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Separation of Outer and Cytoplasmic Membranes of *Fibrobacter succinogenes* and Membrane and Glycogen Granule Locations of Glycanases and Cellobiase

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The outer membrane (OM) of *Fibrobacter succinogenes* was isolated by a combination of salt, sucrose, and water washes from whole cells grown on either glucose or cellulose. The cytoplasmic membrane (CM) was isolated from OM-depleted cells after disruption with a French press. The OM and membrane vesicles isolated from the extracellular culture fluid of cellulose-grown cells had a higher density, much lower succinate dehydrogenase activity, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles different from those of the CM. The OM from both glucose- and cellulose-grown cells and the extracellular membrane vesicles from cellulose-grown cultures exhibited higher endoglucanase, xylanase, and acetylesterase activities than the CM and other cell fractions. Endoglucanase 2 was absent from the isolated OM fractions of glucose- and cellulose-grown cells and from the extracellular membrane vesicles of cellulose-grown cells but was present in the CM and intracellular glycogen granule fractions, while endoglucanase 3 was enriched in the OM. Cellobiosidase was located primarily in the periplasm as previously reported, while cellobiase was mainly present in the glycogen granule fraction of glucose-grown cells and in a nongranular glycogen and CM complex in cellulose-grown cells. The cellobiase was not eluted from glycogen granules by cellobiose, maltose, and maltotriose nor from either the granules or the cell membranes by nondenaturing detergents but was eluted from both glycogen granules and cell membranes by high concentrations of salts. The eluted cellobiase rebound almost quantitatively when diluted and mixed with purified glycogen granules but exhibited a low affinity for Avicel cellulose. Thus, we have documented a method for isolation of OM from *F. succinogenes*, identified the OM origin of the extracellular membrane vesicles, and located glycanases and cellobiase in membrane and glycogen fractions.

Fibrobacter succinogenes S85 is one of the major cellulolytic bacteria in the rumen of animals receiving low-quality forage rations (44). It extensively degrades cellulose after attachment to the insoluble substrate (39), which indicates that the cellulase enzymes are functioning at the cell surface during cellulose digestion. Nevertheless, the mechanism by which the bacterium degrades cellulose is still unknown (18, 44). To elucidate the physiological mechanism of cellulose degradation by the bacterium, three endoglucanases, endoglucanase 1 (EG1), EG2 (46), and EG3 (49), a chloride-stimulated cellobiosidase (31), and a cellodextrinase (28, 29) were purified and characterized. EG1 was released from cells during growth, and EG2 was mainly cell associated, while EG3 was released late during growth (48). The chloride-stimulated cellobiosidase was extracellular and membrane associated, while the cellodextrinase was periplasmic (28), and the cellobiase was mainly in membranes and cytoplasm (28, 30). However, the resolution of these experiments was limited because no method had been developed for separation of the outer membrane (OM) from the cytoplasmic membrane (CM), nor had the presence of a discrete glycogen granule fraction been identified. In this study, we report the isolation of the OM from *F. succinogenes*, the OM origin of membrane vesicles in the extracellular culture fluid, and the distribution of cellulase and xylanase enzymes (glycanases) among the various cellular fractions. The association of cellobiase with glycogen granules has also been examined.

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MATERIALS AND METHODS

Bacterium and growth conditions. *F. succinogenes* subsp. *succinogenes* S85 (ATCC 19169 [51]) was grown at 37°C in a chemically defined medium with ammonium sulfate as the sole source of nitrogen and either 0.5% (wt/vol) glucose or 0.3% (wt/vol) Avicel microcrystalline cellulose PH105 (FMC Corp., Marcus Hook, Pa.) as a carbon source (26).

Isolation of OM. OM from *F. succinogenes* was isolated by a modification of the procedure described by Forsberg et al. (19). Glucose-grown cultures (2.4 liters) with an optical density of about 2.0 at 675 nm were centrifuged at 10,400 × *g* for 10 min at 4°C and washed once with 1.2 liters of a freshly prepared salt solution (pH 6.8) composed of all salts present in the chemically defined medium. All subsequent procedures were at 4°C, and all supernatant solutions were saved. The washed cells were suspended in 200 ml of 0.5 M NaCl in 10 mM piperazine-*N*,*N*′-bis-2-ethanesulfonic acid (PIPES; pH 6.8), gently shaken for 5 min, and then recovered by centrifugation at 10,400 × *g* for 10 min. This step was repeated a second time. The cells were then subjected to osmotic shock by suspension in 100 ml of 10 mM PIPES (pH 6.8) containing 25% (wt/vol) sucrose and 1 mM EDTA and shaking for 10 min and then centrifugation at 21,500 × *g* for 20 min. The sedimented cells were quickly and vigorously resuspended in 200 ml of ice-cold glass-distilled water and shaken for 10 min. The shocked cells were recovered by centrifugation at 27,300 × *g* for 20 min and washed with 100 ml of 17% (wt/vol) sucrose–1 mM EDTA in 10 mM PIPES (pH 6.8) with shaking for 5 min. After the cells were recovered from sucrose by centrifugation at 21,500 × *g* for 20 min, they were resuspended in 60 ml of 10 mM PIPES (pH 6.8)-buffered saline and disrupted by three passages through a chilled French pressure

