously (26). The final solutions obtained were adjusted to a concentration equivalent to 1 mg of original mucin per ml with PBS. Partially deglycosylated mucins were then used to coat the wells in the binding assay described above, and adherence of *Y. enterocolitica* was determined.

RESULTS

In earlier studies, we showed that virulent (plasmid-bearing) *Y. enterocolitica* binds to purified rabbit and human small intestinal mucins to a greater extent than do nonvirulent (plasmid-cured) bacteria (22, 25). To define the nature of the receptor site(s) in the mucin molecule that is responsible for bacterial binding, we investigated whether adherence of *Y. enterocolitica* to purified rabbit small intestinal mucin changes following treatments of the mucin that affect its protein or carbohydrate moiety or overall polymeric structure and/or conformation.

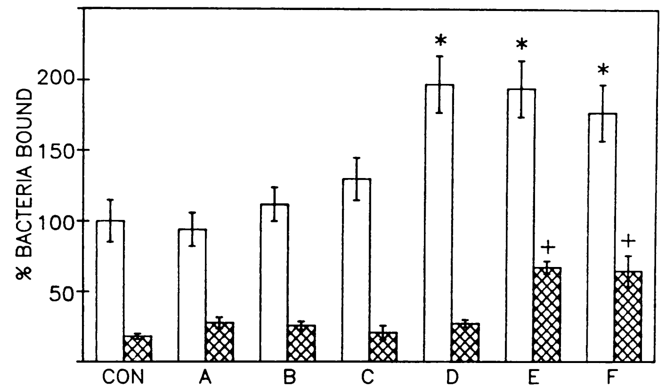

FIG. 1. Adherence of *Y. enterocolitica* to treated preparations of purified rabbit small intestinal mucin. CON, untreated mucin; A, mucin after pronase digestion; B, mucin after digestion with endo F; C, mucin after periodate oxidation; D, mucin after extraction with chloroform-methanol; E, mucin after reduction with 2-mercaptoethanol; F, boiled mucin. Following each treatment, mucins were adjusted to a final concentration equivalent to 1 mg of original mucin per ml and 50-μl aliquots were immobilized on polystyrene microtiter plate wells. The remaining binding sites in the wells were blocked with skim milk (350 μl of a 20-mg/ml concentration) before addition of bacteria (~10^9 organisms per 50 μl). Open bars denote binding of plasmid-bearing *Y. enterocolitica*, and cross-hatched bars show binding of plasmid-cured organisms. Binding of plasmid-bearing *Y. enterocolitica* to untreated mucin was set at 100% (equivalent to ~5×10^6 bacteria), and all of the other data are expressed relative to this. *, significantly different from the binding of virulent bacteria to untreated native mucin ($P < 0.05$). +, significantly different from the binding of nonvirulent bacteria to untreated mucin ($P < 0.05$). In all cases, adherence of plasmid-cured organisms was significantly lower than that of plasmid-bearing bacteria ($P < 0.02$).

Treatments of the mucin substrate. Adherence of both plasmid-bearing and plasmid-cured *Y. enterocolitica* isolates was unaffected by exhaustive pronase digestion of rabbit small intestinal mucin (Fig. 1). Digestion dissociates the polymer into smaller monomers and degrades nonglycosylated or poorly glycosylated peptide regions, including the 118-kDa glycoprotein (23, 27) (Fig. 2). The carbohydrate component of the mucin remains unchanged following pronase digestion, while the protein composition shows enrichment in serine, threonine, and proline at the expense of all other amino acids, as described previously (23). Endo F digestion of mucin similarly had no effect on the binding of either bacterial strain. This enzyme cleaved high-mannose and/or complex-type N-glycans from the 118-kDa glycoprotein of the mucin and reduced its molecular mass to ~100 kDa on sodium dodecyl sulfate-polyacrylamide gels (Fig. 2). Periodate oxidation of the mucin caused a slight but nonsignificant rise in the binding of plasmid-bearing bacteria but had no effect on adherence of plasmid-cured organisms.

In previous studies, we showed that trace amounts of lipid (~5% [by weight]) remain noncovalently bound to human small intestinal mucin during its purification process (24).

