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Biochemical Evidence that Starch Breakdown by *Bacteroides thetaiotaomicron* Involves Outer Membrane Starch-Binding Sites and Periplasmic Starch-Degrading Enzymes

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Bacteroides thetaiotaomicron **can utilize amylose, amylopectin, and pullulan as sole sources of carbon and energy. The enzymes that degrade these polysaccharides were found to be primarily cell associated rather than extracellular. Although some activity was detected in extracellular fluid, this appeared to be the result of cell lysis. The cell-associated amylase, amylopectinase, and pullulanase activities partitioned similarly to the periplasmic marker, acid phosphatase, when cells were exposed to a cold-shock treatment. Two other enzymes associated with starch breakdown, α-glucosidase and maltase, appeared to be located in the cytoplasm. Intact cells of *B. thetaiotaomicron* were found to bind ^{14}C-starch. Binding was probably mediated by a protein because it was saturable and was decreased by treatment of cells with proteinase K. Results of competition experiments showed that the starch-binding proteins had a preference for maltodextrins larger than maltohexaose and a low affinity for maltose and maltotriose. Both the degradative enzymes and starch binding were induced by maltose. These findings indicate that starch utilization by *B. thetaiotaomicron* apparently does not involve secretion of extracellular enzymes. Rather, binding of the starch molecule to the cell surface appears to be a first step to passing the molecule through the outer membrane and into the periplasmic space.**

Bacteroides is one of the numerically predominant genera of bacteria in the human colon (15). Members of this genus are gram-negative, obligate anaerobes that require carbohydrates as a source of carbon and energy. In the colon, polysaccharides probably provide the primary source of carbohydrates for these bacteria. Previous work has shown that the enzymes involved in breakdown of chondroitin sulfate and polygalacturonic acid by *Bacteroides thetaiotaomicron* are cell associated and are not exposed on the cell surface (14, 17). This indicates that large polysaccharides must be able to pass through the outer membrane of *Bacteroides*. Kotarski et al. (9, 10) compared outer membrane protein profiles from *B. thetaiotaomicron* grown on different polysaccharides and found that some proteins were associated with growth on particular polysaccharides. They suggested that the polysaccharide-associated outer membrane proteins might represent polysaccharide receptors. However, no such role for outer membrane proteins in polysaccharide uptake has yet been demonstrated.

Since radiolabeled chondroitin sulfate and polygalacturonic acid are not commercially available, it was not possible to test for binding of these polysaccharides to *Bacteroides* cell surface proteins. A more tractable model system for studying polysaccharide binding and uptake is offered by the starch utilization system, because radiolabeled substrate is commercially available. *B. thetaiotaomicron* can utilize three types of starch: amylose, amylopectin, and pullulan. The utilization process appears to be quite efficient, since growth is nearly as rapid on these polysaccharides as on maltose. However, little is known about the nature and location of starch-degrading enzymes in *B. thetaiotaomicron*, and no previous work has been reported on the association of starch with outer membrane proteins. A *Bacteroides* pullulanase activity has been characterized, and the gene has been cloned (18). Preliminary experiments indicated that although pullulanase and the other starch-degrading enzymes, such as amylase, amylopectinase, and α-glucosidase, were primarily cell associated, some extracellular activity could be detected. This raised the question of whether starch-degrading enzymes in *Bacteroides* species are cell associated or extracellular. If they were cell associated, it should be possible to detect the binding of starch to cell surface receptors.

In this report, we present evidence that the amylase, amylopectinase, pullulanase, and α-glucosidase activities of *B. thetaiotaomicron* are cell associated. Furthermore, we offer evidence that there are starch-binding proteins on the *Bacteroides* cell surface. Finally, we show that the starch-binding activity, like the degradative enzymes, is maltose inducible.

MATERIALS AND METHODS

Organism and culture conditions. *B. thetaiotaomicron* 5482 (ATCC 29148) was obtained from the culture collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg. Stocks were maintained at room temperature in chopped-meat broth (6) under an atmosphere of CO_2. The growth medium for the bacteria was the defined medium of Kotarski and Salyers (10).

Carbohydrates used in this study were as follows: amylopectin, chondroitin sulfate, dextran, glucose, maltose, maltoheptaose, maltohexaose, maltopentaose, maltotetraose, maltotriose, melibiose, and pullulan (Sigma Chemical Co., St. Louis, Mo.); amylose (M_r 4,100; Aldrich Chemical Co., Milwaukee, Wis.); and lactose (Fisher Scientific Co., Pittsburgh, Pa.). All carbohydrates were filter sterilized and added to the medium (0.5%) after autoclaving, except amylose, amylopectin, and pullulan, which were added (0.5%) before autoclaving.