cell at 10,000 lb/in^2. The cell extract was centrifuged at 10,400 $\times$ *g* for 10 min to remove unbroken cells and then at 37,000 $\times$ *g* for 1 h to sediment the membrane fraction. The membrane pellet formed two distinct fractions, a yellow top layer and a white bottom layer which mainly contained glycogen granules. The yellow membrane fraction was separated from the glycogen granules by gently washing off the top layer with 10 mM PIPES (pH 6.8)-buffered saline by using a pipette. Membranes at the interface of the two fractions were saved as a separate fraction for enzyme analysis. The recovered membrane fractions were washed twice with 10 mM PIPES (pH 6.8)-buffered saline and saved. The glycogen granules were washed twice, each time removing traces of yellow CM. This procedure for purifying glycogen granules is called method 1. The supernatants obtained from the NaCl and water washes were centrifuged at 23,300 $\times$ *g* for 20 min to remove any remaining cells, after which they were centrifuged at 100,000 $\times$ *g* for 2 h to sediment the OM fragments. The sedimented OM fragments were washed once with 10 mM PIPES (pH 6.8)-buffered saline. Both the OM fractions and the resulting supernatants free from OM fragments were saved. The procedure to recover OM fractions from the sucrose washes was the same as that described above, except that those from the sucrose washes were diluted twofold with 10 mM PIPES (pH 6.8)-buffered saline prior to the centrifugation. To assess homogeneity of the membrane fractions, isopycnic sucrose gradient centrifugation was performed by using a discontinuous 30 to 50% (wt/wt) sucrose gradient with 5% steps at 36,000 rpm in a SW40 rotor for 15 h. Fractions of 0.25 ml were collected from the bottom of each tube. The density, succinate dehydrogenase (SDH) activity, a cytoplasmic membrane marker, and protein concentration were then determined. Fractions containing each protein band were pooled. For localization of fibrolytic enzymes, all OM fractions released by different washes were pooled. All cell fractions for enzyme analysis were either diluted in/or dialyzed against 0.05 M sodium phosphate buffer (pH 6.7).

For fractionation of cellulose-grown cells, the cells were harvested after 60 h of growth when the cellulose substrate in the culture medium was almost completely digested. Residual cellulose in the medium was first removed by centrifugation at 650 $\times$ *g* for 5 min. Cells were recovered by centrifugation at 10,400 $\times$ *g* for 10 min and then subjected to the fractionation procedure described for glucose-grown cells. After OM fragments were removed, cells were disrupted and centrifuged at 37,000 $\times$ *g* for 1 h to recover the CM as before. In cellulose-grown cells, the glycogen fraction was barely visible compared with the glucose-grown cells, and no attempt was made to physically separate the glycogen from the CM. Proteins in the extracellular culture fluid were concentrated by ultrafiltration through a PM-10 membrane (Amicon Corp., Lexington, Mass.) and dialyzed against 50 mM sodium phosphate buffer (pH 6.7). Membrane vesicles in the extracellular culture fluid were obtained by ultracentrifugation as described for isolation of OM fragments. The membrane vesicle pellets were contaminated by a thin layer of fine cellulose particles presumably derived from incomplete removal of cellulose by the centrifugation procedure. This layer was recovered as an individual fraction for enzyme analysis.

Isolation of glycogen granules by methods 2 and 3. Glycogen granules containing bound cellobiase were also prepared by method 2 from glucose-grown cells as described above, except that OM was not removed prior to disruption of the cells.

Purified glycogen granules freed of all noncovalent materials were isolated by method 3 from a glucose-grown culture (2.5 liters) with an optical density at 675 nm of 3.7. The culture was quickly cooled by pouring the cell suspension into a flask containing ice, and the cells were harvested by centrifugation (10,000 $\times$ *g*, 10 min, 4°C) and resuspended in 250 ml of ice-cold water. Cells, in the presence of 25 mg of bovine pancreatic DNase I (13,500 U; Sigma), were disrupted by using a Braunsonic 2000 sonicator (B. Braun Inc., San Mateo, Calif.) with nine 20-s bursts of power at 200 W with 40-s intervals on ice for cooling between treatments. After removal of unbroken cells by centrifugation (10,000 $\times$ *g*, 10 min, 4°C), the cell extract was centrifuged at 35,000 $\times$ *g* for 60 min at 4°C. Material which sedimented was resuspended in 100 ml of 2% (wt/vol) sodium dodecyl sulfate (SDS) containing 5 mM EDTA (pH 8.0) and homogenized with a Potter-Elvenhjem tissue homogenizer. After 20 min at 67°C with intermittent mixing, glycogen granules were recovered by centrifugation (37,000 $\times$ *g* for 1 h); washed once with 1% (wt/vol) SDS in water, once with 0.85% (wt/vol) NaCl, and three times with water; and then lyophilized.

Release and binding of cellobiase to glycogen granules. To investigate release of cellobiase from glycogen granules, the granules prepared without SDS extraction were extracted with various sugars and detergents in 25 mM sodium phosphate buffer (pH 6.7). Cellobiose, maltose, and maltotriose were at a final concentration of 2% (wt/vol), while 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and Tween 80 were at a final concentration of 0.5% (wt/vol), and Triton X-100 was at a final concentration of 1% (wt/vol). Extraction with various salt solutions was conducted in 25 mM sodium phosphate buffer (pH 6.7) containing 10% (vol/vol) glycerol and 2 mM DL-dithiothreitol (DTT). NaCl and KCl were at a final concentration of 1 M, Na_2SO_4 and $MgCl_2$ were at a final concentration of 0.33 M, and $MgSO_4$ was at a final concentration of 0.25 M. For extraction with EDTA, the final concentration was 2.5 mM and the buffer was 10 mM PIPES (pH 6.5). Extraction with EDTA and detergents was at 22°C for 20 min with gentle vortex mixing at 5-min intervals; other extractions were conducted in an identical fashion, except at 0°C. To examine binding of cellobiase to purified glycogen granules and to Avicel cellulose, enzyme released from glycogen granules by 1 M NaCl (0.5 mg of protein per ml) was diluted 10-fold in 50 mM sodium phosphate buffer (pH 6.7) which contained 2 mM DTT, and then 1 ml was mixed with 30 mg (each) of washed glycogen granules purified from *F. succinogenes* S85 by SDS extraction or washed Avicel crystalline cellulose PH105. Binding was at 22°C for 30 min with gentle vortex mixing at 10-min intervals. The glycogen granules were sedimented by centrifugation in a Beckman Airfuge at 140,000 $\times$ *g* for 5 min at 22°C, washed in 1 ml of the same buffer containing 2 mM DTT, and then assayed for cellobiase activity.