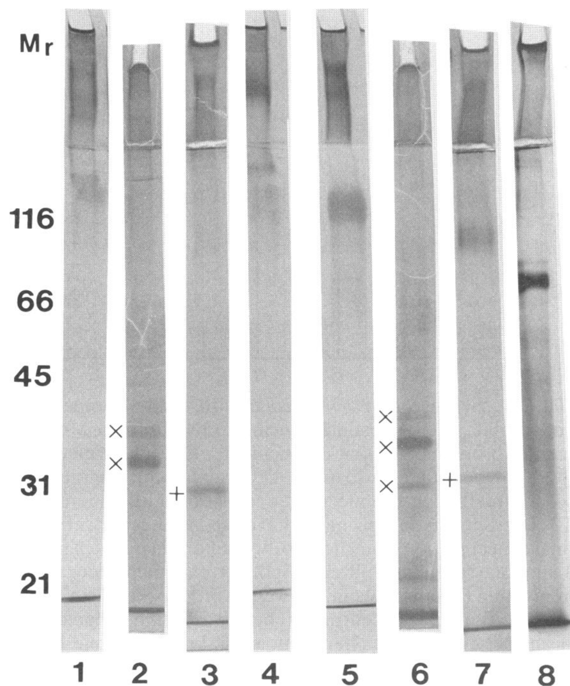

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified rabbit small intestinal mucin. Lanes: 1 and 5, untreated native mucin; 2 and 6, mucin after pronase digestion; 3 and 7, mucin after digestion with endo F; 4 and 8, mucin after treatment with TFMS. Samples 1 to 4 were run under nonreducing conditions, and samples 5 to 8 were reduced with 0.2 M 2-mercaptoethanol. Electrophoresis was performed on 10% separating gels with 5.7% stacking gels, and gels were stained with silver. M_r, molecular weight standards (10^3). Note that the 118-kDa glycoprotein component of the mucin is destroyed by pronase digestion and decreased in molecular mass after endo F or TFMS digestion. The symbols × and + indicate pronase and endo F, respectively.

When rabbit small intestinal mucin was treated with chloroform-methanol, 93% (by weight) of the original mucin was recovered in two separate delipidation experiments and small amounts of lipid were detected in the first (but not subsequent) chloroform-methanol extracts. Following this delipidation, adherence of plasmid-bearing (but not plasmid-cured) organisms to mucin was markedly enhanced (Fig. 1). Cleavage of disulfide bonds by boiling in reducing agents is known to depolymerize small intestinal mucin into its high-molecular-mass glycoprotein monomers and release the 118-kDa glycoprotein (23, 27). Such treatment resulted in a significant increase in the binding of both plasmid-bearing and plasmid-cured *Y. enterocolitica* strains to the mucin. Adherence of both virulent and nonvirulent organisms was also markedly enhanced following just boiling of the mucin (without reduction). With the exception of pronase digestion, none of the above-described treatments alters the amino acid composition of the mucin (23, 24).

By using ^{14}C-radiolabelled mucin to coat the microtiter plate wells, we confirmed that mucin adherence to the wells (as assessed from the counts bound) was unaffected by boiling, reduction and alkylation, or delipidation (2,400 ± 110 dpm bound for untreated mucin, 2,200 ± 60 dpm bound for boiled mucin, 2,300 ± 100 dpm bound for reduced and alkylated mucin, and 2,500 ± 190 dpm bound for delipidated mucin). Thus, the differences in bacterial adherence observed in the above-described experiments were not due to changes in the binding of treated mucin to the microtiter plate wells.

