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In Vitro Utilization of Mucin by *Bacteroides fragilis*

ANTHONY M. ROBERTON†* AND ROGER A. STANLEY

Department of Biochemistry, The University of Auckland, Auckland, New Zealand

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A method for isolating pig colon mucin in a soluble high-molecular-weight form, suitable for addition to bacterial growth media, is described. This preparation was utilized as a sole carbohydrate energy source by two strains of *Bacteroides fragilis*. The extent of degradation was compared with that of commercial pig gastric mucin by the same strains. Gas-liquid chromatographic analysis of the mucin carbohydrates and gel chromatography of the preparations were carried out before and after in vitro degradation. The mucin carbohydrates were utilized only to a very limited extent, colon mucin being more resistant to degradation than gastric mucin. Both mucins chromatographed at or near the excluded volume on Sepharose 4B, and only in the case of ATCC 25285 grown on gastric mucin was a significant degradation peak detected. If mucins are degraded in vivo by the sequential action of several bacteria, a pure culture in vitro might be expected to degrade mucins to a limited extent only. Techniques previously used to examine mucin utilization by pure cultures may have overlooked limited mucin degradation demonstrated by the methods used in this work.

Intestinal mucins are thought to be available as fermentable substrates for many of the colon anaerobic bacteria (11). Investigators have attempted to demonstrate the degradation of mucins by incorporating them into a basal medium and measuring growth (6), fermentation (15, 16), and "mucinase" production (19) by pure cultures or the production of glycosidases by mixed cultures (9).

Mucins are high-molecular-weight complex glycoproteins, and because of this complexity their in vivo degradation in the colon may require the sequential action of a series of bacteria (7). A search for gross utilization of a single mucin by a pure culture may not detect utilization of a small portion of the mucin, which could be ecologically significant in vivo.

In this work we investigated the degradation of two types of mucins by *Bacteroides fragilis*. The loss of specific carbohydrates and the change of molecular size were measured. This approach was developed to yield specific information on the role that a single bacterium may play in mucin degradation. Such information will be necessary to test models of mucin breakdown. A method for extracting pig colon mucin as a soluble high-molecular-weight product in quantities which can be incorporated into bacterial media is also described.

† Present address: Mikrobiologie, Fachbereich Biologie, Philipps-Universitat, 3550 Marburg/Lahn, Lahnberg, West Germany.

MATERIALS AND METHODS

Bacteria. *B. fragilis* strains ATCC 25285 and ATCC 23745 were obtained from the American Type Culture Collection, Rockville, Md. Cultures were maintained on the medium used by Sperry et al. (18) to which 2 μg of heme per ml and 1.5% agar had been added. Strains were kept at 4°C or stored at −70°C when not required for routine use.

Preparation of culture medium. Bacteria were grown in liquid culture on a basal medium modified from medium 10 (2), with the omission of volatile fatty acids and the inclusion of mucin in place of the usual carbohydrates. The medium contained, per liter (final volume): K_2HPO_4, 0.23 g; KH_2PO_4, 0.23 g; $(NH_4)_2SO_4$, 0.23 g; NaCl, 0.46 g; $MgSO_4 \cdot 7H_2O$, 0.09 g; $CaCl_2 \cdot 2H_2O$, 0.04 g; yeast extract (BBL Microbiology Systems, Cockeysville, Md.), 0.5 g; Trypticase (BBL), 2 g; resazurin, 0.001 g; and hemin, 1×10^{-5} g, pH 6.5. These components were sterilized in half the final volume under O_2-free N_2 (121°C for 15 min). Sterile anaerobic solutions of CO_2-equilibrated Na_2CO_3 (4 g), cysteine-HCl (0.5 g), mucin as indicated, and water to volume were subsequently added, and the medium was equilibrated with O_2-free CO_2.

Culture procedures. The bacteria were passaged at least four times on agar containing gastric mucin as an energy source. An inoculum was prepared by stabbing from a fresh agar slope of bacteria into 2.0 ml of broth containing 10 mg (dry wt) of the appropriate mucin per ml. This was incubated for 24 h at 37°C. A 0.1-ml portion was transferred into tubes containing 2.0 ml of broth, and growth was followed by measuring the optical density at 600 nm on a Coleman Junior II spectrophotometer (Coleman Instruments Corp., Ill.). At the conclusion of experiments, the cultures were centrifuged at 10,000 × *g* for 10 min, and the supernatants stored at −10°C for later analysis.