Isolation of cell envelopes from cellulose-grown cells and release of cellobiase. Cells grown on cellulose for 50 h were used for the preparation of cell envelopes as described by Huang and Forsberg (28). To release cellobiase from the cell envelopes, cell envelope samples were treated with CHAPS, Triton X-100, NaCl, and KCl in a fashion identical to that described for glycogen granules. The samples containing 0.55 mg of carbohydrate and 0.35 mg of protein were also digested with 2,500 U of α-amylase (type II, from *Bacillus* species; Sigma). The digestion was at 22°C for 2 h in 25 mM sodium phosphate buffer (pH 6.7) containing 2 mM DTT.

Antibody preparation. Polyclonal antiserum and monoclonal antibodies against EG2 and polyclonal antiserum against EG3 were prepared previously (47, 49). Monospecific polyclonal antibodies were prepared as described previously (29).

SDS-PAGE and Western immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the

method of Laemmli (41). Transblotting after SDS-PAGE was conducted in the buffering system of Towbin et al. (59) at 30 V overnight at 4°C. Western blot analysis was performed according to the instructions in the Bio-Rad immunoblot manual. The first antibodies were appropriately diluted polyclonal antiserum, monospecific polyclonal antibody, or monoclonal antibody (as indicated in the figure legends).

Enzyme assays and analytical methods. Assays for endoglucanase, cellobiosidase, cellobiase, and β-glucosidase were performed as described by Huang and Forsberg (28), with the exception that glucose resulting from cellobiase activity was determined by using Trinder reagent from Sigma. A final concentration of 5 mM rather than 0.5 mM *p*-nitrophenyl-β-glucopyranoside was used for the β-glucosidase assay in the presence and absence of 10 mM cellobiose. Xylanase and acetylesterase were measured as described previously (45) with final concentrations of 1% (wt/vol) birchwood xylan and 1 mM α-naphthyl acetate, respectively.

Zymogram analysis for endoglucanases was conducted in a SDS-polyacrylamide gel containing 0.2% (wt/vol) carboxymethyl cellulose (medium viscosity) as the substrate. The method was essentially that described by Kohring et al. (35), with the exceptions that samples were incubated at 39°C for 30 min in the SDS-PAGE sample buffer lacking β-mercaptoethanol prior to electrophoresis, and SDS in the gel after electrophoresis was removed by 2% Triton X-100 at 22°C for 60 min, and then Triton X-100 was removed by washing the gel with 50 mM sodium phosphate buffer (pH 6.7).

Fumarate reductase and SDH were assayed by using the methods of Dickie and Weiner (14). NADH oxidase was assayed as described by Osborn et al. (52). Lactic dehydrogenase was assayed as outlined by Kornberg (37). Glutamate dehydrogenase (GDH) was chosen as a cytoplasmic marker and assayed as described by McGavin et al. (48). Protein was measured by using the method of Bradford (3), with bovine serum albumin (BSA) as a standard. The refractive index of sucrose gradient fractions was determined at room temperature. The refractive index values were then converted to densities (13). Lipopolysaccharide (LPS) was prepared by using membrane vesicles from the extracellular culture fluid of a cellulose-grown culture by a method similar to that of Chart and Rowe (6). Vesicle samples containing 0.2 mg of protein were digested with 0.05 mg of proteinase K (13 U/mg of solid; Sigma) at 37°C for 3 h in 10 mM Tris hydrochloride buffer (pH 7.5) containing 0.5% (wt/vol) SDS and 5 mM EDTA. The digested samples which originally contained 12 μg of protein were subjected to SDS-PAGE and then silver staining as described by Tsai and Frasch (60). LPS from *Pseudomonas aeruginosa* PAO1 (10) was used as a standard. The LPS indicator 2-keto-3-deoxyoctonic acid (KDO) in membrane samples was released by hydrolysis with 0.19 N H_2SO_4 at 100°C for 20 min. The free KDO was assayed as described by Karkhanis et al. (33) by using authentic KDO (Sigma) as the standard. Total carbohydrate was determined by using the phenol-sulfuric acid method (15). Phosphate in glycogen samples was measured as described by Ames and Dubin (1). Dry weight of the cells was determined as described by Forsberg and Lam (21).

Acid hydrolysis of glycogen and component analysis. Samples for neutral sugar analysis were hydrolyzed in 2 M HCl at 100°C for 2 h, while those for amino sugar analysis were hydrolyzed in 4 N HCl in sealed and evacuated tubes. To serve as a control for the neutral sugar analysis, glucose was treated in an identical fashion. Hydrolysates were evaporated to dryness in a rotary evaporator at 42°C and then redissolved in water and evaporated to dryness twice. The samples for neutral sugar analysis were freed from ions by mixing with the ion-exchange resins Amberlite IR-120 (hydrogen form) and Rexyn 201 (carbonate form), filtered through a 0.45-μm-pore-size filter, and then analyzed with a Waters 510 high-pressure liquid chromatography (HPLC) system equipped with an Aminex HPX-42A carbohydrate column (Bio-Rad) as described previously (28). To determine amino sugars, hydrolysates were analyzed by a Waters 625 LC system (Maxima 820 data system) equipped with a Dionex CarboPac PA1 anion-exchange column and a Waters 464 pulsed electrochemical detector. The analytical method was essentially that described by Clarke et al. (9), with the exception that the pulsed electrochemical detector, operating in the pulse mode, had electron potentials set as follows: $E_1 = +112$ mV, $E_2 = +812$ mV, and $E_3 = -588$ mV, with 216-, 216-, and 299-ms applied durations, respectively.

Samples for diaminopimelic acid analysis were suspended in 6 M HCl at 5 mg/ml, hydrolyzed at 110°C for 24 h in sealed and evacuated tubes, and then centrifuged to remove any sediment. A 200-μl volume of each supernatant was brought to dryness under nitrogen and then derivatized by the method described by MacKenzie (43). The derivatives were analyzed with a Varian 6000 gas chromatograph equipped with a flame ionization detector and an Econocap SE30 column with a 0.25-μm internal diameter and a length of 15 m (0.25-μm coat; Alltech). To examine trace proteins bound to glycogen granules, the granules were hydrolyzed with 6 M HCl at 10 mg/ml in sealed and evacuated tubes at 105°C for 24 h and then analyzed by using a Beckman System Gold amino acid analyzer as described by Clarke (8).