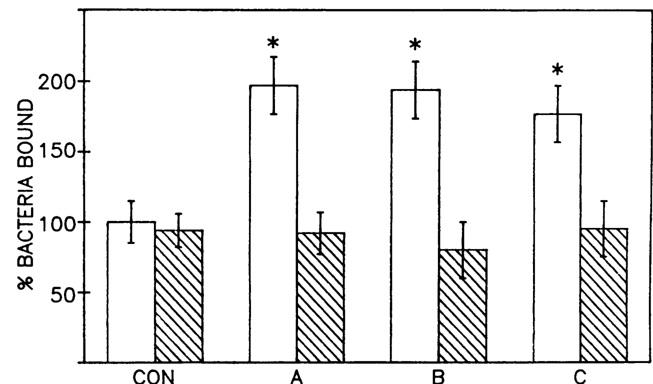

FIG. 3. Adherence of plasmid-bearing *Y. enterocolitica* to delipidated, reduced, and boiled rabbit small intestinal mucin before and after pronase digestion. Preparations of delipidated, reduced, and boiled small intestinal mucin were subjected to exhaustive pronase digestion. Binding of virulent *Y. enterocolitica* to the mucin before and after digestion was then assessed as described in the legend to Fig. 1. CON, native mucin; A, mucin after extraction with chloroform-methanol; B, mucin after reduction with 2-mercaptoethanol; C, boiled mucin. Open bars show binding before pronase digestion, and cross-hatched bars show binding after digestion. *, significantly different from binding to untreated mucin and all pronase-digested preparations ($P < 0.05$).

Effects of TMU on bacterial binding to native and treated mucins. The increased binding of plasmid-bearing *Y. enterocolitica* to small intestinal mucin following delipidation, reduction, or boiling raised the question of whether these treatments reveal potential hydrophobic attachment sites in the mucin for the bacteria. To address this question, bacteria were incubated for 1 h with TMU (a strong inhibitor of hydrophobic interactions) at a final concentration of 0.5 M and then added to mucin-coated wells. Treatment with this concentration of TMU has previously been shown to cause an 80% reduction in the binding of plasmid-bearing *Y. enterocolitica* to polystyrene, a known hydrophobic surface, but does not alter the adherence of bacteria to untreated rabbit small intestinal mucin (25). Binding of virulent *Y. enterocolitica* to either reduced or boiled mucin was unaffected by TMU—adherence remained elevated above adherence to untreated native mucin. However, TMU did decrease bacterial attachment to delipidated mucin by approximately 50% such that binding was essentially the same as that to native mucin (data not shown).

Pronase digestion of treated mucins. To determine whether the increase in bacterial adherence to delipidated, reduced, or boiled mucin was due to exposure of additional binding domains in the mucin protein moiety, these mucins were exhaustively digested with pronase. Digestion decreased binding of plasmid-bearing *Y. enterocolitica* to levels observed in untreated native mucin (Fig. 3). In agreement with previous studies (23), pronase digestion of reduced or boiled mucin (delipidated mucin was not tested) resulted in the production of glycoprotein monomers with the same amino acid and sugar composition as those released following pronase digestion of native mucin. Since all of the pronase digests appeared to be compositionally the same, it is unlikely that the decrease in bacterial adherence to reduced or boiled (or delipidated) mucin after proteolysis resulted from diminished attachment of these mucins to the microtiter plate wells because a similar decrease in bacterial binding was not observed after pronase digestion of native mucin.

Removal of mucin oligosaccharides. Purified rabbit and

TABLE 1. Composition of oligosaccharides from rabbit and human small intestinal mucins[a]

Sugar	Mol% in:			
	Rabbit mucin	Rabbit OS[b]	Human mucin	Human OS
Fucose	9.5	11.8	16.6	16.1
Mannose	Trace	ND[c]	Trace	ND
Galactose	23.9	25.5	20.7	18.4
N-Acetylgalactosamine	33.8	28.4	27.8	27.9
N-Acetylglucosamine	25.6	25.8	22.6	25.1
Sialic acid	7.2	8.4	12.3	12.5

[a] Purified rabbit and human small intestinal mucins were subjected to alkaline borohydride reduction (β elimination of oligosaccharides) by treatment with 0.1 M KOH and 1 M KBH_4 at 45°C for 26 h. After titration to pH 4.5 with 4 M acetic acid, the reaction mixture was passed through an AG50WX8 ion-exchange column. Oligosaccharides were eluted with water, evaporated to dryness, and extracted with methanol to remove borate. Oligosaccharides were dissolved in PBS at a concentration of 3 mg (dry weight)/ml. The protein contents of these preparations were established from amino acid analyses, and the sugar compositions were determined by gas-liquid chromatography. The carbohydrate/protein ratios (weight/weight) were as follows: rabbit mucin, 2.9; rabbit oligosaccharides, 6.8; human mucin, 4.6; human oligosaccharides, 8.7.

[b] OS, oligosaccharides.