Enzyme assays. $CaCl_2$ (2 mM) was added to all assay mixtures because it increased amylase and amylopectinase

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activity almost twofold and pullulanase, α-glucosidase, and maltase activity by 20 to 40%.

Amylase, amylopectinase, and pullulanase activities were measured by a reducing-sugar assay (3). Cell extracts or concentrated extracellular fluids were mixed with amylose, amylopectin (1 mg/ml, 50 mM potassium phosphate buffer [pH 7.0]), or pullulan (4 mg/ml, 50 mM potassium phosphate buffer [pH 6.5]), and $CaCl_2$ (2 mM) and then incubated at 37°C. Reducing-sugar concentrations were measured at 10-min intervals. One unit of enzymatic activity was defined as the release of 1 μmol of reducing sugar (as glucose) per min.

α-Glucosidase activity was measured by determining the rate of hydrolysis of *p*-nitrophenyl-α-D-glucoside at 37°C (4). The reaction mixture contained 0.4 ml of 50 mM potassium phosphate buffer (pH 7.0), 2 mM $CaCl_2$, 50 μl of 20 mM *p*-nitrophenyl-α-D-glucoside, and 50 μl of appropriately diluted enzyme. Increase A_{405} was measured with a Gilford recording spectrophotometer 250 (Gilford Instrument Laboratory, Oberlin, Ohio), and 1 U of activity was defined as 1 μmol of *p*-nitrophenol liberated per min.

Maltase activity was measured by determining the rate of release of glucose from maltose. Cell extracts or concentrated extracellular fluid were mixed with 0.1 M maltose and 2 mM $CaCl_2$ in 50 mM phosphate buffer (pH 7.0) and incubated at 37°C. At 10-min intervals 0.5 ml of the reaction mixture was removed and boiled for 3 min. The concentration of free glucose was then determined by using glucose oxidase as recommended by Sigma (procedure no. 510). One unit of maltase activity was defined as the release of 1 μmol of glucose per min.

Location of enzymatic activity. Cells in defined medium (350 ml) containing maltose were grown at 37°C to the midexponential phase (optical density of 0.7 to 0.8 at 650 nm). A portion (250 ml) was centrifuged (10,000 × *g*, 15 min, 4°C). Extracellular fluid was removed and centrifuged again (10,000 × *g*, 15 min, 4°C) and then concentrated 20-fold at 4°C with an Amicon concentrating cylinder (model 202) and ultrafiltration membranes (PM30; Amicon Corp., Lexington, Mass.) The pelleted cells were washed twice (10,000 × *g*, 15 min, 4°C) with 50 mM potassium phosphate buffer (pH 7.0) containing 0.8% (wt/vol) NaCl and then suspended in 25% sucrose–1 mM EDTA (125 ml). Periplasmic and cytoplasmic fractions were obtained as described by Huang and Forsberg (7) and concentrated a total of 40-fold as described above. The membrane pellet was suspended in 6.25 ml of potassium phosphate buffer (50 mM, pH 7.0). The remaining 100-ml portion of cells was used to prepare a crude cell extract by centrifugation of the cells (10,000 × *g*, 15 min, 4°C) and washing with 0.1 M potassium phosphate buffer (pH 7.0). After cells were suspended in 5 ml of potassium phosphate buffer (50 mM, pH 7.0), they were maintained at 4°C and disrupted by 50% pulsed sonication (4 min) on a Branson sonifier 200 (Branson Sonic Power Co., Danbury, Conn.) at 60% output. Disrupted bacteria were centrifuged twice (16,500 × *g*, 5 min, 4°C) to remove cell debris.

α-Galactosidase (5) and fructose 1,6-diphosphate aldolase (aldolase) (12) activities were used as the cytoplasmic markers (13). Acid phosphatase activity (Sigma; procedure no. 104), phosphoglucose isomerase activity (16), and succinate dehydrogenase activity (8) were used as markers of the periplasmic (2), soluble (14), and membrane (9) fractions, respectively. After dialysis in 50 mM phosphate buffer (pH 7.0) for 16 h at 4°C, the extracellular fluid, cellular crude extract, and cellular fractions were assayed for starch-degrading activities. Activities in cellular fractions were expressed as percentages of total cellular activity. The difference between the sum of these percentages and 100% provides a measure of the recovery of the different enzymes.