Infrared spectroscopy. Glycogen samples prepared by method 3 were ground in Nujol mineral oil (paraffin oil; Fisher Scientific Co.) and then analyzed by using a Nicolet Easy Operation 5DXC infrared spectroscope (Nicolet Analytical Instruments, Madison, Wis.).

Transmission electron microscopy. The methods for transmission electron microscopy and thin-section preparation of cell or membrane samples were essentially those described by Groleau and Forsberg (26). To estimate percentage of the cells without intact envelope, at least 100 cells were counted. Sample preparation for negative staining was performed as described previously (48). Metals were examined with an energy-dispersive X-ray system as described by Harvey et al. (27).

RESULTS

Isolation of the OM and CM. Attempts to separate OM from CM of French press-disrupted cells of *F. succinogenes* S85 by isopycnic sucrose gradient centrifugation by using methods developed for other gram-negative bacteria (32, 38, 52) were unsuccessful. Isolation of OM by extraction of cell envelopes in 10 mM Tris HCl (pH 7.4) containing 0.5% (wt/vol) of Sarkosyl which preferentially solubilized the CM of *Escherichia coli* (16) also failed because the detergent solubilized both the OM and the CM. However, a modification of a method for fractionation of cell envelopes of the marine bacterium *Alteromonas haloplanktis* (19, 40) which involved sequentially washing cells in 0.5 M NaCl and 25% (wt/vol) sucrose was successful. The fractionation was followed by electron microscopy of thin sections of cells.

The morphological characteristics of glucose-grown cells in culture medium, washed cells, and membranes are shown in Fig. 1. *F. succinogenes* possesses a typical gram-negative envelope morphology with an OM, thin peptidoglycan layer, and inner CM (Fig. 1A). No membrane fragments were released

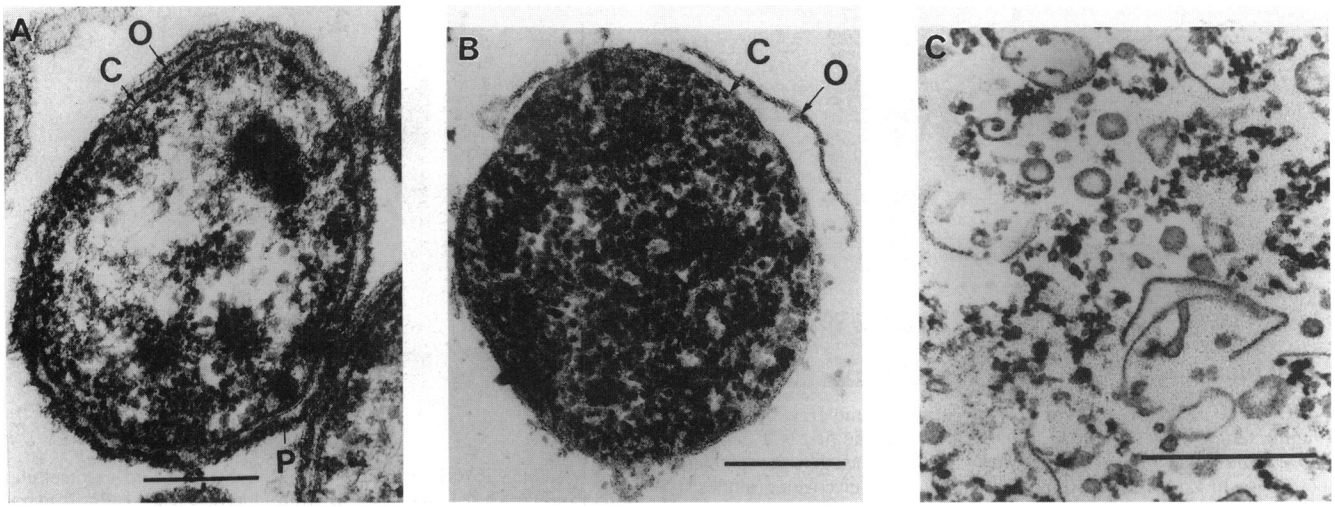


FIG. 1. Morphology of glucose-grown cells at various stages of the cell fractionation process and of OM fragments. (A) Unwashed cells. (B) Cells washed with 0.5 M NaCl and once with 25% (wt/vol) sucrose show OM fragments peeling off. (C) Isolated OM fragments. Abbreviations: O, OM; C, CM; P, peptidoglycan. Bars, 0.2 μm (A and B) and 0.5 μm (C).

from cells at this stage of growth, and when the cells were washed with a mineral solution corresponding in composition to the medium, the OM remained intact. However, OM fragments were released from cells by washes in NaCl, sucrose, and water (Fig. 1B). Cells with OM fragments peeling off accounted for approximately 5 to 10% of the population after the first wash in NaCl and 40 to 45% after the first wash in sucrose. Approximately 90 to 95% of cells lost either most or all of the OM during the water and the second sucrose washes. The released OM fragments assumed both open planar shapes and closed vesicular structures (Fig. 1C). The distribution of isolated OM and CM from glucose-grown cells in sucrose gradients after isopycnic centrifugation is displayed in Fig. 2. The OM fraction released by the first NaCl wash had a density of 1.21 g ml^{-1} and low SDH activity (Fig. 2A), whereas the yellow CM fraction recovered from NaCl-, sucrose-, and water-washed cells after disruption exhibited a density of 1.18 g ml^{-1} and high SDH activity (Fig. 2B). Membrane fractions released by sucrose and water washes showed a profile in the sucrose gradients similar to that in Fig. 2A, with a high density and low SDH activity. A white glycogen granule fraction (characterized later) which formed the bottom half of the CM pellet on centrifugation at 37,000 × *g* was separated by washing off the upper CM layer. The characteristic components of peptidoglycan, diaminopimelic acid, and muramic acid were not detected in this fraction. The SDS-PAGE protein profiles of the OM fractions released by the series washes differed from that of the CM, the glycogen fraction, and the cell envelope fraction which was composed of inner membranes and OMs contaminated with glycogen granules and prepared from sonicated cells (Fig. 3). All the OM fractions gave two major protein bands of approximately 94 kDa, and the OM released by the first sucrose wash also exhibited an additional major protein band of 20 kDa. The CM and cell envelope fractions displayed more complex protein banding patterns. The 94-kDa bands were also detected in the CM, suggesting that it was contaminated with OM. The glycogen fraction had five major protein bands which ranged from 30 to 110 kDa. Thus, the protein profiles are diagnostic of most fractions. Identical protein banding patterns were obtained for the membrane preparations whether or not the membrane fractions had been subjected to sucrose gradient centrifugation. Therefore, gradient centrifugation was not used in subsequent experiments. The low SDH activity and different protein profiles of the membranes released by the washes demonstrate that they are from the OM. In contrast, the yellow membrane fraction with high SDH activity recovered by disruption of cells lacking the OM is the CM.