[c] ND, not detected.

human small intestinal mucins were subjected to alkaline borohydride reduction, and β-eliminated oligosaccharides were recovered for use in hapten inhibition studies. Chemical analyses revealed that the oligosaccharide preparations had essentially the same sugar compositions as the untreated native mucins but a much lower protein content (Table 1). When oligosaccharides were assessed for the ability to inhibit binding of plasmid-bearing *Y. enterocolitica* to purified rabbit small intestinal mucin, those obtained from rabbit mucin decreased bacterial adherence to 28% ± 4% of control values while those obtained from human mucin reduced binding to 19% ± 2% of controls. Binding of plasmid-cured bacteria was not inhibited by either rabbit or human mucin oligosaccharides.

Finally, purified preparations of rabbit, human, and rat small intestinal mucins were treated with TFMS to remove terminal and backbone sugar residues and allow isolation of mucin peptide with only internal core sugars attached. Hydrolysis with TFMS under the conditions used in the present experiments resulted in loss of approximately 80% of the mucin carbohydrate (Table 2) and decreased the molecular mass of the 118-kDa glycoprotein component to ~70 kDa (Fig. 2). Several minor bands with much lower staining intensity than the 118-kDa band were also detected on the gels, suggesting small amounts of breakdown of the mucin peptide during TFMS treatment. In rabbit small intestinal mucin, only *N*-acetylgalactosamine remained attached to the mucin peptide core, as judged from gas-liquid chromatographic analyses (Table 2). In human small intestinal mucin, *N*-acetylgalactosamine and *N*-acetylglucosamine (70 and 20%, respectively, of the amounts originally present in native mucin) were left attached to the peptide core (Table 2). After TFMS hydrolysis of rat small intestinal mucin, *N*-acetylgalactosamine and galactose (70 and 40%, respectively, of the amounts originally present in native mucin) remained bound to the peptide core (Table 2). None of the three mucins showed significant changes in the composition of the peptide moiety following TFMS treatment, and total protein recovery approximated 80%. Adherence of plasmid-bearing *Y. enterocolitica* to partially deglycosylated human mucin was slightly, but not significantly, decreased compared with that to untreated mucin, while significant increases were observed in the binding of virulent bacteria to both partially deglycosylated rabbit and rat mucins (Fig. 4). Since only two of three partially deglycosylated mucins showed enhanced bacterial binding after TFMS treatment, it seems unlikely that those increases were a reflection of

TABLE 2. Compositions of purified rabbit, human, and rat small intestinal mucins before and after treatment with TFMS[a]

Component(s) or parameter	Mol%, ratio, or % in:					
	Rabbit mucin		Human mucin		Rat mucin	
	Before TFMS	After TFMS	Before TFMS	After TFMS	Before TFMS	After TFMS
Amino acids						
Asp, Glu	15.7	18.4	11.3	10.9	11.5	13.6
Ser, Thr, Pro	38.7	38.2	56.8	58.2	61.5	61.8
Gly, Ala, Val, Ile, Leu	28.5	22.0	23.2	23.6	18.2	16.7
Rest	17.1	21.4	8.7	7.3	8.8	7.9
Sugars						
Fucose	9.5	0	16.6	0	13.3	0
Mannose	Trace	0	Trace	0	Trace	0
Galactose	23.9	0	20.7	0	24.3	40
GalNac[b]	33.8	100	27.8	85	22.3	60
GlcNAc[c]	25.6	0	22.6	15	24.7	0
Sialic acid	7.2	0	12.3	0	15.4	0
Carbohydrate/protein ratio (wt/wt)	2.9	1.1	4.6	1.5	4.4	1.3
Recovery (%) of:						
Protein		75		84		85
Sugar		12		20		25

[a] Purified rabbit, human, and rat small intestinal mucins were treated with TFMS for 2 h at 25°C. Partially deglycosylated preparations were then dialyzed against PBS overnight and adjusted to a concentration equivalent to 1 mg of original mucin per ml. Removal of sugars was established by gas-liquid chromatography, and recovery of protein was determined from amino acid analyses.

[b] GalNac, *N*-acetylgalactosamine.

[c] GlcNAc, *N*-acetylglucosamine.

