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Evidence that Polygalacturonic Acid May Not Be a Major Source of Carbon and Energy for Some Colonic *Bacteroides* Species

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Five *Bacteroides* species that are found in the human colon can utilize polygalacturonic acid (PGA) when they are grown in laboratory media: *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Bacteroides ovatus*, *Bacteroides fragilis* subsp. a, and *Bacteroides* sp. strain 3452A (an unnamed DNA-DNA homology group). PGA-degrading enzymes from *B. thetaiotaomicron* have been isolated and characterized previously. To determine whether a PGA lyase activity in human feces could be attributed to any of these species, we first determined the properties of PGA lyases from the other four *Bacteroides* species. PGA lyases from all the *Bacteroides* species were soluble, cell associated, and inducible by PGA. All had similar pH optima (8.4 to 8.8) and similar molecular weights (50,000). All activities were enhanced by calcium. The PGA lyases from the five species differed with respect to isoelectric point: *B. thetaiotaomicron* (pI 7.5), *B. vulgatus* (pI 7.7), *B. ovatus* (pI 5.8, 7.2), *B. fragilis* subsp. a (pI 6.1), and *Bacteroides* sp. strain 3452A (pI 7.7). The PGA lyase activity in human feces resembled those of the *Bacteroides* PGA lyases in that it had a pH optimum of 8.4 to 8.8 and was enhanced by calcium. However, it differed from the *Bacteroides* PGA lyases both with respect to isoelectric point (pI 4.2 to 4.4) and molecular weight (100,000). On the basis of these findings, it appears that the PGA lyase activity in human feces is not produced by any of the *Bacteroides* species surveyed in this survey. Moreover, there was no detectable PGA lyase activity in feces that had the same properties as the *Bacteroides* enzymes. Because the *Bacteroides* PGA lyases were produced at high levels only when they were grown on PGA, the failure to detect *Bacteroides* PGA lyases indicates that PGA may not be a major source of carbon and energy for these species in the colon.

Dietary polysaccharides are degraded extensively in the human colon by the resident microflora (16). A previous survey of human colonic bacteria showed that many of the strains that are capable of degrading polysaccharides are members of the genus *Bacteroides* (14, 15). Thus, *Bacteroides* species could be responsible for the extensive digestion of polysaccharides that occurs in the human colon. The demonstration that an organism can utilize a polysaccharide when it is grown as a pure culture in a laboratory medium does not prove that the organism actually degrades the polysaccharide in the colon. Accordingly, we wanted to find some way to determine directly what polysaccharides are actually being degraded by *Bacteroides* species in the colon. All of the polysaccharides from human colonic *Bacteroides* species that have been studied to date have proven to be regulated by the polysaccharide substrate. That is, the enzyme specific activity is barely detectable, whereas the specific activity is elevated substantially when the organism is grown on the polysaccharide (13). High levels of polysaccharidase activity might thus serve as indicators of polysaccharide utilization by *Bacteroides* species in the colon.

Until recently it has not been feasible to use polysaccharidase activities as an indication of polysaccharide utilization in the colon because relatively little was known about polysaccharidases that are produced by the different *Bacteroides* species. We now have considerable information about the breakdown of one dietary polysaccharide, poly-D-galacturonic acid (PGA), by *Bacteroides thetaiotaomicron*. When this organism is grown on PGA, it produces two degradative enzymes, both of which have been partially purified and characterized (9). One is a polygalacturonate lyase (EC 4.2.2.2) that degrades PGA primarily to unsaturated disaccharides. This enzyme is soluble and cell associated. The second enzyme is a PGA hydrolase (EC 3.2.1.15). This enzyme releases monosaccharides from PGA and is associated primarily with the inner membrane (9). Synthesis of both enzymes is induced by PGA.

In *B. thetaiotaomicron*, the PGA lyase, a soluble enzyme, is the most easily detectable of the two enzymes. Also, the assay for PGA lyase is more specific and less prone to interference than the assay for PGA hydrolase. Thus, PGA lyase activity seemed to be a good indicator of PGA utilization by *B. thetaiotaomicron*. Preliminary experiments indicated that we could detect PGA lyase activity in a bacterial fraction which had been isolated directly from human feces by centrifugation. We wanted to determine whether this PGA lyase activity was produced by *B. thetaiotaomicron* or by one of the other *Bacteroides* species that degrade PGA. Several numerically predominant colonic *Bacteroides* species other than *B. thetaiotaomicron* include strains that can utilize PGA: *B. vulgatus*, *B. ovatus*, *Bacteroides* sp. strain 3452A (an unnamed DNA homology group that was formerly classified as part of *B. distasonis*), and *B. fragilis* subsp. a. However, no information was available about the PGA lyases of these organisms. We determined the cellular location, inducibility, molecular weight, and isoelectric point of the PGA lyases of *B. ovatus*, *Bacteroides* sp. strain 3452A, *B. fragilis* subsp. a, and *B. vulgatus*. Characteristics of these enzymes and of the PGA lyase of *B. thetaiotaomicron* were then compared with the characteristics of a PGA lyase activity that is detectable in human feces.

MATERIALS AND METHODS

Organisms and culture conditions. *Bacteroides* strains were obtained from the culture collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State Univer-

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sity, Blacksburg. These strains included the following: *B. thetaiotaomicron* 5482 (ATCC 29148), *B. fragilis* subsp. a B5-21, *B. ovatus* 0038 (ATCC 8483), *Bacteroides* sp. strain 3452A, and *B. vulgatus* 4245 (ATCC 8482). Stock cultures were maintained at room temperature in chopped meat broth (3) under an atmosphere of CO_2.