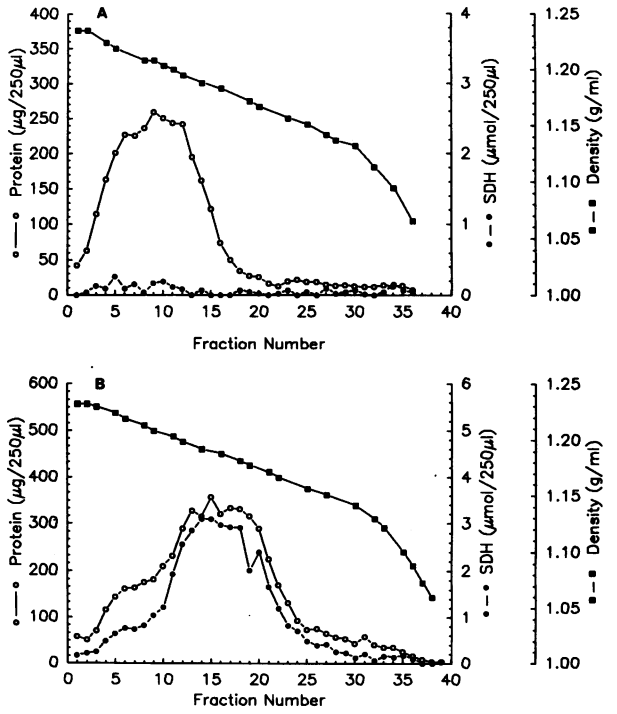


FIG. 2. Distribution of membrane fractions in sucrose density gradients. (A) OM released by the first NaCl wash. (B) CM recovered from the French press extract of cells after removal of the OM by washes in NaCl, sucrose, and water.

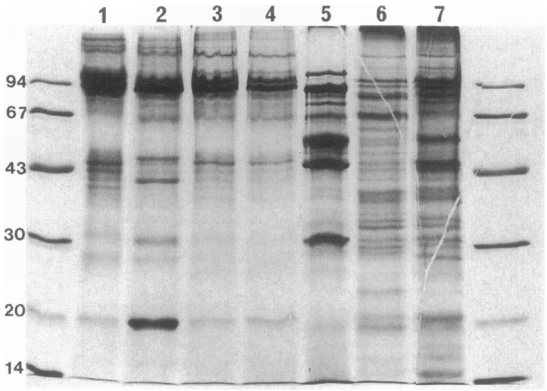


FIG. 3. SDS-PAGE protein profiles of membrane fractions from 5-h glucose-grown cells. Lanes: 1, OM released by the first NaCl wash; 2, OM released by the first sucrose wash; 3, OM released by the second sucrose wash; 4, OM released by the water wash; 5, glycogen granules; 6, CM; 7, cell envelope contaminated with glycogen. About 11 μg of protein was loaded in each lane.

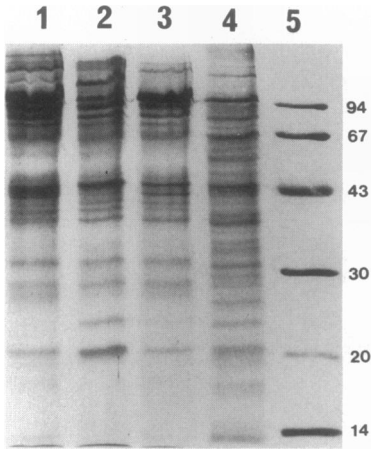


FIG. 4. SDS-PAGE protein profiles of the OM and extracellular membrane vesicles. Lanes: 1, OM from glucose-grown cells; 2, extracellular membrane vesicles from a cellulose-grown culture; 3, OM from cellulose-grown cells; 4, CM from cellulose-grown cells; 5, low-molecular-weight protein standards (Pharmacia). About 10 μg of protein was loaded in each lane.

In contrast to glucose-grown cells, cellulose-grown cells released membrane vesicles into the culture fluid during growth. The membrane vesicles released into the extracellular culture fluid and OM isolated from cellulose-grown cells had identical densities (1.21 g ml^{-1}), low SDH activities (Table 1), and similar SDS-PAGE protein profiles, with the exception that the extracellular membrane vesicles contained a protein band of approximately 160 kDa that was absent from the OM (Fig. 4). The only major difference between the protein profiles of OM from glucose-grown cells and the OM and extracellular membranes of cellulose-grown cells was the absence of a 23-kDa protein from the former (Fig. 4). Because of the shared characteristics of the extracellular membrane vesicles and OM, the vesicles would appear to arise from the OM. Some protein bands of the OM preparations and extracellular membrane vesicles were also present in the CM preparation. This presumably was because of incomplete release of the OM from the CM during the fractionation process.