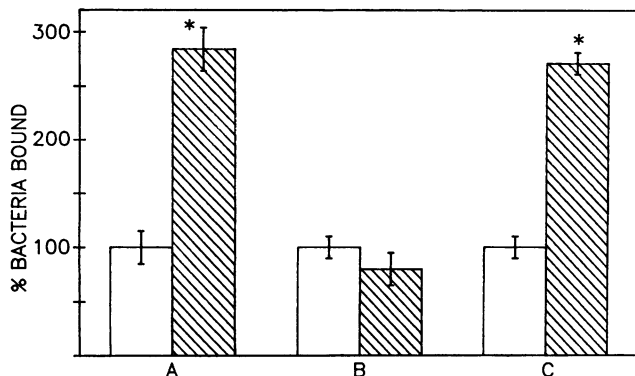

FIG. 4. Adherence of plasmid-bearing *Y. enterocolitica* to TFMS-treated small intestinal mucins. Purified preparations of rabbit, human, and rat small intestinal mucins were hydrolyzed with TFMS for 2 h at 25°C to remove terminal and backbone sugar residues. Partially deglycosylated preparations were dialyzed against PBS and adjusted to a concentration equivalent to 1 mg of original mucin per ml. Binding of virulent *Y. enterocolitica* to the mucins before and after TFMS hydrolysis was then performed as described in the legend to Fig. 1. A, rabbit small intestinal mucin; B, human small intestinal mucin; C, rat small intestinal mucin. Open bars show binding before TFMS treatment, and cross-hatched bars show binding after TFMS treatment. *, significantly different from binding to untreated mucin ($P < 0.01$).

greater attachment of mucin to the microtiter plate wells. Adherence of nonvirulent organisms was unaffected by TFMS treatment of the mucins.

DISCUSSION

The present study was undertaken to elucidate which region(s) of small intestinal mucin is important for the binding of *Y. enterocolitica*. Although it is known that adherence to mucin correlates with bacterial virulence, suggesting that binding is dependent on the virulence plasmid, it is not known which bacterial proteins constitute adhesins for mucin nor which components of the mucin serve as receptors for the bacteria. The findings from the present studies strongly suggest that plasmid-bearing *Y. enterocolitica* adheres primarily to the carbohydrate chains of native mucin but can, under certain circumstances, also interact with mucin protein.

Proteolytic digestion of purified rabbit small intestinal mucin under conditions that are known to degrade nonglycosylated or poorly glycosylated protein regions, including the 118-kDa glycoprotein (23, 27), had no effect on the binding of plasmid-bearing *Y. enterocolitica*, suggesting that naked peptide in the mucin does not contain the major bacterial binding site and, by implication, that attachment involves the mucin carbohydrate moiety. Thus, *Y. enterocolitica* may resemble a number of other microorganisms, including *Streptococcus sanguis* and *S. mutans* (3, 9), *Pseudomonas aeruginosa* (39), *P. cepacia* (40), *Staphylococcus* species (42), and strains of *Escherichia coli* (41, 51), all of which have been reported to interact with mucin carbohydrate. Since periodate oxidation did not alter the extent of bacterial adherence, oxidizable vicinal hydroxyl groups in mucin sugars and terminal fucose and sialic acid residues (15) are unlikely to be part of the binding site. These results are in agreement with studies by Pærregaard et al. (33) showing that periodate oxidation had no effect on the binding of *Y. enterocolitica* to crude mucus from a rabbit ileum.

Although mannose represents only a very small proportion (<1.0%) of the sugars present in native small intestinal mucin (23), it is an important receptor for some bacteria (41, 46). However, removal of mannose-containing oligosaccharide chains by endo F digestion had no effect on the binding of virulent *Y. enterocolitica* to rabbit small intestinal mucin, suggesting that mannose is not involved in adherence of this organism to mucin.