Pig colon mucin preparation. Colon mucin was col-

lected from freshly slaughtered adult pigs of either sex. Approximately 60 cm of colon was excised from the apex of the centripetal and centrifugal coils within 20 min of death. Fecal material was removed by gentle irrigation with cold water, and the colon mucosal surface was scraped with a glass microscope slide to collect the mucus gel (14). The material consisting of mucus, epithelial cells, and bacterial debris was placed into flasks immersed in ice water for transport back to the laboratory, where the mucus was processed immediately.

The gel was dispersed in 2 volumes of 0.1 M NaCl containing 10 mM sodium phosphate, pH 7.0, by making two passes with the pestle in a motor-driven Thomas Teflon-pestle tissue grinder. The mucin precipitate was retained after centrifugation and suspended in a volume of the buffered NaCl equivalent to the volume of the discarded supernatant, and the homogenization and centrifugation were repeated. The mucus was occasionally freeze-dried at this stage, and the purification was continued later by suspending the material in buffered NaCl (1 g/20 ml). Usually, however, further purification was carried out on fresh mucin.

The insoluble mucin was extracted for 12 h at 0°C under N_2 in 4 volumes of 0.1 M NaCl, 0.1 M sodium phosphate, and 0.4 M β-mercaptoethanol, pH 7.5. The debris was removed by centrifuging for 10 min at 10,000 × *g*, and the supernatant containing solubilized mucin was exhaustively dialyzed against distilled water at 4°C. It was then digested with DNase I (type III; Sigma Chemical Co., St. Louis, Mo.), 25 μg per g (wet wt) of original mucus, in 0.02% (wt/vol) NaN_3–10 mM $MgCl_2$–5 mM $CaCl_2$–0.1 M sodium phosphate buffer, pH 7, at 37°C for 6 h. Trypsin (type IX; Sigma), 25 μg per g (wet wt), was then added, and the digestion was continued for 12 h. An equivalent amount of trypsin was again added, and the digestion was continued for a further 12 h. The solution was then exhaustively dialyzed at 4°C against distilled water, insoluble material being removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was freeze-dried and stored. The properties of this colon mucin are discussed in Results.

A modification of the above-described method was introduced to give better mucin solubilization in those preparations with a high content of DNA. The insoluble DNA appeared to form a gel coprecipitating the mucin molecules on centrifugation. DNase I in 0.02% (wt/vol) NaN_3, 10 mM $MgCl_2$, and 5 mM $CaCl_2$ was added to the mucus solution after the β-mercaptoethanol treatment and incubated for 6 h at 37°C followed by centrifugation to remove insoluble debris. Further dialysis and digestion steps were carried out as detailed above.

Pig gastric mucin. Pig gastric mucin was purchased from Sigma as a protease-digested preparation. It was suspended at 50 mg/ml, exhaustively dialyzed against distilled water, and freeze-dried.

Sterilization of mucin. Sigma pig gastric mucin, suspended at 40 mg/ml in water, was sterilized by autoclaving. This has been shown not to break glycosidic bonds (15) but may cause irreversible structural changes due to heating (12). Volumes up to 10 ml were sometimes sterilized by filtration through 0.45-μm membrane filters, using a prefilter to reduce blockage. The pig colon mucin was less viscous and could be sterilized by membrane filtration (0.45-μm pore size) at 40 mg/ml. Monosaccharides used in control experiments were sterilized by autoclaving separately under N_2 in distilled water.

The sterile mucin solutions were entered into a Coy anaerobic chamber, equilibrated with the O_2-free atmosphere for at least 2 h in shallow containers, and then added aseptically to the double-strength basal medium, water being added to give the correct volume. Before use, tubes were gassed with CO_2, and the cysteine-HCl was added finally.

Analytical procedures. The carbohydrates present in mucin were quantitatively determined by gas-liquid chromatography of their trimethylsilyl derivatives after methanolysis (3, 4). Up to 0.1 ml of culture supernatant was analyzed, with 0.1 μmol of *meso*-inositol added as an internal standard. The methanolysis step was carried out in 0.5 ml of 1.5 N methanolic HCl for 24 h under N_2 at 85°C. Trimethylsilylation was carried out in 0.2 ml of a freshly prepared solution of pyridine, trimethylsilane, and hexamethyldisilazane (5:1:1). After 30 min at room temperature, 1 to 3 μl of sample was injected into a Varian 1240 gas chromatograph containing a 78-cm glass column (0.13-cm OD) packed with 5% OV-101 on Chromosorb W. The temperature was held at 140°C for 5 min and then programed to increase to 220°C at 0.5°C per min. The upper limit was held until the *N*-acetylneuraminic acid (NANA) derivative peaks emerged. Relative response factors were determined from peak areas of standard solutions for each sugar.