Bacteria were grown in a defined medium which was similar to the defined medium described by Varel and Bryant (17), except that potassium phosphate buffer (0.1 M, pH 7.0) was substituted for carbonate and histidine-hemin (2.0 μg/ml) was substituted for hemin. In most cases PGA (Sigma Chemical Co., St. Louis, Mo.) was the sole source of carbohydrate. For batch cultures, PGA was added to the medium before autoclaving to a final concentration of 5 mg/ml. For induction experiments, bacteria were grown in defined medium that contained galacturonic acid (5 mg/ml) as the carbohydrate source. Galacturonic acid was filter sterilized and added to the medium after autoclaving. The atmosphere was 20% N_2–80% CO_2. In experiments to determine the effect of generation time on PGA lyase activity, bacteria were grown in continuous culture with PGA (0.3%) as the limiting carbohydrate (5).

PGA lyase assays. In most instances, PGA lyase activity was measured by monitoring the increase in A_{235}, which was due to the release of Δ4,5-unsaturated oligosaccharides. For the isoelectric focusing (IEF) gels, this assay could not be used when Triton X-100 was added to optimize focusing because Triton X-100 absorbs at 235 nm. Accordingly, PGA lyase activity was measured by monitoring the increase in reducing sugar concentration (1). In some cases, to confirm that the detectable activity was PGA lyase, Triton X-100 was omitted and the A_{235} assay was used. The reducing sugar assay was also used to locate PGA lyase activity in fractions from the gel filtration column. In both cases, the PGA lyase assay mixture (1-ml total volume) contained enzyme, 1 mg of PGA, and 0.2 mM $CaCl_2$ in 20 mM Tris phosphate buffer (pH 8.7). PGA lyase activity was linear throughout the assay period. One unit of enzyme activity was defined as an increase in 1 absorbance unit (at 235 nm or, in the case of the reducing sugar assay, at 450 nm) per minute. One unit at 235 nm was approximately equal to 1 unit at 450 nm.

The procedure of Lowry et al. (8) was used to measure the protein concentration, with bovine serum albumin used as the standard.

Localization experiments. Bacteria were grown in 2 liters of PGA defined medium to an optical density at 650 nm (1-cm path length) of 0.6 to 0.9 and harvested by centrifugation at 10,000 × *g* for 20 min (4°C). The supernatant fluid (extracellular fluid) was dialyzed against potassium phosphate buffer (50 mM, pH 7.0) and assayed for polygalacturonase activity. In some cases, the extracellular fluid was concentrated 10-fold on a Centriflo concentrator (Amicon Corp., Lexington, Mass.) and assayed for activity. The harvested bacteria were washed twice and suspended to a final volume of 50 ml in 50 mM potassium phosphate buffer (pH 7.0). A total of 2 mg DNase I and 4 mg of RNase A (Sigma) were added. The cells were disrupted by two passages through a French pressure cell (12,000 lb/in^2) and allowed to stand for 30 min at 4°C. The cellular debris was removed by centrifugation (17,000 × *g* for 15 min at 4°C), and the supernatant fluid (cell extract) was centrifuged at 200,000 × *g* for 2.5 h at 4°C. The supernatant fluid (soluble protein; S1) was saved. The membrane pellet was suspended in 50 mM potassium phosphate buffer (pH 7.0) by homogenization with a 2-ml tissue homogenizer. A portion of the suspended membrane preparation was saved for assay, and the remainder was centrifuged at 200,000 × *g* for 2.5 h at 4°C. The supernatant fluid (membrane wash; S2) and suspended membrane pellet (P2) were saved for assay. All cellular fractions were assayed for PGA lyase; phosphoglucose isomerase, a soluble enzyme; and the membrane-associated enzyme succinate dehydrogenase (9). The soluble fraction (S1), which contained most of the PGA lyase activity, was used in experiments to determine the isoelectric points and the molecular weights of the PGA lyases from the different species.

Induction experiments. To determine whether PGA lyase specific activity was increased when organisms were grown on PGA, bacteria were grown on defined medium that contained D-galacturonic acid (0.5%) or on defined medium that contained PGA (0.5%). To assess the effect of PGA concentration on PGA lyase specific activity, *B. thetaiotaomicron* and *B. fragilis* subsp. a were grown on PGA defined medium which contained different PGA concentrations (0.2, 1, and 5 mg/ml). To assess the effect of growth rate on PGA lyase specific activity, bacteria were grown in PGA-limited continuous cultures at generation times of 15 to 22 h. To assess the stability of PGA lyase, bacteria were grown in batch culture (5 mg/ml) until they reached the stationary phase. Then, portions were removed at 12-h intervals and assayed for PGA lyase activity.

Bacteria were harvested at an optical density at 650 nm (1 cm light path) of 0.7 to 1.0 and centrifuged at 10,000 × *g* for 15 min at 4°C. The bacteria were washed twice, suspended in 50 mM potassium phosphate buffer, and disrupted by sonication (50% pulsed sonication for a total of 4 min). Cell debris was removed by centrifugation at 17,000 × *g* for 15 min at 4°C, and the supernatant fluid (cell lysate) was assayed for PGA lyase activity.

