INFECTION AND IMMUNITY, Oct. 1993, p. 4131–4138
0019-9567/93/104131-08$02.00/0

Vol. 61, No. 10

Growth in and Breakdown of Purified Rabbit Small Intestinal Mucin by *Yersinia enterocolitica*

MICHÈLE MANTLE* AND CHARLANE ROMBOUGH†

Gastrointestinal Research Unit, Departments of Medical Biochemistry and Paediatrics, Health Sciences Centre, 3330 Hospital Drive N.W., Calgary, Alberta, Canada T2N 4N1

Received 10 March 1993/Returned for modification 12 April 1993/Accepted 7 July 1993

The mucus lining of the gastrointestinal tract serves as a protective barrier over the epithelial surface that must be crossed by invading bacteria seeking entry into the mucosa. The gel-forming component of mucus is mucin, a large polymeric glycoprotein. The present study examined the growth of *Yersinia enterocolitica* (with and without its virulence plasmid) in purified rabbit small intestinal mucin and the ability of bacteria to degrade mucin. Both virulent and nonvirulent organisms showed enhanced growth in mucin-supplemented media compared with unsupplemented media, but only at 37°C and not at 25°C. The effects of mucin were not specific because medium supplemented with bovine serum albumin also enhanced bacterial growth at 37°C. Purified mucin was broken down into lower-molecular-weight components (assessed by monitoring its elution profile on a Sepharose CL-2B column) by plasmid-bearing *Y. enterocolitica* but not by plasmid-cured organisms. Culturing virulent *Y. enterocolitica* at 25°C completely suppressed its capacity to degrade mucin, suggesting that this activity depends on plasmid expression. These results were confirmed in similar studies with purified rabbit colonic mucin. Mucin-degrading activity could be demonstrated in spent culture media from virulent *Y. enterocolitica* incubated at 37°C but not in bacterial membrane preparations. Changes in the elution profiles of small intestinal and colonic mucins exposed to plasmid-bearing *Y. enterocolitica* at 37°C were consistent with proteolytic depolymerization. The ability to grow well in mucin may help *Y. enterocolitica* to colonize the intestine, while the production of a mucin-degrading enzyme(s) by plasmid-bearing organisms may assist pathogenic strains to solubilize and penetrate the mucus gel layer.

Yersinia enterocolitica is an enteroinvasive bacterium that causes gastroenteritis in humans. Following oral infection, *Y. enterocolitica* must cross the intestinal epithelium and reach the mucosa in order to produce clinical disease (5, 38). The mammalian gastrointestinal tract is lined with a layer of mucus produced by specialized epithelial cells (goblet cells in the small intestine and colon). The major component of mucus that is responsible for its gel-forming properties is mucin, a large (>2 × 10^6-kDa) polymeric molecule composed of highly glycosylated (~80% [by weight] carbohydrate) glycoprotein monomers that are held together by disulfide bonds located in nonglycosylated (or poorly glycosylated) regions of their peptide cores (2, 7, 20–22). 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, 40). The mucus layer serves as a protective physical barrier over the epithelium such that invading bacteria (like *Y. enterocolitica*) must interact with the gel and be able to pass through it to gain entry into the mucosa.

The clinical symptoms of yersiniosis in humans can be reproduced in rabbits infected with pathogenic strains of *Y. enterocolitica* (31). In previous studies on the early steps of intestinal colonization, we showed that a pathogenic, plasmid-bearing strain of *Y. enterocolitica* (MCH700S, serotype O:3) bound well to rabbit small intestinal and colonic mucus and their purified mucins when cultured at 37°C, conditions which promote expression of its virulence plasmid (19). 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 small intestinal and colonic mucus and mucin was dependent on the production of plasmid-encoded proteins.

The 42- to 50-MDa virulence plasmid of *Y. enterocolitica* encodes a variety of proteins, 16 to 20, whose expression is regulated by both temperature and the availability of calcium (9, 33, 34, 41). A number of these proteins are secreted, and some then associate with the outer membrane of the organism and are referred to as Yops (for yersinia outer membrane proteins) (27, 33, 34). In addition, the plasmid encodes for YadA, a fibrillar adhesin that is a true outer membrane protein (14). When expressed, these plasmid-encoded proteins alter the surface charge and hydrophobicity of the bacterium, promote autoagglutination and mannose-resistant hemagglutination, enhance adherence to cell lines, epithelial cell membranes, collagen, and fibronectin, and confer on the organism the ability to resist phagocytosis by polymorphonuclear leukocytes and the bacteriocidal effects of serum (1, 10, 14, 15, 17, 19, 23, 24, 30, 39, 43). Thus, plasmid proteins may be involved in bacterial attachment to the intestinal wall and penetration of the mucosa and may facilitate survival and proliferation of the organism in the host tissue.

The objective of the present study was to examine whether *Y. enterocolitica* (with and without its virulence plasmid) was capable of growing in and degrading purified rabbit small intestinal mucin. We demonstrate that mucin can serve as a nutrient source for both virulent and nonvirulent organisms, although apparently no more so than a pure protein. In addition, we show that plasmid-bearing bacteria are able to degrade small intestinal and colonic mucin more

* Corresponding author.
† Present address: Department of Microbiology and Infectious Diseases, Health Sciences Centre, Calgary, Alberta, Canada T2N 4N1.

effectively than plasmid-cured organisms, a factor that may assist pathogenic strains to solubilize and penetrate the mucus gel layer in the intestine.