Enzymatic activities of soluble and membrane fractions were determined by using maltose-grown cells that had been suspended in phosphate buffer (50 mM, pH 7.0) and disrupted by sonication as described above. After undisrupted bacteria were removed by centrifugation (16,500 × *g*, 5 min, 4°C), membranes were pelleted by centrifugation at 200,000 × *g* for 2 h at 4°C and sequentially washed with NaCl and Triton X-100 as described by McCarthy et al. (14). The resulting supernatant and membrane fractions were dialyzed in 50 mM potassium phosphate buffer (pH 7.0, 16 h, 4°C) before determination of enzyme activities. Phosphoglucose isomerase and succinate dehydrogenase activities served as soluble protein and membrane markers, respectively.

Effect of growth substrate on enzymatic activity. Crude cell extracts were obtained from cells grown in defined medium (100 ml) containing glucose, chondroitin sulfate, maltose, melibiose, amylose, amylopectin, or pullulan as the energy source. Crude cell extracts were prepared by sonication as described above. These cell extracts were then assayed for enzymatic activity as described above.

Starch binding. ^{14}C-starch (1.9 mCi/mg; Dupont, NEN Research Products, Boston, Mass.) was used to determine the ability of *B. thetaiotaomicron* to bind starch. In all starch-binding experiments, cultures were grown to an optical density of 0.6 to 0.8 at 650 nm. Cells (10 ml) were then harvested by centrifugation (16,500 × *g*, 10 min, 22°C).

(i) Competition. Cells grown on maltose were suspended in 5 ml of amylose, amylopectin, dextran, or pullulan (1-mg/ml solutions). After 5 min at 22°C, ^{14}C-starch (0.5 μCi) was added to 1 ml of each cell suspension, vortexed for 3 s, and allowed to incubate for 1 min at 22°C. Then 0.5 ml of this mixture was centrifuged, and the ^{14}C-starch remaining with the cells was determined as described above.

Preliminary experiments indicated that an amylose concentration of 100 μg of suspended cells per ml eliminated most of the ^{14}C-starch binding to cells. The effect of chain length on competition was determined by adding 100 μg of amylose, maltoheptaose, maltohexaose, maltopentaose, maltotetraose, maltotriose, or maltose to 1 ml of suspended maltose-grown cells and incubated at 22°C for 2 min. ^{14}C-starch (0.5 μCi) was then added, and the mixture was incubated for an additional 1 min at 22°C. After centrifugation, cell-associated ^{14}C-starch was measured as described above.

Competition was reported as the percentage of inhibition by the equation: (1 − counts per minute bound/maximum counts per minute) × 100%. The maximum value for counts per minute was the amount of ^{14}C-starch bound by the cells when no competing starch or maltodextrin was present.

(ii) Saturation curve. Cells grown on maltose were suspended in 5 ml of potassium phosphate buffer (0.1 M, pH 7.0). ^{14}C-starch (0.5 μCi) and unlabeled amylose were added to obtain a final concentration of 0, 5, 10, 20, 40, 50, or 60 μg of unlabeled amylose in 0.5 ml of potassium phosphate buffer (0.1 M, pH 7.0). Suspended cells (0.5 ml) were added to this mixture, vortexed for 3 s, and incubated for 1 min at 22°C. After 1 min, 0.5 ml was removed and centrifuged in an Eppendorf microcentrifuge for 5 min. The resulting pellet was suspended in 0.5 ml of potassium phosphate buffer (0.1 M, pH 7.0). ^{14}C-starch still associated with the cellular suspension was determined by liquid scintillation counting. As a control for nonspecific trapping of label by the cells, ^{14}C-starch was replaced with D-[^{14}C]sorbitol (250 mCi/mM; ICN Pharmaceuticals Inc., Irvine, Calif.).

(iii) Effect of growth substrate. Cells grown on glucose, maltose, lactose, amylose, amylopectin, pullulan, or dextran were suspended in 5 ml of potassium phophate buffer (0.1 M, pH 7.0). The suspended cells (0.5 ml) were then added to 0.5 ml of ^{14}C-starch (0.5 μCi), amylose (20 μg), and potassium phosphate buffer (0.1 M, pH 7.0), vortexed for 3 s, and incubated for 1 min at 22°C. Cell-associated ^{14}C-starch was determined as described above.