Of the enzymes which are frequently used as CM markers, including SDH, fumarate reductase, lactic dehydrogenase, and NADH oxidase, SDH exhibited the highest activity. The distribution of SDH and GDH in various cell fractions of glucose and cellulose-grown cells is shown in Table 1. The OM fraction and extracellular membrane vesicles were low in SDH. The periplasmic fraction contained some GDH, indicative of

TABLE 1. Distribution of SDH and GDH in extracellular and cellular fractions of cells grown on glucose and cellulose

Fraction	Enzyme activity[a] (nmol/min/mg of protein) or protein					
	Glucose-grown cells			Cellulose-grown cells		
	SDH	GDH	Total protein (mg)	SDH	GDH	Total protein (mg)
Extracellular						
Vesicles	–[b]	–	–	0 (0)	12 ± 1.0 (2.4)	22.5 (9.6)
Vesicles[c]	–	–	–	74 ± 12.3 (3.5)	0 (0)	11.0 (4.7)
NS[d]	–	–	–	0 (0)	71 ± 0.8 (20.1)	31.3 (13.3)
Cellular						
OM	6 ± 2.1 (0.1)	0 (0)	3.4 (1.7)	0 (0)	10 ± 0.5 (<0.1)	0.7 (0.3)
Periplasm[e]	0 (0)	45 ± 10.0 (29.0)	64.8 (32.0)	0 (0)	32 ± 0.2 (10.1)	35.3 (15.0)
Glycogen	197 ± 2.1 (2.0)	0 (0)	2.4 (1.2)	–	–	–
CM	555 ± 16.8 (72.9)	0 (0)	31.5 (15.5)	216 ± 2.0 (70.9)	9 ± 2.2 (6.3)	75.8 (32.1)
Cytoplasm	50 ± 7.1 (20.5)	72 ± 1.7 (71.0)	98.6 (48.7)	100 ± 4.2 (25.6)	115 ± 2.2 (61.1)	58.9 (25.0)
Glycogen + CM	534 ± 2.1 (4.4)	0 (0)	2.0 (1.0)	–	–	–
Recovery (%)	80	88	86	94	99	96

[a] Enzyme activity is expressed as mean ± standard deviation. Values in parentheses for cellulose-grown cells are the percentages of the total activity or protein found in the culture, while for glucose-grown cells values in parentheses are the percentages of the total cell-associated activity or protein. Recovery was calculated on the basis of the total activity or protein of the French press-disrupted cells in the case of glucose-grown cells and French press-disrupted whole culture in the case of cellulose-grown cells.

[b] –, not determined because no membrane vesicles were released from glucose-grown cells and no glycogen granules were isolated from cellulose-grown cells.

[c] Membrane vesicles contaminated with fine cellulose particles.

[d] NS, nonsedimentable at 100,000 × *g* for 2 h.

[e] Pooled nonsedimentable fractions derived from the NaCl, sucrose, and water washes of cells. Protein in the nonsedimentable NaCl fractions accounted for only 1% of the total protein in these fractions.

TABLE 2. Distribution of fibrolytic enzymes in glucose-grown cells

Fraction	Enzyme activity[a] (nmol/min/mg of protein)				
	Endoglucanase	CBSase[b]	Cellobiase	Xylanase	Acetylesterase
Extracellular culture fluid	0	–	–	0	–
OM	732 ± 19 (9.5)	2 ± 0.2 (0.1)	0 (0)	991 ± 20 (20.3)	316 ± 8 (17.0)
Periplasm[c]	34 ± 8 (8.3)	55 ± 0.8 (93.4)	0 (0)	12 ± 11 (4.6)	7 ± 1 (6.9)
Glycogen	88 ± 8 (0.8)	6 ± 0.2 (0.4)	6,709 ± 202 (58.5)	33 ± 7 (0.5)	10 ± 1 (0.4)
CM	286 ± 31 (34.0)	2 ± 0.2 (0.7)	109 ± 8 (12.4)	245 ± 15 (46.0)	95 ± 2 (46.6)
Cytoplasm	123 ± 2 (45.8)	2 ± 0.2 (5.3)	68 ± 12 (24.5)	44 ± 5 (26.0)	17 ± 0 (26.4)
Glycogen + CM	213 ± 6 (1.6)	2 ± 0.1 (0.1)	660 ± 68 (4.7)	227 ± 9 (2.7)	85 ± 1 (2.6)
Recovery (%)	122	125	99	88	81

[a] See footnotes *a* and *b* of Table 1.
[b] CBSase, cellobiosidase.
[c] See footnote *e* of Table 1.

lysis during the procedure, but most was in the cytoplasmic fraction. Similar results were obtained for cellulose-grown cells in which some GDH activity was detected in both the periplasmic fraction and the nonsedimentable fraction of the extracellular culture fluid. More than 70% of the SDH activity was associated with the CM fraction, and practically none was associated with the OM fraction (Fig. 2; Table 1). The SDH activity associated with vesicles in the fraction contaminated with cellulose presumably was due to bound membranes derived from a few cells lysed during growth.

LPS is frequently used as an OM marker in gram-negative bacteria. When the membrane vesicles from the extracellular culture fluid of cellulose-grown *F. succinogenes* S85 were treated with proteinase K, analyzed by SDS-PAGE, and silver stained for the characteristic ladder profile of LPS (10), only one fast-migrating band which was not stained by Coomassie blue was detected at the bottom of the gels. The membrane vesicles, OM, and cell envelope fractions were further examined for the presence of KDO. KDO exhibits a pink color with an absorbance maximum at 549 nm. However, an orange color with an absorbance maximum at 529 nm was produced instead, suggesting the absence of unsubstituted KDO. If KDO is phosphorylated, it does not react efficiently in the thiobarbituric acid assay (61), but because of obviously interfering substances, this marker was not pursued further.