Hapten inhibition studies using oligosaccharides isolated from either human or rabbit small intestinal mucins supported the notion that plasmid-bearing *Y. enterocolitica* interacts with mucin carbohydrate chains since both oligosaccharide preparations were capable of causing a significant reduction in bacterial binding to mucin. The observed increases in adherence of virulent *Y. enterocolitica* to TFMS-treated rabbit and rat small intestinal mucins (which had lost ~80% of their sugars) suggest that plasmid-bearing bacteria bind to internal regions of the mucin oligosaccharide side chains that can become more exposed after TFMS hydrolysis and removal of terminal and backbone sugar residues. Our findings are similar to those reported in studies on the binding of *P. cepacia* to human small intestinal mucin (40)—bacterial adherence was enhanced after partial deglycosylation of mucin with TFMS, apparently because of exposure of *N*-acetylgalactosamine and *N*-acetylglucosamine receptors in the core region of the oligosaccharide chains. With *Y. enterocolitica*, *N*-acetylgalactosamine bound to the peptide core (either alone or with galactose attached, as in rabbit and rat small intestinal mucins, respectively) seems to be important for adherence while substitution with *N*-acetylglucosamine (as in human small intestinal mucin) does not contribute to bacterial binding. These conclusions support our previous hapten inhibition studies showing that both *N*-acetylgalactosamine and galactose may be involved in bacterial adherence to small intestinal mucin (25). Binding of *Y. enterocolitica* to the mucin carbohydrate moiety again appeared to be plasmid mediated, since adherence of plasmid-cured organisms to rabbit small intestinal mucin was not inhibited by rabbit and human mucin oligosaccharides and their low level of binding was unaffected by partial deglycosylation of rabbit, human, and rat mucins.

Studies by Pærregaard et al. (33) could not demonstrate the involvement of carbohydrate in the binding of virulent *Y. enterocolitica* to rabbit ileal mucus. However, crude mucus contains not only mucin but also proteins, DNA, lipid, and membrane fragments from shed epithelial cells. Since *Y. enterocolitica* may interact with a variety of these components by different mechanisms, hapten inhibition studies with individual monosaccharides may not be capable of causing a detectable change in bacterial binding to this complex substrate. The differences between our findings and those of Pærregaard et al. (33) may reflect our use of purified mucin and not crude mucus.

Removal of noncovalently bound lipid from rabbit small intestinal mucin by chloroform-methanol extraction increased the adherence of plasmid-bearing *Y. enterocolitica*, suggesting that the minor amounts of contaminating lipid present in purified mucin (~7% [by weight]) may normally shield receptor sites for the bacteria in the native molecule. In earlier studies on the binding of *P. cepacia* and *E. coli* (serotype O157:H7, strain CL-49) to human and rat small intestinal mucins, respectively, increased adherence was also observed after delipidation of mucin because of exposure of buried binding sites for the bacteria (40, 41). Since glycolipids reportedly associate noncovalently with the carbohydrate moiety of mucin while phospholipids attach to the protein component (52, 53), delipidation could have unmasked potential bacterial binding sites in either the mucin protein or its carbohydrate. The fact that pronase digestion of delipidated rabbit small

intestinal mucin decreased bacterial binding to levels observed in the native mucin suggests that the additional binding sites are in the mucin protein. Studies on a variety of mucins have shown that the nonglycosylated regions of the protein core contain hydrophobic domains that are susceptible to proteolytic digestion (10, 48, 49). Furthermore, the 118-kDa glycoprotein component of small intestinal mucin is relatively enriched in hydrophobic amino acids compared with the mucin monomers (23), suggesting that it could potentially participate in hydrophobic interactions with bacteria. Since TMU treatment of plasmid-bearing *Y. enterocolitica* prevented the increase in their binding to delipidated small intestinal mucin, it seems possible that the bacteria (which are known to be hydrophobic) are capable of interacting with regions of the mucin protein that are also hydrophobic but may normally be covered by noncovalently bound lipid. The fact that adherence of plasmid-cured *Y. enterocolitica* did not increase after delipidation of small intestinal mucin suggests that the adhesin in plasmid-bearing organisms responsible for binding to hydrophobic regions in the nonglycosylated mucin peptide is plasmid encoded.

When purified rabbit small intestinal mucin was reduced and alkylated or boiled, adherence of both plasmid-bearing and plasmid-cured *Y. enterocolitica* isolates was significantly enhanced, suggesting that dissociation of the polymer into its constituent glycoprotein monomers and 118-kDa glycoprotein or heat denaturation of the mucin without depolymerization resulted in the appearance of new binding sites for the bacteria. Presumably, these binding sites are normally buried in the native mucin and become exposed because of conformational changes in the macromolecule. Again, these findings resemble those for *P. cepacia* and *E. coli* CL-49 binding to human and rat small intestinal mucins (40, 41). The increase in the binding of plasmid-bearing *Y. enterocolitica* to both reduced mucin and boiled mucin was approximately twice that seen for plasmid-cured organisms, suggesting that two adhesins are involved, one chromosome encoded and the other plasmid encoded. The additional binding sites revealed by reduction-alkylation and boiling were susceptible to pronase digestion, suggesting that they are likely to be situated in the naked regions of the peptide core. Since TMU treatment of plasmid-bearing *Y. enterocolitica* did not affect their binding to either reduced or boiled mucin, the initial increase in adherence to these substrates was not due to hydrophobic interactions. As no partial changes in adherence were detected in the above-described series of studies (which may have allowed us to distinguish chromosomal from plasmid-encoded factors), we did not carry out similar experiments with plasmid-cured *Y. enterocolitica*.