MATERIALS AND METHODS

Preparation of mucin. Mucin was purified in the presence of proteolytic inhibitors from the small intestines of New Zealand White rabbits (jejunum and ileum combined, from the ligament of Trietz to the ileo-cecal junction) as described previously (22). Mucosal homogenates were subjected to equilibrium density gradient centrifugation in CsCl (twice) and then to 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) (22). For degradation assays, purified mucin was 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 2 mg (dry weight)/ml. Purified rabbit small intestinal mucin was used as a model throughout our studies because it is known to be similar in composition and structure to human small intestinal mucin (20, 22).

Bacteria and growth conditions. Two isogenic strains of *Y. enterocolitica* (MCH700S, plasmid bearing, and MCH700L, plasmid cured, obtained from C. Pai) 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 (30, 31). The isogenic strain MCH700L was cured of its plasmid, as described by Pai and DeStephano (30), and is known not to produce disease in rabbits (16, 30). The presence or absence of the virulence plasmid was confirmed by agarose gel electrophoresis (18, 33). Samples from frozen stocks of both strains were cultured as described previously (19). After overnight growth at 25°C in Dulbecco's modified Eagle medium (MEM; no. 320-1965, GIBCO Laboratories, Grand Island, N.Y.), bacteria were diluted (1:5) into fresh medium and incubated at 37°C for 1 h. At the end of incubation, bacteria were pelleted by centrifugation, washed with sterile medium, and finally suspended in the same buffer at a concentration of 5×10^7 organisms per ml (adjusted spectrophotometrically at 540 nm and confirmed by dilution and colony counting). In some cases, bacterial cultures were not temperature shifted but were maintained at 25°C throughout.

Growth in small intestinal mucin. Bacterial suspensions (0.1 ml) were mixed with MEM (0.4 ml), 0.001% Triton X-100 (0.1 ml), and either (i) mucin (0.4 ml), (ii) bovine serum albumin (BSA; ICN Biochemicals, Cleveland, Ohio; 0.4 ml of a 2-mg/ml solution in PBS), (iii) chondroitin sulfate (CS; Sigma Chemical Co., St. Louis, Mo.; 0.4 ml of a 2-mg/ml solution in PBS), or (iv) PBS only (0.4 ml). In these experiments, BSA (a pure protein) and CS (a carbohydrate-rich proteoglycan) served as controls for studies on mucin, allowing us to assess separately the effects of protein and carbohydrate on bacterial growth. Mixtures were incubated aerobically in a rotary shaker at either 37 or 25°C, and aliquots were taken at 0.5, 1, 2, 4, and 6 h for determination of bacterial numbers (assessed spectrophotometrically at 540 nm and confirmed by plating onto blood agar). Growth experiments were performed five times, in duplicate. Data are expressed as the mean ± standard error of the mean. Means were compared by one-way analysis of variance and unpaired, two-tailed *t* tests. Values for *P* of <0.05 were considered statistically significant.

To determine whether bacteria could use mucin as the sole source of carbon and nitrogen, an additional series of experiments was performed with a chemically defined synthetic medium base (50 mM NaCl, 10 mM NH_4Cl, 0.4 mM K_2HPO_4, 2.5 mM $Na_2S_2O_3$, 40 mM morpholinepropanesulfonic acid, 10 mM Tricine; pH 7.0) (3). Both plasmid-bearing and plasmid-cured strains of *Y. enterocolitica* were cultured at 37°C exactly as described above, except that MEM was replaced with the medium base or with the base supplemented with either 0.1% fructose and 0.2% Casamino Acids or purified small intestinal mucin (1 mg).

Degradation assays. Bacterial suspensions (0.1 ml) were mixed with MEM (1.3 ml), 0.001% Triton X-100 (0.1 ml), and mucin (0.5 ml) and incubated aerobically in a rotary shaker for up to 36 h at either 37 or 25°C. After incubation, cultures were centrifuged and the supernatant was collected. The pellet was resuspended in PBS containing 0.001% Triton X-100 and 0.02% NaN_3, vortexed, and recentrifuged: the resulting supernatant was pooled with the first supernatant and stored at −20°C until analyzed. Supernatant samples were applied to a Sepharose CL-2B column (1.6 by 70 cm) equilibrated with 0.2 M NaCl. Fractions (2 ml) were collected from the column and analyzed for their mucin content by using a nitrocellulose slot blot assay with blots stained with periodic acid-Schiff (PAS) reagent (44). Stained blots were scanned on a laser densitometer, and the amount of mucin in each column fraction was determined by comparing its peak area with standard curves obtained by applying purified rabbit small intestinal mucin (0.3 to 25 μg) to the nitrocellulose blot. From the derived column profiles, the amount of mucin eluting in the void volume (polymeric material) and the amount eluting partially included (degraded mucin) were calculated to determine the degree of breakdown of the macromolecule at each time period studied. Appropriate controls (MEM alone and spent medium without mucin obtained from bacterial cultures incubated at 37°C for 36 h) were also analyzed as described above to ensure that the medium and any bacterial products it contained at the end of incubation did not react in the PAS assay.

A limited series of studies was performed to examine whether plasmid-bearing *Y. enterocolitica* was able to degrade rabbit colonic mucin. Mucin was purified from the first 25 cm of the proximal colon of rabbits as described above and in reference 22. Purified mucin was then ^{3}H-radiolabelled on its carbohydrate component (terminal, exposed galactose and *N*-acetylgalactosamine residues) with galactose oxidase by the method of Gahmberg and Hakomori (8) but without prior neuraminidase treatment. Degradation assays were performed as in the case of small intestinal mucin except that column fractions were counted to establish the distribution of radiolabel in the void and partially included volumes and hence to calculate the extent of mucin breakdown at each different time period.