Distribution of fibrolytic enzymes in cell fractions. In glucose-grown cells, there were high endoglucanase, xylanase, and acetylesterase activities in the OM fraction, although these enzyme activities were present in all cell fractions (Table 2). The extracellular culture fluid which had a low protein content (0.008 mg/ml) did not contain either endoglucanase or xylanase activities. Cellobiosidase was primarily located in the periplasmic fraction. This was most likely because of the periplasmic cellodextrinase, as reported previously (28, 29). The stimulation of cellobiosidase by added sodium chloride was not detected in the extracellular culture fluid and cell extract, suggesting that chloride-stimulated cellobiosidase was not expressed. Cellobiase was mainly found in the cytoplasm, bound to glycogen granules. The distribution of fibrolytic enzymes in cellulose-grown cells is shown in Table 3. The extracellular vesicles exhibited enzyme activities similar to those of OM from the same cells and from glucose-grown cells, except that the vesicles exhibited higher specific activities. Twenty-three percent of the total GDH activity was found in the extracellular culture fluid (Table 1), indicating release of some cellular enzymes, which may explain why there were 37 and 14% of the total activities of cellobiosidase and cellobiase detected in the extracellular culture fluid, respectively. Activity of the chloride-stimulated cellobiosidase was mainly found in the extracellular culture fluid. The ratio of the enzyme activity in the presence of chloride to that in the absence of chloride was 1.65 in the sedimented fraction and 1.96 in the nonsedimented fraction of the extracellular culture fluid but 1.09 in the

TABLE 3. Distribution of fibrolytic enzymes in cellulose-grown cultures

Fraction	Enzyme activity[a] (nmol/min/mg of protein)				
	Endoglucanase	CBSase	Cellobiase	Xylanase	Acetylesterase
Extracellular					
Vesicles	1,290 ± 76 (34.6)	7 ± 0.8 (3.8)	90 ± 15 (5.1)	1,134 ± 1 (27.4)	352 ± 7 (26.0)
Vesicles[b]	737 ± 29 (9.6)	23 ± 1.3 (5.7)	279 ± 14 (7.8)	664 ± 6 (7.8)	127 ± 8 (4.5)
NS	836 ± 11 (31.2)	38 ± 3.3 (27.5)	13 ± 1 (1.0)	1,159 ± 7 (38.9)	448 ± 18 (45.9)
Cellular					
OM	585 ± 19 (0.5)	4 ± 1.4 (<0.1)	25 ± 1 (<0.1)	781 ± 3 (0.5)	148 ± 6 (0.3)
Periplasm	77 ± 16 (3.2)	46 ± 0.2 (36.7)	132 ± 15 (11.9)	79 ± 4 (3.0)	19 ± 1 (2.3)
CM	146 ± 40 (13.3)	4 ± 0.7 (7.4)	349 ± 15 (67.2)	186 ± 1 (15.1)	46 ± 1 (11.4)
Cytoplasm	109 ± 14 (7.6)	14 ± 0.8 (18.8)	47 ± 13 (7.0)	115 ± 9 (7.3)	50 ± 0 (9.6)
Recovery (%)	87	119	123	105	84

[a] See footnotes *a* and *b* of Table 1. Abbreviations: NS, nonsedimented fraction of the extracellular culture fluid. CBSase, cellobiosidase.
[b] See footnote *c* of Table 1.

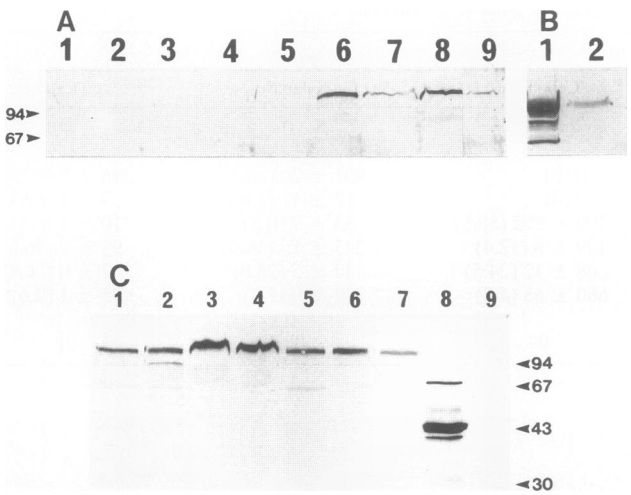


FIG. 5. Immunoblots of cell fractions from cells grown on glucose and cellulose by using polyclonal antibodies against EG2 and EG3. (A) Immunoblot of membrane fractions from 5-h glucose-grown cells probed with 200-fold-diluted polyclonal antiserum against EG2. Lanes: 1, OM released by the first NaCl wash; 2, OM released by the second NaCl wash; 3, OM released by the first sucrose wash; 4, OM released by the second sucrose wash; 5, OM released by the water wash; 6, glycogen granules; 7, CM; 8, cell envelope contaminated with glycogen granules; 9, unwashed sonicated cells. About 17 μg of protein of each sample was loaded in each lane. (B) Immunoblot of EG2 and NaCl extract of cell envelopes from glucose-grown cells probed with 100-fold-diluted monoclonal antibodies against EG2. Lanes: 1, purified EG2 and degradation products, 1.5 μg; 2, proteins released by 1 M NaCl, 13 μg. (C) Immunoblot of cell fractions from 60-h cellulose-grown cells probed with monospecific antibodies against EG 3. Lanes: 1, cell extract; 2, nonsedimentable fraction from the extracellular culture fluid; 3, sedimentable fraction from the extracellular culture fluid; 4, OM; 5, periplasm; 6, CM; 7, cytoplasm; 8, 0.8 μg of EG3 isolated previously by affinity purification with an anti-EG3 affinity column from *E. coli* K-12 strain RR1 containing the *cel-3* gene (49); 9, 1.5 μg of EG2. About 16 μg of protein of each sample was loaded in each lane.

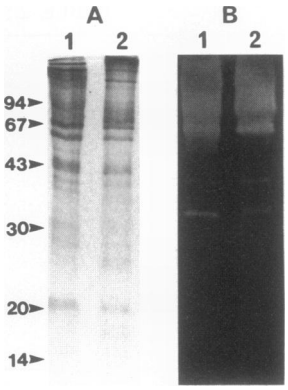


FIG. 6. Zymogram analysis of endoglucanases from the extracellular membrane vesicles of a cellulose-grown culture and the OM from glucose-grown cells. (A) SDS-PAGE; (B) zymogram. Lanes: 1, extracellular membrane vesicles; 2, OM. A total of 17 μg of protein of each sample was loaded for SDS-PAGE, and 4 μg of protein (each sample) was loaded for zymogram analysis. Samples in panels A and B were treated identically before electrophoresis.

OM fraction and 0.96 in the periplasm, suggesting little of the enzyme in either the OM or periplasmic fractions.