Electrophoresis. For IEF, tube gels (9 mm by 13 cm) were used. The composition of the IEF gels was 5% acrylamide, 12% glycerol, 1% Triton X-100, and 2% ampholytes from Serva, Garden City Part, N.Y. (ratio of pH 3 to 10/pH 5 to 8/pH 7 to 9 was 3:1:1 [vol/vol/vol]). The anode and cathode solutions were 0.1 M H_3PO_4 and 0.1 M NaOH, respectively. Soluble protein from sonically disrupted pure cultures or suspended fecal bacteria was loaded onto the gel. Electrophoresis was carried out as described by Righetti and Drysdale (11). Triton X-100 was added to the gels to ensure that all the activity in the soluble fraction migrated into the gel. In some cases, however, Triton X-100 was omitted so that the A_{235} assay for PGA lyase could be used. After electrophoresis, gels were cut into 0.5-cm slices, placed into 0.7 ml of potassium phosphate buffer (20 mM, pH 7.0), and allowed to stand at 4°C overnight. Eluant from each gel slice was mixed with PGA, and PGA lyase activity was detected by measuring the increase in reducing sugar concentration after incubation for 1 h at 37°C. Approximately 10 to 20% of the activity that was loaded on the gel was recovered from gel slices. For determination of the isoelectric point, slices of a parallel gel were eluted with distilled water (at 4°C overnight), and the pH of the eluant was measured.

Gel filtration chromatography to estimate native molecular weights of the PGA lyases. A Sephacryl S-300 column was used to estimate the molecular weight of the fecal PGA lyase. A Sephadex G-100 column was used to estimate the molecular weights of the *Bacteroides* enzymes. The Sephadex G-100 gel filtration column (1.5 by 70 cm) was equilibrated with 50 mM potassium phosphate buffer (pH 7.0), as was the Sephacryl S-300 column (1.5 by 71 cm). In both cases the soluble protein fraction (S1) was applied to the column, and 1.5-ml fractions were collected. The flow rate was approximately 25 ml/h. Column fractions were assayed

for PGA lyase activity by mixing 0.3 ml of each column fraction with 0.3 ml of PGA (1 mg/ml, pH 8.7, 0.2 mmol $CaCl_2$) and by incubating at 37°C for 1 h. The concentration of reducing sugar in each mixture was compared with the concentration of reducing sugar in a parallel mixture to which boiled enzyme was added to substrate. The following proteins were used as molecular weight standards: thyroglobulin (670,000), yeast alcohol dehydrogenase (150,000), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and RNase A (13,700). Recovery of enzyme activity from column fractions was at least 75 to 80% for all samples.

Preparation of fecal samples for IEF or gel filtration. Freshly passed stool samples, from two healthy adult volunteers, were collected in plastic bags and thoroughly kneaded to ensure as homogeneous a preparation as possible. Fecal samples were suspended in cold potassium phosphate buffer (50 mM, pH 7.0) by homogenization with a Teflon- (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) coated pestle and vigorous vortexing. Ten milliliters of buffer was used to suspend each gram (wet weight) of feces. The suspension was centrifuged at 10,000 × *g* for 15 min at 4°C to pellet the bacteria, and the resulting pellets were suspended in an equal volume (10 ml/g [wet weight] of feces) of potassium phosphate buffer. Suspended bacteria were disrupted by sonication (4 min at 50% pulsed sonication).

To estimate the percent efficiency of sonication for bacteria in the fecal specimens, intact cells of *B. thetaiotaomicron* grown on PGA were added to a fecal preparation, to a final bacterial concentration that approximated the level of this species in feces; the mixture was sonicated as described above. The PGA lyase activity in the mixture was approximately equal to the sum of the two controls, feces alone or *B. thetaiotaomicron* cells alone which had been suspended to the same volume and sonicated. The percent efficiency of sonic disruption for bacteria in fecal preparations was at least 90% of that observed with pure cultures.

TABLE 1. Cellular location of PGA lyase activity

Species	Percentage of total activity of the following[b]:			
	Fraction[a]	PGA lyase	PGI[c]	SDH[d]
B. thetaiotaomicron	S1	71	73	5
	P1	17	10	83
	S2	8	6	4
	P2	<2	2	78
B. fragilis subsp. a	S1	74	72	4
	P1	11	9	85
	S2	8	7	3
	P2	<1	<1	83
B. ovatus	S1	62	64	3
	P1	16	13	78
	S2	10	9	2
	P2	<1	<2	75
Bacteroides sp. strain 3452A	S1	64	78	2
	P1	11	16	89
	S2	7	12	3
	P2	3	<1	82
B. vulgatus	S1	69	87	2
	P1	21	17	88
	S2	14	14	1
	P2	<1	<1	79

[a] S1 and S2 refer to the soluble (cytoplasmic) fraction remaining after membranes were removed following the first and second ultracentrifugations, respectively. P1 and P2 refer to the pelleted membrane fractions resulting from the first and second ultracentrifugations, respectively.

[b] Variation from one experiment to another was between 5 and 10%.

[c] Phosphoglucoisomerase (PGI) was used as a marker for soluble protein.

[d] Succinate dehydrogenase (SDH) was used as a marker for membranes.

TABLE 2. Effect of carbohydrate source on PGA lyase specific activity

Species	Sp act (U/mg of protein[a]) of PGA lyase in bacteria grown on:	
	Galacturonic acid	PGA
B. thetaiotaomicron	0.13	3.5
B. fragilis subsp.	0.19	8.0
B. ovatus	0.56	6.0
Bacteroides sp. strain 3452A	0.05	1.7
B. vulgatus	<0.02	0.4

[a] One unit is defined as a change of 1 absorbance unit at 235 nm per min.