Isolation of spent medium and bacterial membranes. Plasmid-bearing *Y. enterocolitica* was cultured at 37°C for 24 h and centrifuged, and the pellet and supernatant were separated. The supernatant (spent medium) was concentrated 60-fold by ultrafiltration through a PM10 membrane (molecular weight cutoff of 10,000). An aliquot of the concentrated medium (0.1 ml) was then added to MEM (1.3 ml) containing 0.001% Triton X-100 (0.1 ml) and small intestinal mucin (0.5 ml) and incubated for 24 h at 37°C. After incubation, the

mixture was applied to a Sepharose CL-2B column as described above, and fractions were analyzed by the slot blot assay to quantitate their mucin content.

Bacterial membranes were isolated by a modification of the method of Carniel et al. (3). The bacterial pellet described above was suspended in 5 mM $MgCl_2$–25 mM K_2HPO_4 (pH 7), sonicated, and centrifuged at 5,000 × *g* for 10 min at 4°C to remove cellular debris. Bacterial membranes were then pelleted by centrifugation at 50,000 × *g* for 30 min at 4°C. Membranes were suspended in 10 mM Tris-HCl buffer at pH 8.0 at a concentration of 3 mg/ml (determined by the Bio-Rad protein assay [Bio-Rad Laboratories, Richmond, Calif.]). Membranes (0.4 ml) were incubated with MEM (1 ml), 0.001% Triton X-100 (0.1 ml), and small intestinal mucin (0.5 ml) for 24 h at 37°C. After centrifugation, the supernatant was applied to a Sepharose CL-2B column, and fractions were analyzed for their mucin content by a solid-phase (slot blot) immunoassay, using antibodies specific for rabbit small intestinal mucin (22, 25). The PAS assay was not used for these experiments because the bacterial membranes themselves interfered with the assay.

RESULTS

Growth of *Y. enterocolitica* in small intestinal mucin. To examine the ability of *Y. enterocolitica* (plasmid-bearing and plasmid-cured isogenic strains) to grow in purified rabbit small intestinal mucin, organisms were cultured at 37 or 25°C for up to 6 h in MEM with and without added mucin. Triton X-100 was added to all of these (and subsequent) experiments in order to prevent autoagglutination of plasmid-bearing organisms and to inhibit binding of mucin to bacteria during the course of the incubations; the lowest concentration of detergent capable of accomplishing these ends was used.

In the presence of mucin, plasmid-bearing bacteria grew significantly better than in unsupplemented medium when cultured at 37°C (Fig. 1A). Increased growth in mucin was detected within 2 h of the start of incubation. At 25°C, the same organisms showed no increased growth in mucin compared with growth in unsupplemented medium (Fig. 1C). Similar trends were observed for plasmid-cured organisms: in the presence of mucin, bacteria grew better than in unsupplemented medium at 37°C, although the difference only reached statistical significance after 6 h of incubation (Fig. 1B). At 25°C, plasmid-cured organisms grew to the same extent in both mucin and medium alone (Fig. 1D). When the two bacterial strains were compared, it was noted that growth of plasmid-bearing organisms at 37°C was slightly but significantly greater than that of plasmid-cured organisms in mucin after 6 h of incubation (Fig. 1A and B).

To determine whether the effects of mucin on the growth of *Y. enterocolitica* at 37°C were specific, exactly the same experiments were performed with medium supplemented with either a protein (BSA) or a carbohydrate-rich molecule (the proteoglycan CS) in place of mucin. Enhanced growth in BSA was detected at 37°C but not at 25°C for both plasmid-bearing and plasmid-cured organisms (Fig. 1). Addition of CS to the culture medium caused a slight (but not significant) increase in bacterial growth at 37°C that was not as great as that elicited by either mucin or BSA (data not shown). Extending the incubation time of these experiments to 24 h made no difference to our findings: growth at 37°C in mucin and BSA remained the same and was greater than that in

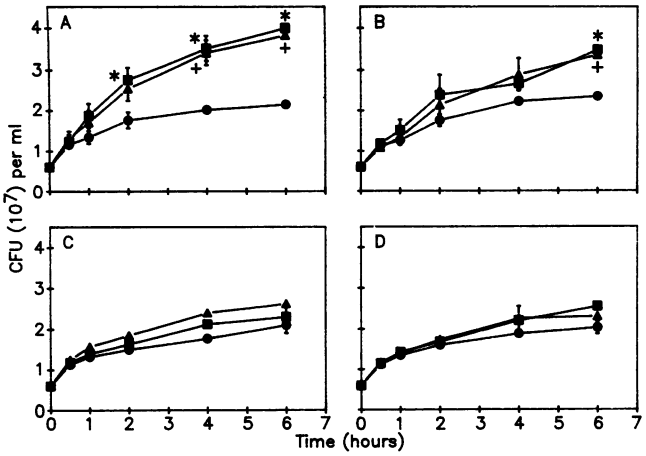

FIG. 1. Growth of *Y. enterocolitica* in unsupplemented medium and medium containing purified rabbit small intestinal mucin or BSA. Plasmid-bearing and plasmid-cured bacteria (5 × 10^6 organisms) were inoculated into MEM alone (●) or into medium supplemented with either mucin (■) or BSA (▲). Mixtures were incubated aerobically at either 37 or 25°C, and aliquots were taken at the times indicated for determination of bacterial numbers. (A and C) Plasmid-bearing *Y. enterocolitica* incubated at 37 and 25°C, respectively; (B and D) plasmid-cured bacteria incubated at 37 and 25°C, respectively. * and +, growth in mucin and BSA, respectively, is significantly different from that in unsupplemented medium ($P < 0.05$).

medium alone (or with CS) for both plasmid-bearing and plasmid-cured organisms.