(iv) Effect of proteinase K. Cells grown on maltose were suspended in 5 ml of potassium phosphate buffer (0.1 M, pH 7.0). Suspended cells (2 ml) were mixed with 0.5 mg of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), vortexed for 5 s, and incubated at 22°C. Samples (250 μl) were then removed after 0, 10, 20, 30, and 50 min and mixed with 250 μl of amylose (10 μg) and ^{14}C-starch (0.25 μCi) in potassium phosphate buffer (0.1 M, pH 7.0). After incubation for 1 min at 22°C followed by centrifugation, cell-associated ^{14}C-starch was determined as described above.

To determine whether starch interfered with proteinase K action, another portion of suspended cells (1 ml) was added to an equal volume of ^{14}C-starch (1 μCi) and amylose (40 μg) in potassium phosphate buffer (0.1 M, pH 7.0) and vortexed for 3 s. A 250-μl sample was removed and centrifuged as previously described. Proteinase K (Boehringer) was then added (0.5 mg) to the mixture, vortexed for 5 s, and incubated at 22°C. Samples (250 μl) were then removed and centrifuged at 10, 20, 30, and 50 min after the addition of the proteinase K. ^{14}C-starch associated with the cells was determined as described above.

As a positive control, the remaining suspended cells were incubated at 22°C. After 0, 10, 20, 30, and 50 min, 250 μl of cells was mixed with equal volumes of the amylose, ^{14}C-starch and potassium phosphate buffer mixture and incubated for 1 min at 22°C, and the cell-associated ^{14}C-starch was determined as described above.

(v) Binding to membranes. Crude cell extracts from sonically disrupted cells were prepared as described above from cells (100 ml) grown on glucose, maltose, amylose, or pullulan. The cell extracts, in 50 mM potassium phosphate buffer (pH 7.0), were centrifuged at 200,000 × *g* for 2 h at 4°C to pellet the membranes. Membrane pellets were then suspended in 4 ml of 0.1 M potassium phosphate buffer (pH 7.0). ^{14}C-starch (0.5 μCi) was added to 1 ml of suspended membranes, vortexed for 3 s, and allowed to set for 1 min at 22°C. Two samples (175 μl each) of this mixture were then centrifuged on an airfuge ultracentrifuge (Beckman Instruments, Palo Alto, Calif.) at 24 lb/in^2 for 30 min. Membrane pellets were suspended in 0.1 M phosphate buffer and assayed for membrane-associated ^{14}C-starch as described above.

Analysis of membrane proteins. Outer membranes were obtained by the sucrose gradient procedure of Kotarski and Salyers (10), except the final gradient purification step was omitted. The final outer membrane fraction was collected by centrifugation at 200,000 × *g* for 3 h at 4°C. The membrane pellet was suspended in 0.1 M potassium phosphate buffer (pH 7.0).

SDS-PAGE. Proteins from outer membranes, crude cell extracts, and concentrated extracellular fluid were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Kotarski and Salyers (10). Proteins were then stained with Coomassie brilliant blue R-250. The molecular weights of the protein standards were 97,400, 66,200, 42,699, 31,000, 21,500, and 14,400.

Protein determination. Protein concentrations were determined by the method of Lowry et al. (11) with bovine serum albumin as the standard.

TABLE 1. Cellular location of enzymatic activity

Cell fraction	% of total enzymatic activity[a]									
	PGI	SDH	AcP	α-Gal	AD	Aase	Apase	Pase	α-Glu	Mase
Extracellular[b]	2	25	ND[c]	7	18	22	15	11	15	11
Cell extract[b]	98	75	100	93	82	78	85	89	85	89
Periplasmic[d]	72	<1	81	10	11	39	27	50	17	13
Cytoplasmic[d]	30	6	7	20	16	16	16	25	10	8
Membrane[d]	3	93	ND	67	68	65	64	40	75	74

[a] PGI, Phosphoglucose isomerase; SDH, succinate dehydrogenase; AcP, acid phosphatase; α-Gal, α-galactosidase; AD, aldolase; Aase, amylase; APase, amylopectinase; Pase, pullulanase; α-Glu, α-glucosidase; Mase, maltase.

[b] Percentage of total enzymatic activity of cells grown on maltose.

[c] ND, None detected.

[d] Percentage of cell-associated enzymatic activity.