Free neuraminic acids were determined by the procedure of Warren (20). Total sialic acids were measured after hydrolysis for 1 h at 80°C in 0.05 M H_2SO_4. Standard Sigma type VII NANA and 2-deoxyribose were included in each set of readings, and the interference by DNA was corrected for, using simultaneous equations. Pretreatment of mucins with 0.1 M KOH to remove *O*-acetyl groups resulted in less than a 5% increase in NANA yield and was therefore not used routinely.

Ascending gel filtration chromatography was performed with a column (59 by 0.9 cm; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) packed with Sepharose 4B. Samples were eluted with 0.1 M NaCl containing 1 mM sodium phosphate buffer, pH 7.5. Fractions of 2 ml were collected, and their carbohydrate content was ascertained by periodic acid-Schiff stain (13). The excluded column volume (V_0) eluted at 20 ml; the total column volume ($v_0 + v_i$) eluted at 60 ml.

RESULTS

Properties of pig colon mucin and pig gastric mucin preparations. More than 60% of the NANA in the crude mucosal scrapings was recovered bound to the soluble colon mucin preparations described. The main contaminants removed were DNA, soluble proteins, and cell debris. When the soluble mucin preparation was chromatographed on Sepharose 4B, over 70% of the dry weight and greater than 95% of the carbohydrates were found at or near the excluded peak, showing that the bulk of the material was bound to high-molecular-weight molecules. The molar ratios of carbohydrates found in the

TABLE 1. Utilization of pig gastric mucin[a] by *B. fragilis*

Mucin	Concn of mucin carbohydrate[b] (mM)						
	Fucose	Mannose	Galactose	GalNAc	GlcNAc	NANA	Total[c]
Undigested	3.1	0.1	10.3	7.0	10.7	0.3	31.5
ATCC 25285 digested	3.0	0	7.9	6.3	9.6	0.1	26.9
ATCC 23745 digested	3.0	0	9.1	6.3	10.4	0.1	28.9

[a] Added at 10 g (dry wt)/liter. The mucin was sterilized by autoclaving.
[b] Determination error is within 5%. Results are from two experiments, with analyses done in duplicate. Different batches of mucin had slightly different carbohydrate compositions and gave internally consistent results.
[c] Total carbohydrate present attached to mucin. Basal medium without mucin contained 0.2 mM carbohydrate. *B. fragilis* 25285 grown in glucose basal medium resulted in less than 1 mM carbohydrate not utilized or excreted into the medium by the bacteria.

colon mucin preparation were: *N*-acetylgalactosamine (GalNAc), 1.0; *N*-acetylglucosamine (GlcNAc), 1.5; galactose, 1.4; fucose, 0.6; NANA, 0.3; and mannose, 0.02. These values are typical of a high-molecular-weight colon mucin (5).

Analysis of the dialyzed commercial gastric mucin gave molar ratios of: GalNAc, 1.0; GlcNAc, 1.5; galactose, 1.5; fucose, 0.4; NANA, 0.04; and mannose, 0.014. Greater than 80% of the dry weight and over 95% of the carbohydrates were found at or near the excluded peak on Sepharose 4B. This analysis is in close agreement with that of List et al. (12) for dialyzed Sigma pig gastric mucin.

Glucoronic acid was not detected in either preparation by gas-liquid chromatography, implying less than 0.01% (wt/wt) contamination by connective tissue glycosaminoglycans.

Bacterial utilization of mucin. Tables 1 and 2 show the mucin sugars present in the medium, before inoculation with *B. fragilis* and after full bacterial growth. Gastric mucin was used more extensively than colon mucin even though the actual compositions were not dissimilar. From 10 g of mucin per liter, about 5 mmol (8 to 15%) of the gastric mucin carbohydrate and about 2 mmol (6 to 7%) of the colon mucin carbohydrate was used by the pure cultures of bacteria.

Most of the small amounts of mannose and neuraminic acid disappeared from the gastric mucin during bacterial growth. By contrast, about 75% of the neuraminic acid(s) in the colon mucin was resistant to removal by bacteria. The Warren assay procedure (20) did not detect any free sialic acid in the medium, indicating that this neuraminic acid was still bound to the glycoprotein. Small amounts of galactose, GalNAc, and GlcNAc were usually utilized, with the effect being more obvious in the gastric mucin because of the greater degradation. Within the limits of the analytical methods, only fucose appeared to be completely resistant to removal by both strains of *B. fragilis*.