In preliminary studies to establish the appropriate conditions for these growth experiments, we used one-half and one-tenth of the mucin concentration finally selected. At these levels, we could not detect significantly increased growth of either plasmid-bearing or plasmid-cured *Y. enterocolitica* at 37°C in mucin-supplemented medium compared with unsupplemented medium. Thus, a final concentration of 1 mg of mucin in a total volume of 2 ml was the minimum at which growth enhancement occurred. Whether higher amounts of mucin could promote growth to a greater extent was not assessed because purified mucin produces viscous solutions at concentrations of >1 mg/ml and finally gels at 8 to 10 mg/ml. Since the high viscosity of more concentrated mucin solutions could potentially interfere with measurements of bacterial growth, we did not investigate whether growth enhancement was dependent on the mucin dose.

All of the above growth experiments were performed in the presence of commercially available MEM (a complex medium containing a variety of amino acids and vitamins and glucose) because we thought that this "background" more closely mimicked the situation faced by the organisms in the intestinal tract. However, to determine whether the bacteria could use mucin as the sole source of carbon and nitrogen, we carried out additional experiments with a chemically defined minimal medium base supplemented with either 0.1% fructose–0.2% Casamino Acids or purified small intestinal mucin (1 mg). Neither plasmid-bearing nor plasmid-cured strains of *Y. enterocolitica* grew in the unsupplemented medium base alone. However, when the base was supplemented with fructose and Casamino Acids, both strains grew well (as expected), while intermediate levels of growth were observed in the medium base containing mucin (data not shown). In these particular experiments, plasmid-cured organisms showed better growth than plasmid-bearing

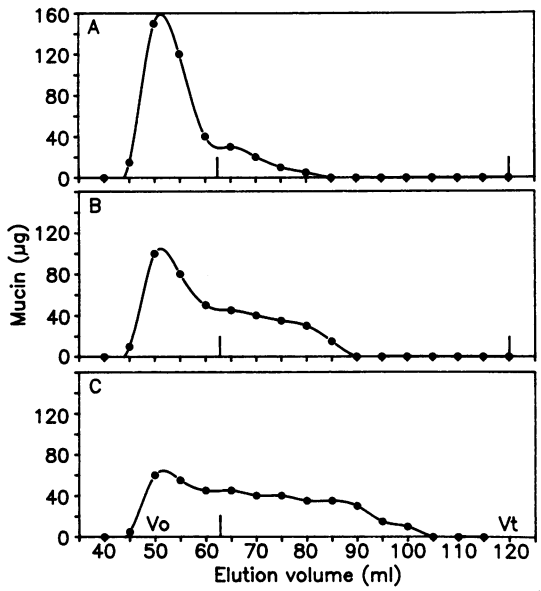

FIG. 2. Gel filtration profiles of purified rabbit small intestinal mucin following exposure to *Y. enterocolitica*. Plasmid-bearing *Y. enterocolitica* was inoculated into MEM containing mucin (1 mg) and incubated aerobically for up to 36 h at 37°C. After incubation, culture supernatants were applied to a Sepharose CL-2B column. Fractions (2 ml) were collected from the column and analyzed for mucin by using a PAS assay. (A) Column profile for mucin not exposed to bacteria; (B and C) column profiles after incubation with bacteria for 4 and 36 h, respectively. Vo, void volume; Vt, total volume of the column.

TABLE 1. Breakdown of purified rabbit small intestinal mucin by *Y. enterocolitica*[a]

Bacterial sample	Time (h)	% Mucin in void vol	% Mucin partially included
Plasmid-bearing *Y. enterocolitica* incubated at 37°C			
	0	85	15
	4	62	38
	8	46	54
	16	40	60
	24	39	61
	36	39	61
Plasmid-cured *Y. enterocolitica* incubated at 37°C			
	8	73	27
	24	73	27
	36	75	25
Plasmid-bearing *Y. enterocolitica* incubated at 25°C			
	4	80	20
	24	72	28

[a] Bacteria (5×10^6 organisms) were added to MEM containing mucin (1 mg) and incubated aerobically for the times indicated at either 37 or 25°C. Cultures were centrifuged, and the supernatants were applied to a Sepharose CL-2B column. Fractions were collected and were analyzed for their mucin content with a PAS assay. From the column profiles, the amount of mucin eluting in the void volume (polymeric material) and the amount eluting partially included (degraded mucin) were calculated to determine the degree of breakdown of the macromolecule at each time period studied.

organisms in the presence of either fructose-Casamino Acids or mucin. The fact that both virulent and nonvirulent organisms grew in the presence (but not the absence) of small intestinal mucin in these experiments illustrates that they are capable of utilizing this material as their sole source of carbon and nitrogen.