RESULTS

Location of starch-degrading enzymes. Some polysaccharidase activity (11 to 22% of total cellular activity) was detectable in the extracellular fluid, even when cells were harvested in the midexponential phase (Table 1). This extracellular activity could have resulted from cell lysis, since similar amounts of the cell-associated enzymes succinate dehydrogenase, α-galactosidase, and aldolase were also found in the extracellular fluid (Table 1). Only 2% of the total phosphoglucose isomerase activity was found in the extracellular fluid. However, this enzyme was less stable than the other enzymes used as markers and may have been more readily lost in the extracellular fluid. Acid phosphatase activity, the periplasmic marker, was also not detected in extracellular fluid, but levels of this enzyme in *B. thetaiotaomicron* were so low that 10 to 15% of the total cellular activity was close to the limit of detection. Levels of acid phosphatase might have been increased by growing the cells in low-phosphate medium, but previous experiments had shown that *B. thetaiotaomicron* lyses more readily in Tris- or *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid-buffered media than in phosphate-buffered media.

To determine whether release of the starch-degrading enzymes into the extracellular fluid was the result of cell lysis, we compared the protein profile of the concentrated extracellular fluid with that of outer membranes and concentrated cellular extract. A number of bands that comigrated with outer membrane proteins and some bands that comigrated with cell extract proteins appeared in the concentrated extracellular fluid (Fig. 1). This would be expected if release were due to cell lysis. Centrifugation of concentrated extracellular fluid at 200,000 × *g* (2 h, 4°C) did not pellet the starch-degrading enzymes. Thus release was probably not due to blebbing of membranes.

A cold-shock procedure was used to release periplasmic contents. Most of the acid phosphatase and phosphoglucose isomerase activities, but only 10 to 13% of the α-galactosidase and aldolase activities, were released by this procedure (Table 1). The α-glucosidase and maltase activities partitioned similarly to the cytoplasmic markers aldolase and α-galactosidase. Thus, the α-glucosidase and maltase activities are probably located in the cytoplasm. A higher percentage (27 to 50%) of the polysaccharidase activities were released by the cold-shock treatment, although the percentage was not as high as that for acid phosphatase or phoso-

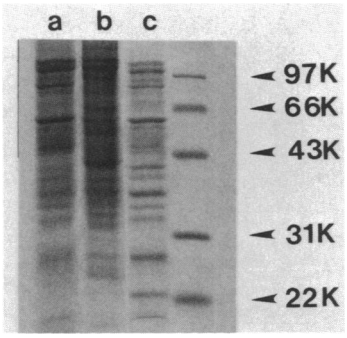

FIG. 1. Comparison of SDS-PAGE profiles from concentrated extracellular fluid (a), crude cell extract (b), and purified outer membrane (c). Approximately 25 μg of protein was loaded in each lane. Mobilities of molecular weight standards are indicated.

glucose isomerase. Accordingly, the polysaccharidases could be periplasmic enzymes.

A high percentage of all enzymes, except phosphoglucose isomerase and acid phosphatase, partitioned with the membrane fraction obtained by the cold-shock procedure. This partitioning seems to be an artifact of the particular conditions used for the cold-shock procedure, since 65 to 76% of the polysaccharidase activities remained soluble when cells in 50 mM phosphate buffer (pH 7.2) were disrupted by sonication and centrifuged to pellet membranes. Washing this membrane pellet with 1 M NaCl released virtually all of the remaining polysaccharidase activities, leaving less than 10% of the original enzyme activities associated with the membrane fraction.

Effect of growth substrate on enzymatic activity. Synthesis of all of the starch-degrading enzymes was regulated. Specific activities were higher in cells grown on maltose, and especially amylose, amylopectin, or pullulan, than in cells grown on glucose, melibiose, or chondroitin sulfate (Table 2).

Starch binding by intact cells. The finding that the polysaccharidases were probably periplasmic enzymes suggested that there might be cell surface proteins that bind starch and facilitate its passage through the outer membrane. Accordingly, we tested cells for the ability to bind labeled starch. When maltose-grown cells were incubated with ^{14}C-starch (no added unlabeled carbohydrate), the amount of bound label was 7.0×10^5 cpm/mg of protein. Incubation of cells with either unlabeled amylose or amylopectin (1 mg/ml) before the addition of labeled starch reduced the level of ^{14}C-starch binding 100-fold (to 5.9×10^3 protein and 8.7×10^3 cpm/mg of protein, respectively). By contrast, preincubation with unlabeled pullulan (1 mg/ml), a linear polymer