Although our studies on delipidated, reduced, and boiled small intestinal mucin suggested that these treatments exposed binding sites for plasmid-bearing *Y. enterocolitica* in the mucin protein moiety, an alternative interpretation of our findings is that all of the interactions between the bacteria and mucin involve carbohydrate and that denaturation of the mucin (by boiling or thiol reduction) or delipidation may enhance bacterial binding by improving the accessibility of internal regions of the oligosaccharide chains, as in the case of TFMS treatment. However, one would then also expect to see an increase in bacterial adherence following pronase digestion of native mucin because this should improve exposure of internal sugar residues at least to the same extent as thiol reduction and probably more so than boiling. Since proteolysis of mucin did not affect adherence of *Y. enterocolitica*, it seems reasonable to conclude that other treatments enhanced the availability of mucin protein (rather than carbohydrate) and promoted bacterial binding by allowing greater interaction between the organism and the mucin peptide.

The significance of the additional binding sites for *Y. enterocolitica* in the protein moiety of small intestinal mucin is unclear, especially if these are normally buried in the native molecule. In studies on the binding of *E. coli* CL-49 to rat small intestinal mucin, it was proposed that bacterial attachment to mannose residues in N-glycans of the 118-kDa glycoprotein may be stabilized by interactions between the organism and mucin protein (41). A similar situation may occur with mucin-*Y. enterocolitica* interactions: initial binding may take place between the microbe and O-glycans of the mucin monomers, and then interactions with mucin peptide may stabilize the bonding. It is also possible that erosion of the mucus layer by the mechanical and enzymatic forces of digestion and release of partially degraded and/or denatured mucin into the intestinal lumen may enhance mucosal protection by binding to more bacterial adhesins and preventing the organism from attaching to components of the mucus gel and the epithelial surface.

The adhesins that mediate the binding of *Y. enterocolitica* to small intestinal mucin are not known, but several may be involved to account for adherence to the carbohydrate moiety and to the protein component by hydrophobic and nonhydrophobic interactions. Both plasmid- and chromosome-encoded outer surface proteins which promote bacterial attachment to and invasion of cell lines have been identified, including YadA, the *inv* gene product invasin, and the *ail* gene product (12–14, 17, 31). The latter two chromosomal proteins are only synthesized by pathogenic strains of *Y. enterocolitica* (31, 36). However, expression of invasin does not correlate with mucin binding since production is high at 25°C (when binding to mucin is low) and decreased at 37°C (when mucin binding is high) (14, 22). Although the *ail* gene is expressed at 37°C, its receptor has not been defined. YadA is thought to be responsible for many of the plasmid-mediated changes in the surface properties of *Y. enterocolitica*, including increased hydrophobicity (17), and also appears to be involved in the binding of *Y. enterocolitica* to HEp-2 cells (12), collagen (44), fibronectin (50), and intestinal brush border membranes (22, 32). We therefore speculate that YadA is responsible for the hydrophobic interactions with mucin protein. However, it remains to be determined whether the other plasmid- and chromosome-mediated attachments to mucin carbohydrate involve YadA, the *ail* gene product, or some other (unidentified) adhesin.

In summary, we have shown that virulent *Y. enterocolitica* is capable of interacting with purified rabbit small intestinal mucin in a variety of ways, most of which are plasmid dependent, although some may be chromosomally mediated. Bacterial attachment occurs primarily to the carbohydrate component of native mucin, but interactions with the protein moiety are also possible under certain circumstances. Such interactions may, on the one hand, protect the host by preventing the organism from attaching to the mucosa but may also benefit the pathogen, providing it with a means of anchoring to the gut wall, thereby delaying its clearance from the intestinal tract and improving its ability to colonize the gut.

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