Degradation of mucin by *Y. enterocolitica*. To determine whether *Y. enterocolitica* was capable of degrading purified rabbit small intestinal mucin, bacteria were inoculated into MEM containing mucin and incubated for up to 36 h at 37°C. After incubation, culture supernatants were applied to a Sepharose CL-2B column, and fractions were analyzed for their mucin content. As shown in Fig. 2 and Table 1, mucin not exposed to bacteria eluted largely in the void volume fractions of the column, with very little appearing in the included fractions. Incubation with plasmid-bearing organisms resulted in a progressive decrease in the amount of polymeric mucin detected in the void volume of the column and a corresponding increase in the amount of smaller, degraded mucin eluting in the partially included fractions. Most of the breakdown occurred within the first 8 h of incubation. In contrast, incubation with plasmid-cured organisms caused only minimal degradation (~10%) of the mucin polymer (Table 1). When incubations of plasmid-bearing bacteria with mucin were performed at 25°C, degradation was markedly diminished (Table 1). The amount of mucin breakdown observed with virulent *Y. enterocolitica* at 25°C or with plasmid-free organisms at 37°C was also seen when purified mucin was incubated without added bacteria. Thus, this level of degradation is unlikely to be due to enzyme activity but may reflect some instability in the mucin molecule itself when it is maintained in solution for long periods of time at a temperature of 25 or 37°C.

To confirm that mucin was not lost through adherence to the organisms during the course of the above experiments, bacteria were harvested at the end of each incubation period and resuspended in PBS containing a high concentration of detergent (0.1% Triton X-100). After vigorous vortexing, bacteria were pelleted by centrifugation, and the supernatant was analyzed for mucin by a specific immunoassay (22, 25). Only 5.3% ± 0.2% of the mucin originally added to the incubation mixtures was found associated with the bacteria in all experiments, which was not enough to result in a substantial difference in the amount of mucin lost from the culture supernatants and subsequently applied to each Sepharose column run.

Similar studies were performed on purified rabbit colonic mucin (Table 2). Again, incubation with plasmid-bearing *Y. enterocolitica* at 37°C resulted in the loss of polymeric mucin from the void volume of the column and the appearance of smaller, degraded mucin eluting in the partially included fractions. In contrast, incubation with virulent organisms at 25°C caused markedly less degradation (~20%) of the mucin polymer. It appeared from these experiments that rabbit colonic mucin was broken down at a faster rate and to a greater extent than small intestinal mucin by plasmid-bearing *Y. enterocolitica* at 37°C (cf. 4- and 24-h values in Tables 1 and 2).

Mucin breakdown by spent medium and bacterial membranes. We next investigated whether the mucin-degrading activity of virulent *Y. enterocolitica* was present in spent culture medium or was associated with bacterial membranes. Aliquots of concentrated medium or bacterial membrane preparations were incubated with purified small intestinal mucin for 24 h at 37°C. After incubation, the mixtures

TABLE 2. Breakdown of purified rabbit colonic mucin by *Y. enterocolitica*[a]

Time (h)	% Mucin in void vol	% Mucin partially included
0	90	10
4	38	62
8	33	67
24	18	82

[a] Plasmid-bearing *Y. enterocolitica* (5×10^6 organisms) was added to MEM containing radiolabelled colonic mucin (~20,000 dpm) and incubated aerobically for the times indicated at 37°C. Cultures were centrifuged, and the supernatants were applied to a Sepharose CL-2B column. Fractions were collected and counted. The distribution of mucin eluting in the void volume (polymeric material) and the partially included volume (degraded mucin) was calculated to determine the extent of breakdown of the macromolecule at each time period.

were applied to a Sepharose CL-2B column as described above, and fractions were analyzed for their mucin content. As shown in Table 3, incubation with the spent culture medium resulted in a substantial breakdown of the mucin (as evidenced by a decrease in the amount eluting in the void volume of the column and an increase in the amount eluting in the included fractions), while very little degradation was observed in mucin incubated with bacterial membranes.

Finally, we examined whether the ability to break down mucin was constitutively expressed by virulent *Y. enterocolitica* or whether a higher degree of expression could be induced by growing the organisms in the presence of mucin. Plasmid-bearing bacteria were cultured for 24 h at 37°C in the presence or absence of 10 μg of small intestinal mucin per ml. Culture supernatants were harvested and concentrated, and then aliquots were added to MEM containing 0.001% Triton X-100 and mucin (essentially as described for the above experiments) and incubated for a further 24 h at 37°C. Gel filtration profiles of the incubation mixtures revealed that mucin was degraded to the same extent regardless of whether the supernatants were obtained from bacteria that had or had not been exposed previously to the macromolecule (Table 4).

In both of the above experiments, the amount of mucin breakdown observed (~47%) was not as great as that seen in experiments with live bacteria (~60%). The reasons for this are not known, but it is likely that either some enzyme activity was lost during processing of the culture supernatants or the concentration of enzyme added to these incubations was lower than that achieved in the actual bacterial cultures.

TABLE 3. Breakdown of purified rabbit small intestinal mucin by spent culture medium and bacterial membranes[a]

Prepn	% Mucin in void volume	% Mucin partially included
Control	85	15
Medium	53	47
Membranes	73	27

[a] Plasmid-bearing *Y. enterocolitica* was cultured at 37°C for 24 h and centrifuged, and the pellet and supernatant were separated. The supernatant (spent culture medium) was concentrated 60-fold, and an aliquot was added to MEM containing small intestinal mucin (1 mg) and incubated for 24 h at 37°C. Membranes were isolated from the bacterial pellet, and an aliquot (containing ~1 mg of protein) was incubated with MEM and mucin (1 mg) for 24 h at 37°C. After incubation, the mixtures were applied to a Sepharose CL-2B column and fractions were analyzed for mucin. The amount of mucin eluting in the void volume and the amount eluting partially included were calculated to determine the degree of breakdown of the macromolecule by the medium and membrane preparations.