TABLE 2. Effect of growth substrate on enzyme specific activity

Growth substrate	Sp act (U/mg of protein)[a]				
	Amylase	Amylopectinase	Pullulanase	α-Glucosidase	Maltase
Glucose[b]	0.02	0.02	0.01	0.07	0.04
Maltose	0.19	0.08	0.27	0.98	0.92
Amylose	0.36	0.29	0.43	1.32	1.30
Amylopectin	0.34	0.21	0.43	1.66	1.50
Pullulan	0.33	0.20	0.40	1.63	1.55

[a] One unit is defined as 1 μmol of glucose equivalent released per minute.
[b] Specific activities of enzymes from cells grown on melibiose or chondroitin sulfate were the same as those shown above for glucose.

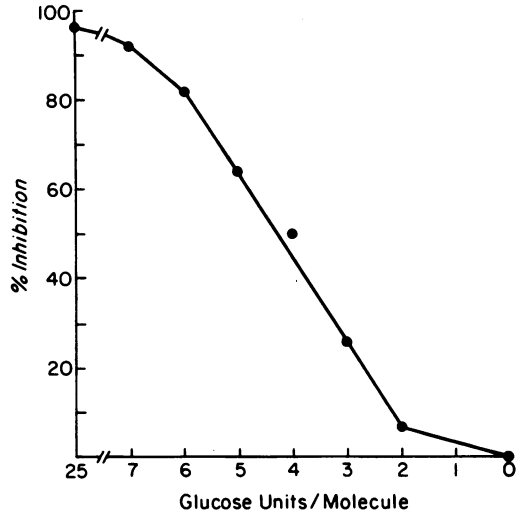

FIG. 2. Effect of incubating intact cells with unlabeled maltodextrins before the addition of labeled starch. The extent to which the unlabeled maltodextrins are able to compete with starch for binding to the cell surface is indicated by the percent inhibition of binding by labeled starch.

of alternating α(1 → 4) and α(1 → 6) linkages, only decreased the amount of labeled starch bound less than fourfold (to 1.9×10^5 cpm/mg of protein). Also, preincubation with unlabeled dextran (1 mg/ml), a linear α(1 → 6)-linked glucan, had no effect on binding of labeled starch (7.2×10^5 cpm/mg). These results indicate that binding is specific for polysaccharides containing long stretches of α(1 → 4)-linked glucose residues and is not simply nonspecific binding of neutral polysaccharides.

The importance of chain length in binding was determined by experiments in which bacteria were incubated with different-sized, unlabeled maltodextrins (1 mg/ml) before the addition of ^{14}C-starch. The ability of the unlabeled maltodextrin to inhibit subsequent binding of labeled starch reflects the ability of the maltodextrin to compete with labeled starch for binding to the cell surface. Preincubation with maltoheptaose and maltohexaose inhibited subsequent binding of labeled starch nearly as effectively as did preincubation with amylose (Fig. 2). However, shorter maltodextrins were much less effective.

If binding were due to specific cell surface proteins, it should be saturable. To determine whether saturation occurred, we needed to dilute labeled starch with a functionally identical unlabeled polymer. The ^{14}C-starch preparation used in these experiments probably contained both amylose and amylopectin. However, since both amylose and amylopectin competed equally with the labeled starch, these two forms of starch could be considered equivalent with respect to binding experiments. Accordingly, we mixed labeled starch with unlabeled amylose to determine how increasing the concentration of starch affected the total amount of starch bound. Saturation occurred at approximately 13 μg of starch per mg of cell protein (Fig. 3). To confirm that binding was mediated by proteins, the effect of proteinase K on binding was determined. Treatment of intact cells with proteinase K decreased starch binding (Fig. 4), whereas cells not treated with proteinase K but incubated for the same length of time did not exhibit a loss of starch binding. The addition of unlabeled starch to the incubation mixture before the addition of proteinase K provided partial protection