After the final sonication, the sonicated suspension was centrifuged (17,000 × *g* for 15 min at 4°C) to pellet the debris. The supernatant (crude cell extract) was dialyzed overnight against 50 mM potassium phosphate buffer (pH 7.0) at 4°C. The dialyzed crude cell extract was then centrifuged at 200,000 × *g* for 2.5 h at 4°C to pellet the membranes. The resulting soluble protein fraction was concentrated in a Centriflo concentrator (Amicon) (10- to 20-fold), or in some cases by lyophilization, for use in gel filtration experiments and IEF. Samples were dialyzed for 10 h against 4 liters of distilled H_2O at 4°C prior to loading of the IEF gels. Samples extensively dialyzed against distilled water lost between 10 and 20% of their original enzyme activity.

RESULTS

Growth on PGA. Except for *B. vulgatus* 4245, all of the strains grew to an optical density at 650 nm (1-cm path length) of at least 1.2 in defined medium that contained either galacturonic acid or PGA as the sole carbon source. *B. vulgatus* grew well in defined medium that contained glucose or galacturonic acid, but the maximum optical density at 650 nm that was attained in PGA defined medium was between 0.4 and 0.5. We tested 10 other *B. vulgatus* strains, and in all cases the maximum optical density at 650 nm that was attainable on PGA defined medium was less than 0.6.

Cellular location and inducibility of PGA lyase activity. No PGA lyase activity was detected in extracellular fluid from any of the *Bacteroides* species tested, even after the extracellular fluid was concentrated 10-fold. In all of the species tested, cell-associated PGA lyase activity partitioned with phosphoglucose isomerase activity in the soluble cellular fraction (Table 1). Less than 5% of the total PGA lyase activity remained with the membrane pellet after the membranes were washed with potassium phosphate buffer (0.05 M, pH 7.0). Accordingly, if PGA lyase activity in feces were being produced by one or more of these *Bacteroides* species, it should localize with the soluble protein fraction from sonically disrupted fecal bacteria. This is an important characteristic because such a bacterial extract is largely free from substances that could potentially interfere with the PGA lyase assay or with IEF.

All of the *Bacteroides* PGA lyases were inducible; i.e., the specific activity was 10- to 40-fold higher (depending on the species) if bacteria were grown on PGA defined medium rather than galacturonic acid defined medium (Table 2). Thus, we would expect to detect PGA lyase activity in bacteria that were obtained from feces by centrifugation only if they were actually degrading PGA in the colon.

Because the concentration of PGA in the colon is probably much lower than the concentration used in the experiments

TABLE 3. Stability of *Bacteroides* species PGA lyase activity after growth on PGA had stopped

Species[a]	Time (h) after growth on PGA had stopped	Culture without added chondroitin sulfate			Culture with added chondroitin sulfate[b]		
		Optical density at 650 nm	PGA lyase sp act (U/mg)[c]	Chondroitin lyase sp act (U/mg)[c]	Optical density at 650 nm	PGA lyase sp act (U/mg)[c]	Chondroitin lyase sp act (U/mg)[c]
Bacteroides sp. strain 3452A	0	1.5	1.9	0.05	1.5	1.8	0.07
	12	1.3	1.6	0.06	1.5	1.4	0.7
	24	1.1	1.5	0.07	1.6	1.7	0.9
	36	1.1	1.3	0.07	1.2	1.3	0.8
	48	1.0	1.2	0.06	1.0	1.2	1.1
B. thetaiotaomicron	0	1.8	3.8	0.06	1.8	3.6	0.07
	12	1.5	3.1	0.06	1.8	2.7	1.8
	24	1.4	3.4	0.07	1.7	1.8	1.7
	36	1.3	3.6	0.08	1.7	2.4	2.0
	48	1.2	3.2	0.06	1.6	2.9	2.0
B. ovatus	0	1.4	6.0	0.04	1.4	6.8	0.05
	12	1.3	6.4	0.07	1.6	4.6	2.6
	24	1.2	6.2	0.06	1.6	5.6	1.8
	36	1.1	4.2	0.06	1.5	5.2	1.8
	48	1.1	5.2	0.06	1.5	4.3	2.3

[a] *B. fragilis* subsp. a and *B. vulgatus* 4245 were not included in this experiment because they do not grow on chondroitin sulfate.
[b] Chondroitin sulfate was added to the culture to a final concentration of 5 mg/ml after growth on PGA had ceased.
[c] Enzyme specific activities are the mean of at least duplicate determinations. Variation was less than 10%.

described in Table 2 (5 mg/ml), we tested the effect of lower concentrations of PGA (0.2 and 1.0 mg/ml) on the PGA lyase specific activity of *B. thetaiotaomicron* and *B. fragilis* subsp. a. In the case of *B. thetaiotaomicron*, the PGA lyase specific activity of bacteria grown in medium containing 1.0 and 0.2 mg of PGA per ml was 95 and 85%, respectively, of the activity in bacteria grown in medium containing 5.0 mg of PGA per ml. In the case of *B. fragilis* subsp. a, the PGA lyase specific activity in bacteria grown in medium containing 1.0 and 0.2 mg of PGA per ml was 70 and 60%, respectively, of the specific activity in bacteria grown on 5 mg of PGA per ml. Thus, even when these organisms were growing on relatively low concentrations of PGA, the PGA lyase specific activity decreased by 40% or less.