TABLE 4. Expression of mucin-degrading activity by *Y. enterocolitica*[a]

Culture	% Mucin in void vol	% Mucin partially included
Control	85	15
Uninduced	53	47
Induced	55	45

[a] Plasmid-bearing *Y. enterocolitica* was cultured for 24 h at 37°C in the presence or absence of 10 μg of small intestinal mucin per ml. Culture supernatants were harvested and concentrated, and then aliquots were added to MEM containing small intestinal mucin (1 mg) and incubated for a further 24 h at 37°C. After incubation, the mixtures were applied to a Sepharose CL-2B column, and fractions were analyzed for their mucin content. The distribution of mucin in the void and partially included volumes was calculated to quantitate breakdown of the macromolecule.

DISCUSSION

The present study shows that purified rabbit small intestinal mucin can enhance the growth of *Y. enterocolitica* at physiologic temperatures (37°C). Our findings indicate that mucin can exert this effect on both virulent (plasmid-bearing) and nonvirulent (plasmid-cured) organisms even in the presence of a complex background rich in other nutrients (amino acids and glucose) and, in addition, that mucin can serve as the sole nutrient (carbon and nitrogen source) for the bacteria. However, the effects of mucin were not specific to this molecule, because BSA also enhanced the growth of plasmid-bearing and plasmid-cured bacteria to exactly the same extent as mucin. Since addition of CS (a carbohydrate-rich molecule) to the culture medium only caused a slight increase in bacterial growth at 37°C that was not as great as that induced by either mucin or BSA, it would appear that growth enhancement may be a function of protein rather than carbohydrate and therefore may involve a proteolytic enzyme(s). When organisms were cultured at 25°C, neither mucin nor BSA (or CS) had any significant effect on bacterial growth, which may reflect decreased enzymatic activity at this lower temperature. Although both virulent and nonvirulent strains of *Y. enterocolitica* grew well in MEM containing small intestinal mucin or BSA at 37°C, plasmid-bearing organisms did show slightly better growth than plasmid-cured organisms after 6 h of incubation. This small difference between the two strains persisted even after 24 h but did not become any more marked than at 6 h. Perhaps this ability of plasmid-bearing bacteria to reproduce in a nutrient-rich, mucin-containing medium gives them a slight growth advantage over their plasmid-cured counterparts in the environment of the small intestinal mucus layer.

The ability of our indigenous flora and pathogenic bacteria to grow in mucus (and in mucin) may facilitate colonization of the intestinal tract. For colonization to occur, these organisms must multiply at a rate greater than that at which they are expelled through turnover and erosion of the mucus layer. Several species of bacteria (including avirulent *Escherichia coli* [45], *Salmonella typhimurium* [26], *Clostridium perfringens* [42], *Bacteroides* species [36, 42], and virulent *Shigella flexneri* [35]) are known to grow well in crude mucus or pure mucin preparations. Our present findings support the earlier studies of Pærregaard et al. (29) showing that crude ileal mucus could support the growth of *Y. enterocolitica* and extend that work by demonstrating for the first time that the

purified gel-forming component of mucus (mucin) may be responsible for at least some of the enhanced growth of these organisms in mucus preparations.

The second objective of our study was to investigate the potential of plasmid-bearing and plasmid-cured strains of *Y. enterocolitica* to degrade purified rabbit small intestinal mucin. Our experiments clearly showed that plasmid-bearing bacteria were able to degrade small intestinal mucin into lower-molecular-weight components far more effectively than plasmid-cured organisms. That mucin-degrading activity depends on plasmid expression was confirmed by culturing plasmid-bearing *Y. enterocolitica* at 25°C (which suppresses the plasmid) and showing a markedly diminished capacity to degrade mucin. Essentially the same results were obtained in similar studies performed with purified rabbit colonic mucin as a substrate for *Y. enterocolitica*. Overall, our findings strongly suggest that the enzyme(s) responsible for breaking down mucin may be plasmid encoded, but we cannot rule out the possibility that the enzyme(s) is chromosomally encoded and expression is up-regulated by factors produced by the virulence plasmid.

Since mucin-degrading activity could be demonstrated in the spent media from cultures of *Y. enterocolitica* incubated at 37°C but not in bacterial membrane preparations, it appears that the enzyme(s) involved in mucin breakdown is largely secreted into the medium. However, our experiments were conducted under conditions designed to prevent autoagglutination of bacteria and binding of mucin to the organisms. These conditions may also have prevented the enzyme(s) from associating with the bacterial outer surface. Whether in vivo the enzyme(s) remains in the milieu surrounding the organism or becomes attached to its outer membrane (as in the case of some plasmid-encoded Yops [27]) requires further investigation. Attempts to enhance the mucin-degrading activity of plasmid-bearing *Y. enterocolitica* by culturing the organisms overnight in the presence of a low concentration of small intestinal mucin suggested that the enzyme(s) was constitutively expressed at 37°C, and higher levels of production could not be further induced. Our findings parallel those of Hoskins et al. (11), who have shown that mucin oligosaccharide-degrading enzymes produced by human colonic bacteria are largely extracellular and are expressed even in the absence of mucin substrate.