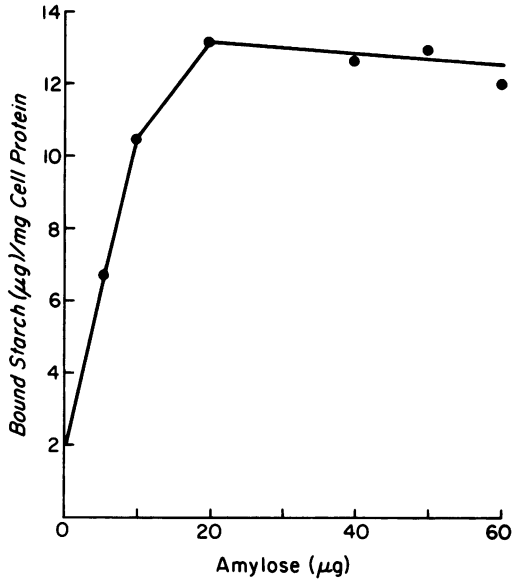

FIG. 3. Effect of starch concentration on starch binding to intact cells. Different amounts of unlabeled amylose were mixed with ^{14}C-starch before the binding assay, and the amount of starch bound was computed on the basis of the specific activity of each mixture.

against proteinase K action. These results support the hypothesis that binding is mediated by proteins.

Effect of growth substrate on starch binding. Synthesis of proteins involved in starch binding was inducible. Whereas cells grown on maltose bound 13.1 μg of starch per mg of cell protein, cells grown on glucose, lactose, or dextran bound 1.1 to 1.3 μg of starch per mg of cell protein. Cells grown on the grade of amylose, amylopectin, and pullulan used for media did not bind ^{14}C-starch at levels higher than the basal levels seen with glucose-grown cells. However, these substrates contain some insoluble polysaccharide that cosedimented with bacteria and could have interfered with starch binding. To determine whether this was the case, we filter sterilized pullulan and added it to the medium after autoclaving, thus eliminating insoluble polysaccharide from the medium. Cells grown in this medium did bind starch at the induced level (15.5 μg of starch per mg of cell protein), in contrast to cells grown in medium containing unfiltered pullulan.

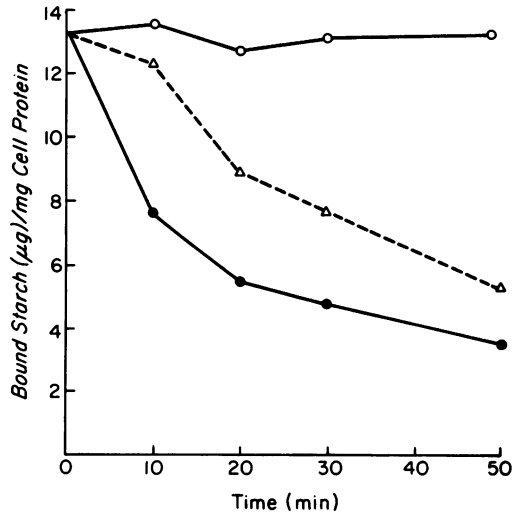

FIG. 4. Amount of starch bound at various time intervals after incubation of maltose-grown cells with proteinase K (●), proteinase K and amylose (△), or no proteinase K or amlylose (○).

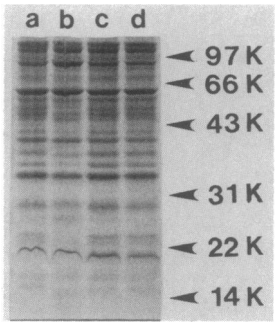

FIG. 5. Comparison of SDS-PAGE profiles of outer membrane proteins of cells grown on glucose (a), maltose (b), amylose (c), or pullulan (d). Approximately 25 μg of protein was loaded in each lane.

Starch uptake by intact cells. Although intact cells of *B. thetaiotaomicron* could bind starch, there was no evidence for uptake and accumulation of starch by the cells under the aerobic conditions used to study binding. That is, when maltose-grown cells were incubated with ^{14}C-starch for up to 1 h, no further increase in cell-associated label was detectable above the level detectable after the first 1 to 2 min. However, accumulation of labeled starch could be demonstrated if cells were harvested under a nitrogen atmosphere and then suspended in defined maltose medium and incubated at 37°C under a nitrogen atmosphere. Under these conditions, cells accumulated starch at a rate of 0.2 μg/min per mg of protein.

Binding of starch by membranes. Membranes from cells grown on maltose, amylose, or pullulan bound 1.1, 1.0, or 1.2 μg of starch per mg of protein, respectively. This was not significantly different from the amount of starch bound by membranes from glucose-grown cells (1.2 μg/mg of protein). This level of binding was comparable to that bound by an equivalent amount of intact cells grown on glucose (1.2 μg of starch per mg of protein) and was 10-fold lower than the level seen with intact cells grown on maltose.

Comparison of outer membrane protein profiles. SDS-PAGE of outer membrane proteins (Fig. 5) did not reveal any differences associated with growth on glucose, maltose, amylose, or pullulan.