The results in Table 2 were obtained with batch cultures. In batch cultures, bacteria grew with generation times that could be as short as 2 to 3 h. In the colon, where generation times may be much longer, the specific activity of *Bacteroides* PGA lyase could be lower than that seen in batch culture. To assess the effect of long generation times, we measured PGA lyase specific activity of bacteria that had been grown in PGA-limited continuous cultures (3 mg of PGA per ml) at generation times of 15 to 22 h. In the case of *B. thetaiotaomicron*, *B. fragilis* subsp. a., *B. ovatus*, and *Bacteroides* sp. strain 3452A, the PGA lyase specific activity at a generation time of 15 h was comparable to those shown in Table 2. At longer generation times (19 to 22 h), the PGA lyase specific activity was somewhat lower. It was 50 to 60% of the specific activity at 15 h in the case of *B. thetaiotaomicron* (at a generation time of 22 h), *B. fragilis* subsp. a (generation time of 22 h), and *B. ovatus* (generation time of 19 h) and 25 to 30% in *Bacteroides* sp. strain 3452A (generation time of 19 h). We did not test *B. vulgatus* because the growth of this organism on PGA was too poor for continuous culture studies.

Growth rates in the colon may not be as constant as they are in carbohydrate-limited continuous cultures which are held at a constant dilution rate. Instead, *Bacteroides* species could grow relatively rapidly on PGA in the ascending colon but could deplete the available PGA supply and then cease growing or switch to utilization of another substrate during the remainder of its time in the colon (24 to 48 h). To test the stability of PGA lyase activity under these conditions, we monitored PGA lyase specific activities in batch cultures at intervals after the bacteria had stopped growing on PGA. After 24 h, the PGA lyase specific activity was unchanged or decreased no more than 10 to 20% for the species tested (Table 3). After 48 h, the PGA lyase specific activity decreased at most by 30 to 40%. In other experiments, bacteria were grown to stationary phase in batch culture on defined PGA medium, and a second carbohydrate, chondroitin sulfate, was added to the medium at a concentration equal to the starting PGA concentration (5 mg/ml). The fact that the bacteria were able to switch to chondroitin sulfate, even though the turbidity of the culture did not increase, was seen by the induction of chondroitin lyase activity (Table 3). Under these conditions, the decrease in PGA lyase specific activity occurred at about the same rate as in batch cultures without added chondroitin sulfate, i.e., a 30 to 40% decrease during the 48-h incubation (Table 3). These experiments indicate that *Bacteroides* PGA lyases are not quickly inactivated or degraded when the inducing substrate is no longer present.

Characteristics of the *Bacteroides* PGA lyases. PGA lyase activity in crude cell extracts from all five *Bacteroides* species had pH optima similar to that of the PGA lyase from *B. thetaiotaomicron* (pH 8.4 to 8.8). All of the PGA lyases were enhanced maximally by 0.2 mM $CaCl_2$. All had native molecular weights of 50,000, as determined by gel filtration. The molecular weight of the purified PGA lyase from *B. thetaiotaomicron* was previously estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 74,000 (9). This discrepancy between estimates of molecular weight obtained by gel filtration and estimates obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis could be due to the presence of large hydrophobic regions that affect binding of sodium dodecyl sulfate. Although the enzyme behaves as a soluble protein under the conditions used to generate the data in Table 1, it associates with membranes when other conditions are used (9).

The PGA lyases from the different *Bacteroides* species differed with respect to isoelectric point values. A typical

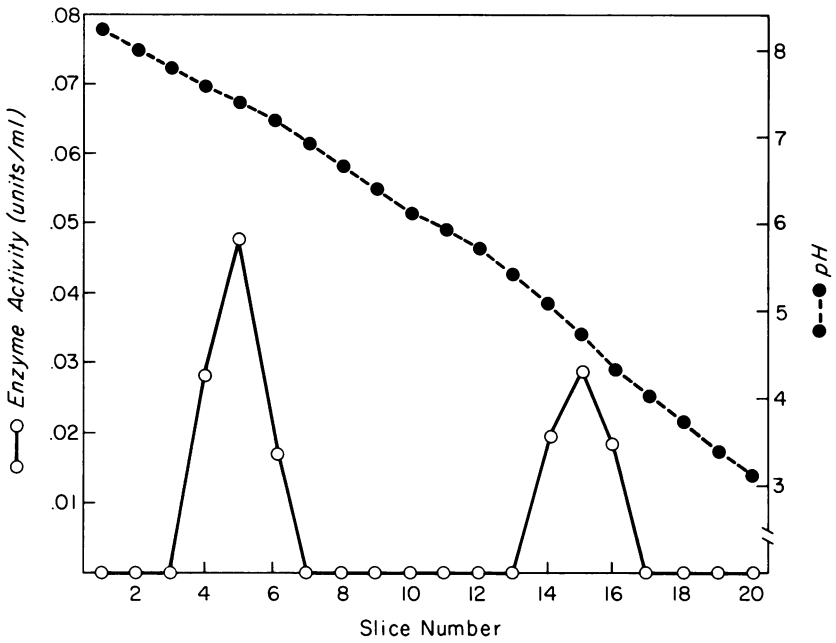

FIG. 1. IEF gel profile of PGA-degrading activity from *B. thetaiotaomicron*. One unit is defined as a change of 1 absorbance unit at 450 nm per min.