Figures 1 and 2 show the molecular sizes of the mucins before and after digestion. The gastric mucin elution volume was not altered by *B. fragilis* 23745, but *B. fragilis* 25285 consistently gave a peak in the included volume in three separate experiments (Fig. 1). The colon mucin did not show any marked alteration in elution volume after digestion by either strain of bacteria (Fig. 2).

Bacterial growth on mucin. Both strains of *B. fragilis* grew more slowly on colon mucin and gastric mucin than on glucose (Fig. 3). The maximal cell optical density reached by both strains was greater for gastric mucin than for colon mucin at 10 mg/ml. For each mucin, the optical density was approximately proportional to the amount added for the individual mucin type.

DISCUSSION

The isolation of a pig colon mucin preparation has been described. It is soluble and of high

TABLE 2. Utilization of pig colon mucin[a] by *B. fragilis*

Mucin	Concn of mucin carbohydrate[b] (mM)						
	Fucose	Mannose	Galactose	GalNAc	GlcNAc	NANA	Total[c]
Undigested	3.4	0.1	7.8	5.5	8.4	1.9	27.1
ATCC 25285 digested	3.3	0	7.3	4.8	8.3	1.6	25.3
ATCC 23745 digested	3.3	0	7.8	5.1	7.8	1.5	25.5

[a] Added at 10 g (dry wt)/liter. The mucin was filter sterilized.
[b,c] See Table 1.

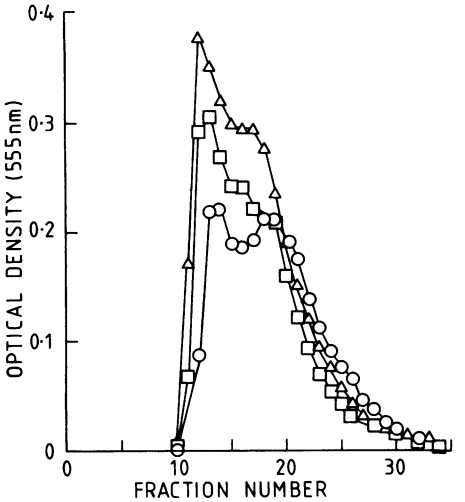

FIG. 1. Molecular size distribution of gastric mucin before (□) and after digestion by *B. fragilis* ATCC 25285 (○) and ATCC 23745 (△).

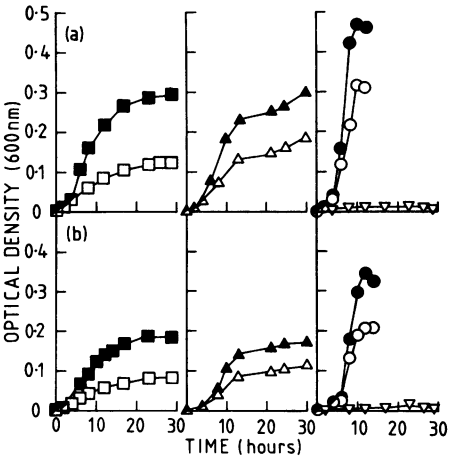

FIG. 3. Growth of *B. fragilis* on gastric mucin, 5 (□) and 10 (■) mg/ml; on colon mucin, 10 (△) and 20 (▲) mg/ml; on glucose, 3 (○) and 6 (●) mM; or with no added carbohydrate (▽). (a) Strain ATCC 25285. (b) Strain ATCC 23745. Inocula were grown on pig gastric mucin. The mucins were sterilized by filtration.

molecular weight, resembles the carbohydrate content of other mucins, can be sterilized by filtration, and can be incorporated into bacterial medium in an optically clear form suitable for studies of mucin utilization. Its digestion by two strains of *B. fragilis* has been compared with that of a commercial preparation of pig gastric mucin.

B. fragilis was chosen for this study because it is among the most successful of the colon bacteria numerically, and growth studies (Fig. 3) showed that strains ATCC 25285 and ATCC 23745 must possess enzymes capable of utilizing a portion of pig gastric and pig colon mucin for growth. There is, however, no evidence to indicate whether *Bacteroides* is the main mucin-degrading genus in the colon. Since the two strains used were unable to grow on the medium when mucin was omitted, they must produce glycosidases capable of cleaving some sugars from the high-molecular-weight mucin. Analysis of the sugars by gas-liquid chromatography showed that 15% or less of the total mucin sugars were used, and a 1% mucin solution grew approximately 10^9 cells per ml (data not shown). If the carbohydrate chains of the mucin contain up to 18 sugar units (17), this would represent one or two terminal sugars being used on average from each chain.