DISCUSSION

Previous studies of polysaccharidases produced by human colonic *Bacteroides* species have shown that these enzymes are usually cell associated rather then extracellular (14, 17). In the present study, some starch-degrading activity was found in the extracellular fluid of cells harvested in the exponential phase. This appeared to be a result of cell lysis, because comparable levels of cytoplasmic and membrane proteins were also detected in the extracellular fluid, but we could not rule out the possibility that release of cell-associated enzymes by cell lysis masked secretion of a truly extracellular enzyme. In the accompanying paper we present genetic evidence that none of the degradative enzymes is extracellular (1).

The α-glucosidase and maltase appeared to be located in the cytoplasm and may represent activities of the same

enzyme. Since these activities partitioned differently from amylase activity, the α-glucosidase and maltase activities are probably not associated with the same enzyme as the amylase activity. The amylase, amylopectinase, and pullulanase activities partitioned like periplasmic enzymes. However, interpretation of these localization results was complicated by the substantial proportion of these activities remaining with the membrane fraction during the cold-shock procedure. Although the starch-degrading enzymes could have an affinity for membranes, these were clearly not integral membrane proteins, since washing with phosphate buffer and NaCl caused them to be released from the membrane fraction. Interpretation of the localization data was also complicated by the fact that we do not know how many enzymes contribute to each of the activities measured. Since there are at least two pullulanases (18) and possibly more than one amylase or amylopectinase, different enzymes with the same activity could be localized differently. Nonetheless, it appears that at least some of the degradative enzymes are in the periplasmic space.

If the starch-degrading enzymes are separated from their substrate by the outer membrane, starch must be bound and transported across the outer membrane, presumably by an outer membrane receptor. Our results are consistent with the hypothesis that cell surface starch-binding sites exist. These putative binding sites appear to be proteins, because binding was saturable and reduced by proteinase K treatment. Moreover, the level of starch binding was highest in cells grown on media that induced synthesis of the starch-degrading enzymes. Thus, the genes coding for binding site components appear to be coregulated with genes coding for the polysaccharidases. Also, the putative starch-binding sites had a greater affinity for large oligomers of starch than for small oligomers of glucose. This suggests that the starch-binding sites are distinct from the sites involved in uptake of maltose and small maltodextrins. Since pullulan was less efficient than amylose at competing with labeled starch for the binding sites, there are probably different binding sites for amylose and pullulan.

Assuming that a single starch molecule is bound by each binding site and that the molecular weight of the labeled starch is 7,000, we estimate there are 4×10^5 sites per cell. Despite the high number of calculated receptors, we did not see any outer membrane proteins that were enhanced under growth conditions that enhanced binding activity. However, the outer membrane protein profile of *B. thetaiotaomicron* is quite complex, and the receptor protein(s) could have comigrated with major outer membrane proteins. Alternatively, the binding site may not have been solubilized by boiling in SDS and β-mercaptoethanol. Still another possibility is that the binding site is actually a complex consisting of one or more outer membrane proteins that are constitutively expressed and a periplasmic protein that is regulated. Since membranes of *B. thetaiotaomicron* did not exhibit maltose-inducible starch binding, this indicates that proteins other than outer membrane proteins may be involved in starch binding. Further work is needed to determine whether periplasmic proteins are involved in starch binding.

Uptake of starch by intact cells was found to require anaerobic conditions and the presence of a carbohydrate source (maltose). Since cells incubated aerobically without a carbohydrate source could bind starch, binding alone apparently is not sufficient for uptake. Therefore, there appear to be energy-dependent or oxygen-sensitive factors involved in uptake that are not required for binding.

The specific activities of all of the starch-degrading enzymes were higher in cells grown on maltose than in cells grown on glucose. This difference is probably not due simply to glucose repression, since low specific activities were also found in cells grown on melibiose and chondroitin sulfate. Although maltose appears to be an inducer of the starch-degrading system in *B. thetaiotaomicron*, specific activities associated with growth on amylose, amylopectin, or pullulan were consistently twofold higher than specific activities associated with growth on maltose. The reason for the higher levels induced by polysaccharides is not known, but it may indicate larger oligomers are better inducers or that breakdown of maltose results in higher levels of intracellular glucose than does breakdown of amylose, amylopectin, or pullulan. It is also possible that residual polysaccharide in the cell extract stabilized the enzymes.

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