IEF gel profile is shown in Fig. 1. In most experiments there were two peaks of PGA-degrading activity, one that migrated to pH 5.8 or higher and one that migrated to pH 4.5 to 4.7 (slices 14 to 16, Fig. 1). The relative size of the peak at pH 4.5 to 4.7 varied from one species to another. This PGA-degrading enzyme was determined to be a PGA hydrolase rather than PGA lyase, because it had a pH optimum of 5.5 and did not require calcium (data not shown). The *Bacteroides* PGA lyases had no detectable activity at pH 5.5 when assayed without $CaCl_2$. Moreover, when the IEF gels were run without Triton X-100 and the fractions were assayed by the reducing sugar assay and by the A_{235} assay, both peaks were detected by the reducing sugar assay but only one peak was detected by the A_{235} assay. Because production of 235 nm-absorbing products is characteristic of PGA lyase, this peak was identified as the PGA lyase. The presence of PGA hydrolase activity was probably due to the fact that the soluble cellular fraction, loaded onto the IEF gel, contained some membranes and thus some hydrolase activity (9). Hydrolyase activity was detectable under the reducing sugar assay conditions because ampholines that eluted in the pH 4 to 6 region reduced the pH of the assay mix so that it approached the pH optimum of the hydrolase. Also, the 1-h incubation at 37°C allowed for detection of low levels of activity.

The pI values for the PGA lyases from the five *Bacteroides* sp. were as follows: 7.5 for *B. thetaiotaomicron*, 6.1 for *B. fragilis* subsp. a, 7.7 for *B. vulgatus*, 7.7 for *Bacteroides* sp. strain 3452, 5.8 and 7.2 for *B. ovatus*.

PGA lyase activity in feces. PGA lyase activity was detectable in the soluble fraction of sonicated bacteria from several different stool samples taken from two donors. The activity of the fecal PGA lyase ranged from 0.15 to 0.25 U/ml in the fecal bacterial suspensions that were tested. We did not attempt to measure PGA lyase activity in extracellular fluid. However, more than 90% of the PGA lyase activity in a sonicated fecal bacterial preparation remained soluble after centrifugation at 200,000 × *g* for 2.5 h (4°C). Thus, the PGA lyase activity partitioned similarly to those of the *Bacteroides* enzymes. The fecal activity had a pH optimum of 8.4 to 8.8, which was the same as the pH optima of the *Bacteroides* enzymes. Also, the fecal PGA lyase activity was enhanced by calcium, as was the activity of the *Bacteroides* enzymes. However, the pI of the PGA lyase activity from feces was much lower (pI of 4.2 to 4.4) than the pI of any of the *Bacteroides* PGA lyases. A typical IEF profile of the fecal PGA lyase activity is shown in Fig. 2. This fecal PGA-degrading activity was shown to be PGA lyase activity and not PGA hydrolase activity because it was not detectable at pH 5.5 (no added calcium). Also, when the IEF gel was run without Triton X-100, the fecal PGA-degrading enzyme migrated to the same position, and lyase activity was detectable by the A_{235} assay, as well as by the reducing sugar assay.

There was no detectable PGA lyase activity in the region of the IEF gel to which the *Bacteroides* PGA lyases normally migrate. To determine whether some component(s) of the fecal preparation might have altered the migration properties of one of the *Bacteroides* PGA lyases, we added the soluble cellular fraction (S1) of *B. fragilis* subsp. a to the soluble protein fraction of a freshly processed fecal sample. We show results for this PGA lyase, rather than for PGA lyases from one of the other *Bacteroides* species, because the pI of the *B. fragilis* subsp. a PGA lyase was close to the pI of the fecal lyase. Thus, the *B. fragilis* subsp. a PGA lyase should provide a good test of the resolving power of the IEF gel system. The mixture of the two PGA lyases was loaded onto an IEF tube gel and electrophoresed. The IEF gel pattern is shown in Fig. 3. The migration pattern for the PGA lyase from *B. fragilis* subsp. migrated to the same pI (6.1) that was seen when this lyase was electrophoresed alone (data not shown) and was clearly separable from the fecal PGA lyase. Similar results were obtained when other PGA lyases, such as that from *B. thetaiotaomicron* (Fig. 1), were mixed with the fecal suspension; i.e., the pI values of the *Bacteroides* PGA lyases were not altered when mixed with the fecal

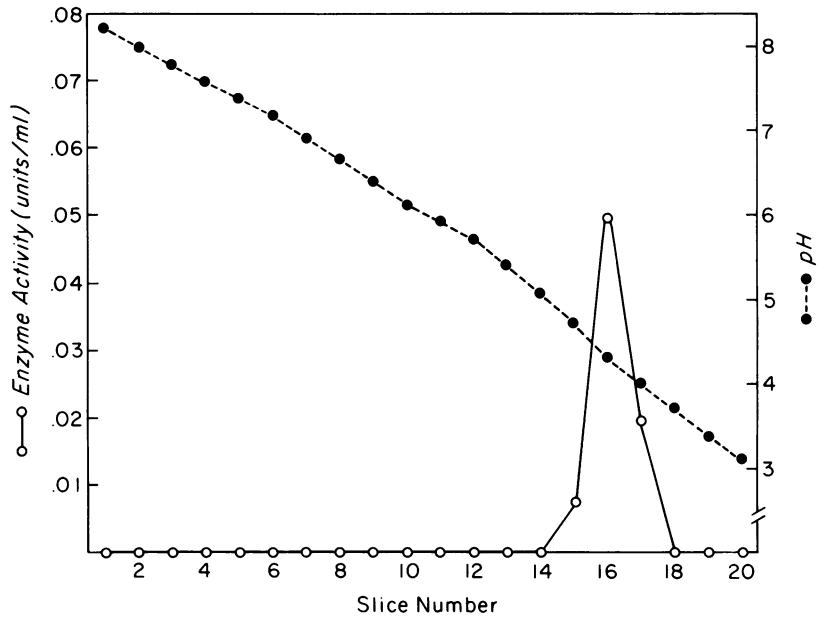

FIG. 2. IEF gel profile of PGA lyase activity from sonicated fecal bacteria.

preparation. The results of these experiments also indicate that proteases released by sonication of fecal bacteria were not degrading the *Bacteroides* PGA lyases under the conditions used to analyze fecal enzyme activity. Recovery of PGA lyase activity from the IEF gel was between 10 and 20% for all fecal samples tested. This was consistent with the recovery values obtained for pure cultures of *Bacteroides*.