Studies on colonic mucin indicated that it was more readily broken down by plasmid-bearing *Y. enterocolitica* at 37°C than small intestinal mucin, since degradation occurred at a faster rate and to a greater extent. The reason for the increased susceptibility of colonic mucin to breakdown by *Y. enterocolitica* is not clear, but we have noted a similar phenomenon in our earlier studies: when rabbit colonic mucin was treated with either pronase or thiol-reducing agents, it depolymerized more rapidly than rabbit small intestinal mucin, likely because of differences between the two mucins in terms of their compositions and structures (22).

Although it would be tempting to suggest that the ability of mucin to enhance the growth of *Y. enterocolitica* may be related to the ability of the bacteria to break down mucin, this does not appear to be the case. While both plasmid-bearing and plasmid-cured organisms grew well in mucin, only the former were capable of degrading the macromolecule. These observations imply that the effects of mucin on the growth of *Y. enterocolitica* may not necessarily depend on the ability of the bacteria to degrade mucin and also that mucin breakdown may not be a prerequisite for enhanced bacterial growth. Further studies are required to elucidate the means by which mucin promotes bacterial growth and how mucin is degraded by plasmid-bearing organisms in order to establish whether there is any link between the two phenomena.

As yet, we do not know whether *Y. enterocolitica* produces more than one enzyme that is active in degrading mucin. The glycoprotein nature of small intestinal and colonic mucins makes them susceptible to attack by both glycosidases and proteases. Several species of bacteria indigenous to the human and porcine intestinal tracts (including *Clostridium perfringens* [42] and strains of *Bacteroides* [36, 42], *Ruminococcus*, and *Bifidobacterium* [11] spp.) have been shown to utilize mucin as a nutrient source. These organisms each produce a limited number of highly specific exoglycosidases that act in concert to sequentially remove terminal sugar residues (for example, galactose, *N*-acetylgalactosamine, and sialic acid) from mucin oligosaccharide chains (4, 11, 12, 28, 36, 42). However, removal of only terminal sugar residues would not be expected to cause a major decrease in the molecular weight of the mucin polymer, as seen in the present studies. Furthermore, terminal galactose and *N*-acetylgalactosamine residues were radiolabelled in our experiments on colonic mucin, and this label was not lost (or converted into very low molecular weight species) during incubations with *Y. enterocolitica*. While we cannot exclude the possibility of some glycosidase activity, it would seem more likely that the major mucin-degrading enzyme(s) elaborated by *Y. enterocolitica* may be proteolytic.

Many studies on gastrointestinal mucins have shown that the native polymer can be dissociated into glycoprotein monomers by proteolysis of nonglycosylated (or poorly glycosylated) peptide regions (2, 20–22). Depolymerization of mucin is known to change its elution profile on a Sepharose CL-2B column: the mucin polymer elutes in the void volume of the column, while the glycoprotein monomers are retarded and partially included. The changes seen in the elution profiles of rabbit small intestinal and colonic mucins exposed to plasmid-bearing *Y. enterocolitica* at 37°C in the present studies are consistent with proteolytic depolymerization. We therefore speculate that the organism elaborates a protease that is capable of degrading mucin, thereby assisting pathogenic strains to solubilize and penetrate the mucus gel layer in the intestinal tract. Whether such a protease has other actions at different stages of the disease (for example, colonization of the mucosa) cannot be ruled out and may be possible.

Degradation of the protein component of gastrointestinal mucins with the resulting collapse of their polymeric structures has been achieved with human fecal extracts (13), *Pseudomonas aeruginosa* elastase (32), *Campylobacter pylori* protease (37), and *Vibrio cholerae* metalloproteinase (6). The last enzyme is secreted and digests nonglycosylated peptide regions of small intestinal mucin, causing a 57% drop in its viscosity. After solubilizing the mucus gel in this manner, the organism and its toxin can presumably reach the epithelial surface more readily. On the basis of the ability of virulent *Y. enterocolitica* to degrade small intestinal and colonic mucin, we suggest that the major enzyme involved may be a plasmid-encoded (or plasmid-regulated) protease whose activity may be important in the establishment of infection by "dissolving" the normal protective mucus barrier and allowing the organism to gain access to the underlying mucosa.

ACKNOWLEDGMENTS

This work was supported by the Natural Sciences and Engineering Research Council. M.M. is the recipient of a Scholarship Award from the Alberta Heritage Foundation for Medical Research.

We thank J. Bagley for performing the studies on rabbit colonic mucin.