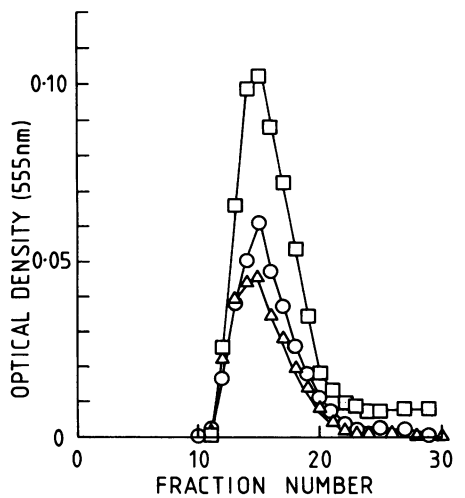

FIG. 2. Molecular size distribution of colon mucin before (□) and after digestion by *B. fragilis* ATCC 25285 (○) and ATCC 23745 (△).

Previous studies have failed to show or have detected only low numbers of bacteria capable of utilizing mucins as energy sources (1, 11, 16). Several factors make it difficult to determine whether pure cultures of bacteria can use mucins. The proposed structure of pig gastric mucin indicates that the carbohydrate side chains are likely to be of a heterogeneous nature, with up to 18 sugar residues, sulfate groups, and multiple linkage types (17). Thus, many different glycosidases and a sulfatase would be required for complete degradation. It is suggested that, in vivo, there may be a cooperative process with several bacteria sequentially or competitively cleaving the glycosidic bonds (16). Isolated pure cultures in vitro may therefore be able to utilize only part of the mucin. Because most mucins isolated from the gastrointestinal tract are heterogeneous with partly degraded or incomplete side chains (5), many different terminal groups

are probably exposed. Bacteria with an enzyme armament capable of cleaving normally interior linkages will grow to only a small extent on isolated mucin. Similarly, bacteria capable of attacking an external linkage also will grow only to a limited extent. Lindstedt et al. (11) found that only a mixed conventional flora would completely degrade the mucus constituents of germfree rat stools.

Measurement of mucin degradation by a fermentative pH drop may overlook bacteria capable of using only one or two terminal sugars. Salyers et al. (15) did not detect any *B. fragilis*, type I or type II (10) strains, capable of using pig gastric mucin among 46 strains tested by this method. The two *B. fragilis* strains used in the present work were type I (10). An example demonstrating that the ability to remove a single sugar group may influence the composition of fecal flora was the study by Hoskins and Boulding (8) showing a 50,000-fold increase in numbers of bacteria capable of cleaving the B-antigenic determinant (α-D-galactose) in type B secretors over type A or H secretors.

Our results detected differences between the degradation of commercial pig gastric mucin and colon mucin as prepared in this work. *B. fragilis* ATCC 25285 increased the proportion of gastric mucin within the included peak on Sepharose 4B, but this was not observed with the colon mucin. As both mucins were prepared by proteolytic digestion, it is unlikely that the difference is a simple proteolytic cleavage of the peptide backbone. The magnitude of the size change, however, indicates the formation of molecules with a smaller volume than would be expected by the removal of a few outer sugars. The pig colon mucin has a higher proportion of neuraminate molecules than the gastric mucin. Our results indicate that only about 20% of the colon mucin neuraminate could be removed by either *B. fragilis* strain, yet most of the gastric mucin neuraminate disappeared when incubated with these bacteria. In contrast, Sigma *Clostridium perfringens* neuraminidase was able to release 70 to 80% of the neuraminate from colon mucin without prior removal of *O*-acetyl groups by alkaline hydrolysis (data not shown).

The differences in degradation of commercial pig gastric and pig colon mucin by these *B. fragilis* strains may be that mucins isolated from different sites may have different susceptibilities to catabolism despite having similar carbohydrate types. This could be due to variations in the type and proportion of the charged neuraminate and sulfate, different terminal carbohydrates, or differences in the linkages between carbohydrate monomers. There are, however, other possible explanations, such as differences in the preparation methods or in the in vivo degradation of mucin before isolation. It is important, therefore, to exercise care in extrapolating the results from experiments using gastric mucin as a model mucin substrate for colon bacteria.

The role of mucins in the ecology of the gastrointestinal bacteria will be better understood when more information is available on the sugars utilized by the major types of bacteria involved, the bonds cleaved, and the molecular size of the products formed. If many different bacteria have the potential to participate in a stepwise degradation of mucin, then this factor may exert a significant influence over the composition of the intestinal flora, particularly near the mucosal surface.

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