Fecal PGA lyase molecular weight. The apparent molecular weight of the fecal PGA lyase activity, as measured by gel filtration, differed from the molecular weights of the *Bacteroides* enzymes. The fecal PGA lyase activity eluted near the void column of the Sephadex G-100 column but within the fractionation range of a Sephacryl S-300 column. The molecular weight of the PGA lyase activity from feces was estimated to be approximately 100,000. By contrast, the *Bacteroides* PGA lyases had molecular weights of about 50,000 and were clearly separable from the fecal PGA lyase activity on the Sephadex G-100 column (Fig. 4). When a fecal extract was chromatographed on the Sephadex G-100 column, no PGA lyase activity was detectable in the fractions in which the *Bacteroides* PGA lyases eluted (Fig. 4).

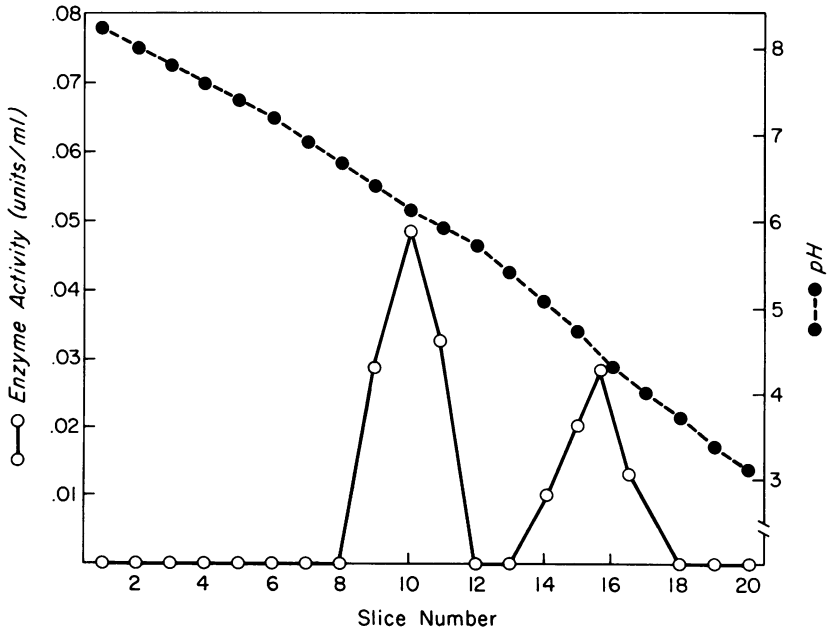

FIG. 3. IEF gel profile of PGA lyase activity in a mixture of equal portions of soluble protein from *B. fragilis* subsp. a and soluble protein from suspensed fecal bacteria (pI 4.2 to 4.4). The PGA lyase activity of *B. fragilis* subsp. a (pI 6.1) was recovered from slices 9 to 11 when this part of the mixture was electrophoresed alone (data not shown).

DISCUSSION

Our results show that the PGA lyase activity in feces is not produced by any of the known numerically predominant PGA-utilizing colonic *Bacteroides* species. None of the PGA lyases from the *Bacteroides* species which are capable of degrading PGA in laboratory medium had the same pI or molecular weight as the fecal PGA lyase activity. It is possible that the fecal PGA lyase is produced by some other colon bacteria. For example, some strains of *Eubacterium eligens* can grow on PGA (15). Moreover, Jensen and Canale-Parola (4) recently reported the isolation of anaerobic pectinolytic organisms from human feces which appear not to belong to any of the major colonic *Bacteroides* species. Nothing is known about the properties of PGA lyases from these other organisms or about their regulation. It is also possible that the fecal PGA lyase is not a bacterial enzyme but rather is released during the sonication step from plant cells that copelleted with bacteria.

Our results not only show that the fecal PGA lyase is not produced by any of the major *Bacteroides* species but also indicate that PGA may not be an important source of carbon and energy for at least some species of colonic *Bacteroides*. Using available information about the concentrations of the different *Bacteroides* species in fecal specimens (2, 6, 7, 10), we can estimate the amount of PGA lyase activity that we would expect to find in a suspended bacterial pellet from a fecal sample if the *Bacteroides* PGA lyases were fully induced in the colon (Table 4). In the case of *B. vulgatus*, *B. thetaiotaomicron*, and *Bacteroides* sp. strain 3452A, concentrations of these organisms in feces were determined for one of the fecal specimens used in this study (6, 7). As estimates of the concentrations of the other two species, we took average values from the work of Moore and Holdeman (10) and Holdeman et al. (2). A PGA lyase activity of 0.02 U/ml could have been detected either by gel filtration or on the IEF gels (cf. Fig. 3 and 4). Given our finding that sonic disruption of *Bacteroides* species added to feces is as effective as sonic disruption of pure cultures, the PGA lyases of all five *Bacteroides* species should have been detected if they were fully induced. Despite this, there was no detectable PGA lyase activity with a pI or molecular weight corresponding to those of the *Bacteroides* enzymes.