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. **Bell, A. E., L. A. Sellers, A. Allen, W. J. Cunliffe, E. R. Morris, and S. B. Ross-Murphy.** 1985. Properties of gastric and duodenal mucus: effect of proteolysis, disulfide reduction, bile, acid, ethanol, and hypertonicity on mucus gel structure. Gastroenterology **88:**269–280.
3. **Carniel, E., J.-C. Antoine, A. Guiyoule, N. Guiso, and H. H. Mollaret.** 1989. Purification, location, and immunological characterization of the iron-regulated high-molecular-weight proteins of the highly pathogenic yersiniae. Infect. Immun. **57:**540–545.
4. **Corfield, A. P., S. A. Wagner, J. R. Clamp, M. S. Kriaris, and L. C. Hoskins.** 1992. Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, *N*-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. Infect. Immun. **60:**3971–3978.
5. **Cover, T. L., and R. C. Aber.** 1989. *Yersinia enterocolitica*. N. Engl. J. Med. **321:**16–24.
6. **Crowther, R. S., N. W. Roomi, R. E. F. Fahim, and J. F. Forstner.** 1987. *Vibrio cholerae* metalloproteinase degrades intestinal mucin and facilitates enterotoxin-induced secretion from rat intestine. Biochim. Biophys. Acta **924:**393–402.
7. **Fahim, R. E. F., G. G. Forstner, and J. F. Forstner.** 1983. Heterogeneity of rat goblet cell mucin before and after reduction. Biochem. J. **209:**117–124.
8. **Gahmberg, C. G., and S. I. Hakomori.** 1973. External labeling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes. J. Biol. Chem. **248:**4311–4317.
9. **Gemski, P., J. R. Lazere, and T. Casey.** 1980. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. **27:**682–685.
10. **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.
11. **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 group antigens and oligosaccharides from mucin glycoproteins. J. Clin. Invest. **75:**944–953.
12. **Hoskins, L. C., and E. T. Boulding.** 1981. Mucin degradation in human colon ecosystems. Evidence for the existence and role of bacterial subpopulations producing glycosidases as extracellular enzymes. J. Clin. Invest. **67:**163–172.
13. **Hutton, D. A., J. P. Pearson, A. Allen, and S. N. E. Foster.** 1990. Mucolysis of the colonic mucus barrier by faecal proteinases: inhibition by interacting polyacrylate. Clin. Sci. **78:**265–271.
14. **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.
15. **Lachica, R. V., and D. L. Zink.** 1984. Plasmid-associated cell surface charge and hydrophobicity of *Yersinia enterocolitica*. Infect. Immun. **44:**540–543.
16. **Lian, C.-J., W. S. Hwang, J. K. Kelly, and C. H. Pai.** 1987. Invasiveness of *Yersinia enterocolitica* lacking the virulence plasmid: an in-vivo study. Med. Microbiol. **24:**219–226.
17. **Lian, C.-J., W. S. Hwang, and C. H. Pai.** 1987. Plasmid-mediated resistance to phagocytosis in *Yersinia enterocolitica*. Infect. Immun. **55:**1176–1183.
18. **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.
19. **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.
20. **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.
21. **Mantle, M., D. Mantle, and A. Allen.** 1981. Polymeric structure of pig small intestinal mucus glycoprotein. Biochem. J. **195:**277–285.
22. **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.
23. **Martinez, R. J.** 1983. Plasmid-mediated and temperature-regulated surface properties of *Yersinia enterocolitica*. Infect. Immun. **41:**921–930.
24. **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.
25. **McCool, D. J., M. A. Marcon, J. F. Forstner, and G. G. Forstner.** 1990. The T84 human colonic adenocarcinoma cell line produces mucin in culture and releases it in response to various secretagogues. Biochem. J. **267:**491–500.
26. **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.
27. **Michiels, T., and G. R. Cornelis.** 1990. Secretion of Yop proteins by yersiniae. Infect. Immun. **58:**2840–2849.
28. **Miller, R. S., and L. C. Hoskins.** 1981. Mucin degradation in human colon ecosystems. Fecal population densities of mucin-degrading bacteria estimated by a "most probable number" method. Gastroenterology **81:**759–765.
29. **Pærregaard, 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.
30. **Pai, C. H., and L. DeStephano.** 1982. Serum resistance associated with virulence in *Yersinia enterocolitica*. Infect. Immun. **35:**605–611.
31. **Pai, C. H., V. Mors, and T. A. Seemayer.** 1980. Experimental *Yersinia enterocolitica* enteritis in rabbits. Infect. Immun. **28:**238–244.
32. **Poncz, L., N. Jentoft, M.-C. D. Ho, and D. G. Dearborn.** 1988. Kinetics of proteolysis of hog gastric mucin by human neutrophil elastase and by *Pseudomonas aeruginosa* elastase. Infect. Immun. **56:**703–704.
33. **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.
34. **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.
35. **Prizont, R., and W. P. Reed.** 1991. Differences in blood group B-specific mucinase activity between virulent and avirulent *Shigella flexneri* 2a strains. Microb. Pathog. **11:**129–135.
36. **Roberton, A. M., and R. A. Stanley.** 1982. In vitro utilization of mucin by *Bacteroides fragilis*. Appl. Environ. Microbiol. **43:**325–330.
37. **Sarosiek, J., J. Bilski, V. L. N. Murty, A. Slomiany, and B. L. Slomiany.** 1989. Colloidal bismuth subcitrate (De-Nol) inhibits degradation of gastric mucus by *Campylobacter pylori* protease. Am. J. Gastroenterol. **84:**506–510.
38. **Schiemann, D. A.** 1989. *Yersinia enterocolitica* and *Yersinia*

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

39. **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.
40. **Sellers, L. A., A. Allen, E. R. Morris, and S. B. Ross-Murphy.** 1987. Mechanical characterisation and properties of gastrointestinal mucus gel. Biorheology **24:**615–623.
41. **Skurnik, M.** 1985. Expression of antigens encoded by the virulence plasmid of *Yersinia enterocolitica* under different growth conditions. Infect. Immun. **47:**183–190.
42. **Stanley, R. A., S. P. Ram, R. K. Wilkinson, and A. M. Roberton.** 1986. Degradation of pig gastric and colonic mucins by bacteria isolated from the pig colon. Appl. Environ. Microbiol. **51:**1104–1109.
43. **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.
44. **Thornton, D. J., D. F. Holmes, J. K. Sheehan, and I. Carlstedt.** 1989. Quantitation of mucus glycoproteins blotted onto nitrocellulose membranes. Anal. Biochem. **182:**160–164.
45. **Wadolkowski, E. A., D. C. Laux, 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.