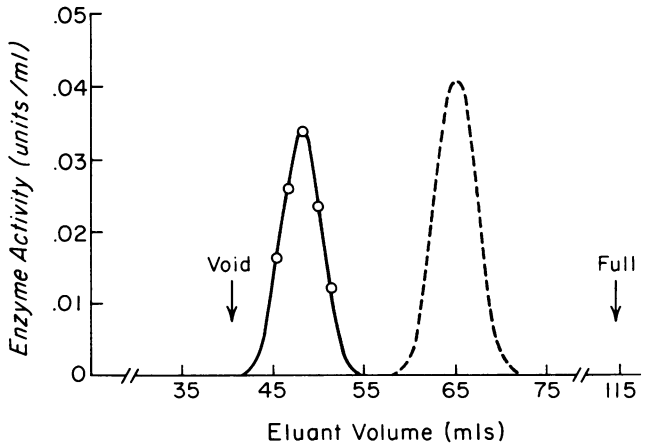

FIG. 4. Chromatography on Sephadex G-100 of the PGA lyase activity from disrupted fecal bacteria (——). The elution pattern of the *Bacteroides* PGA lyases is indicated (– – –). One unit is defined as a change of 1 absorbance unit at 450 nm per min.

TABLE 4. Estimates of fecal *Bacteroides* PGA lyase activity (as units per ml of resuspended bacteria) if synthesis of PGA lyase was fully induced

Species	Bacterial concn (organisms/g [wet wt] of feces)[a]	Estimated protein concn (μg/ml) in resuspended fecal bacteria[b]	Estimated PGA lyase activity (U/ml) in resuspended fecal bacteria[c]
B. vulgatus	4×10^9–6×10^9	67–100	0.03–0.04
B. thetaiotaomicron	1×10^9–2×10^9	17–33	0.06–0.12
B. fragilis subsp. a	0.8×10^9–1×10^9	13–17	0.10–0.14
Bacteroides sp. strain 3452A	0.7×10^9–1×10^9	12–17	0.02–0.03
B. ovatus	2×10^8–5×10^8	3–8	0.02–0.05

[a] Values shown for *B. vulgatus*, *B. thetaiotaomicron*, and *Bacteroides* sp. strain 3452A were determined previously with DNA-specific hybridization probes (6, 7). Values shown for *B. ovatus* and *B. fragilis* subsp. a are those determined by Holdeman and co-workers (2, 10).

[b] Because the fecal bacteria suspension was diluted 10-fold compared with the original fecal specimen, the bacterial concentrations in feces were divided by 10. To convert to the approximate concentration of protein for each species, this value was divided by 6×10^9 bacteria per mg of protein, a conversion factor obtained by averaging results from previous studies in which *Bacteroides* species were grown in carbohydrate-limited continuous culture at a generation time of 14 h (5).

[c] The estimated values for the PGA lyase activities were computed by multiplying the estimated protein concentrations in column 2 by the PGA lyase specific activities given in Table 2.

The estimates of PGA lyase activities in Table 4 were based on PGA lyase specific activities in *Bacteroides* which had been grown in batch cultures on relatively high concentrations of PGA (5 mg/ml). Judging from the results of the experiments in which *Bacteroides* species were growing on PGA with generation times ranging from 15 to 22 h, PGA lyase activity could have been as much as 75% lower if the growth rate of the bacteria were restricted to generation times of 20 h or longer. If the *Bacteroides* species had been growing on PGA but had depleted the supply of PGA in the ascending colon, the specific activity could be 40% lower than the fully induced value (Table 2). A further drop could occur as a result of dilution as the organisms grew on other substrates during passage through the colon. Because bacteria probably cannot divide more than a few times as they pass through the transverse and descending colon, at least 20% of the original PGA lyase specific activity should still be detectable in feces. Thus, PGA lyase activity from some *Bacteroides* species, such as *B. thetaiotaomicron* or *B. fragilis* subsp. a, should have been detectable in feces if PGA were a major growth substrate for these organisms in the colon. In the case of *B. vulgatus*, *Bacteroides* sp. strain 3542A, and *B. ovatus*, the predicted level of fully induced PGA lyase in feces is low enough that we might have failed to detect activity if it dropped by more than 50% of the fully induced value as a result of some of the factors discussed above.

It is possible that the amount of PGA that enters the colon is too low to cause full induction of *Bacteroides* PGA lyase activity. At present, no information is available about how much PGA or pectin enters the colon. From the results of experiments in which we grew bacteria on low concentrations of PGA, it is clear that PGA lyase specific activity decreases with lower PGA concentration in the medium. If preferred sources of carbohydrate are available along with PGA, full induction of *Bacteroides* PGA lyases is even less likely. Nonetheless, our data strongly support the hypothe-

sis that PGA is probably not a major source of carbohydrate for at least two species of colonic *Bacteroides* (*B. thetaiotaomicron* and *B. fragilis* subsp. a). This leaves unanswered the question of why these organisms synthesize PGA lyases at all. It is possible that they survive by scavenging small amounts of many different types of polysaccharides, none of which are present at high enough concentrations to fully induce the relevant polysaccharidases.

This study is the first attempt to determine what individual species of bacteria are actually doing in the colon. Although the results are not conclusive, it is clear that because of the inducibility and stability of *Bacteroides* polysaccharidases it is feasible to determine what substrates these organisms are using in the colon by measuring activities in feces. The main limitation of the present study is that measurements of enzyme activity have limited sensitivities. One way to increase sensitivity would be to use antibodies as probes to detect polysaccharidases in feces. This approach would be much more difficult than the approach described in this report because it would require that purified polysaccharidases be obtained for use as antigens. Also, the possibility of cross-reactivity with other proteins that might be found in feces would have to be ruled out. Further investigations of this sort are under way in our laboratory.

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