Distribution of EG2 and EG3 in cell fractions. When the different membrane fractions from glucose-grown cells were probed with polyclonal antiserum against the 118-kDa intact EG2 and monoclonal antibodies against the binding domain of EG2 (47), the enzyme was not detected in any of the OM fractions, except for a weak reaction with those released by the water wash and second sucrose wash. EG2 was present in the NaCl, sucrose, and water washes that made up the periplasmic fraction and the cytoplasm (data not shown) and was in the glycogen-, CM-, and glycogen-contaminated cell envelope fractions (Fig. 5A). EG2 in the unfractionated cells could be eluted from cell membranes with 1 M NaCl (Fig. 5B), although NaCl concentrations as low as 0.25 M were also shown to release it. Cellulose-grown cells had the same membrane location of EG2 as glucose-grown cells. The enzyme was absent from the OM and extracellular membrane vesicles but present in the CM and the nonsedimentable fraction of the extracellular culture fluid (data not shown). The distribution of EG3 in the various cellular fractions was shown by immunoblot analysis with monospecific polyclonal antibodies against EG3 (Fig. 5C). While EG3 corresponding to the reported molecular mass of 118 kDa (49) was present primarily in the OM of cells and OM fragments released into the extracellular culture fluid, lesser amounts were detected in the nonsedimentable fraction of the extracellular culture fluid, periplasm, and the CM, and only a trace of the enzyme was present in the cytoplasm. When different cellular fractions of glucose-grown cells were probed with the monospecific polyclonal antibodies against EG3, a weak immunoreaction was also observed in the extracellular culture fluid and all cell fractions, but none was observed with the glycogen fraction (data not shown). Since the enzyme remained bound to the OM during the NaCl washes, this suggests that it is more firmly anchored than EG2.

Zymogram analysis of endoglucanases. Enzyme patterns of the OM from glucose-grown cells and the membrane vesicles from the extracellular culture fluid of a cellulose-grown culture were examined by SDS-PAGE and zymogram analysis with carboxymethyl cellulose as the substrate. Both samples exhibited an identical enzyme pattern (Fig. 6). Three endoglucanase bands of 33, 41, and 67 kDa were visible. However, most activity was associated with proteins of a size greater than 67 kDa, which could result from activity of EG3 and other high-molecular-weight enzymes but may, in part, be due to aggregation as a consequence of the gentle treatment of the samples in the SDS-PAGE sample buffer lacking β-mercaptoethanol prior to electrophoresis. Irrespective of whether there was aggregation, the fact that identical zymogram profiles are seen further demonstrates the OM origin of the extracellular membrane vesicles.

Release of cellobiase from glycogen granules of glucose-grown cells and rebinding to glycogen granules. The association of cellobiase with glycogen granules prepared by method 2 from glucose-grown cells was first investigated by incubation of the glycogen granule fraction at 37°C for 3 h in buffer without DTT or glycerol and then by assaying for the enzyme activity bound to the granules and determination of glucose released from the granules. After the incubation, no inactivation of the cellobiase was observed. More than 88% of the enzyme activity still remained bound to the glycogen, and less than 0.46% (wt/wt) of the glycogen was hydrolyzed to glucose. The glycogen-bound cellobiase was also examined for β-glucosidase activity by using *p*-nitrophenyl-β-D-glucoside as the substrate, but no cleavage of the substrate was detected when it was assayed in either the absence or the presence of 10 mM cellobiose, demonstrating that the cellobiase is a different

TABLE 4. Elution of cellobiase from glycogen granules of *F. succinogenes* S85[a]

Treatment	Final concn	Enzyme released[b] (%)	Recovery (%)
Maltose	2.0% (wt/vol)	4.6 ± 1.4	103.8
Maltotriose	2.0% (wt/vol)	2.4 ± 0.4	96.8
Cellobiose	2.0% (wt/vol)	0	90.5
CHAPS	0.5% (wt/vol)	2.1 ± 0.5	102.9
Triton X-100	1.0% (vol/vol)	0	99.9
Tween 80	0.5% (vol/vol)	0.2 ± 0.4	100.5
EDTA	2.50 mM	3.8 ± 0.1	79.2
NaCl	1.00 M	96.1 ± 4.2	104.5
KCl	1.00 M	95.7 ± 6.3	104.0
Na_2SO_4	0.33 M	94.9 ± 8.7	89.6
$MgSO_4$	0.25 M	93.6 ± 4.4	106.2
$MgCl_2$	0.33 M	94.4 ± 1.9	114.2

[a] Summary of separate experiments. Each included an untreated sample taken as 100% which had a specific activity in the range from 2,716 to 3,074 nmol of glucose min^{-1} mg of $protein^{-1}$. Glycogen samples were prepared by method 2.
[b] Expressed as means ± standard deviations.

enzyme from the β-glucosidases reported by Buchanan and Mitchell (5). Elution of cellobiase from the glycogen granules with various salt solutions was first tested in 10 mM PIPES (pH 6.5). Greater than 61% of the total cellobiase activity of the granules was inactivated after any salt elution and ultracentrifugation for recovery of the granules. Protection from enzyme inactivation was then tested by extraction of the glycogen samples with 1 M NaCl in 25 mM sodium phosphate buffer (pH 6.7) containing 0.5% (vol/vol) Tween 80, 10% (vol/vol) glycerol, 1% (wt/vol) BSA, or 2 mM DTT. The recoveries of released cellobiase activity by these treatments were 57.0, 76.9, 75.2, and 105.1%, respectively, of the activity present on the granules. Elution with the salt solutions was then performed in 25 mM sodium phosphate buffer (pH 6.7) containing 10% (vol/vol) glycerol and 2 mM DTT. Elution of cellobiase from the glycogen granules is shown in Table 4. Sugars and detergents were ineffective at releasing cellobiase, whereas all salts including NaCl, KCl, Na_2SO_4, $MgSO_4$, and $MgCl_2$ efficiently eluted cellobiase from the glycogen granules. Binding to purified *F. succinogenes* glycogen prepared by method 3 and to Avicel crystalline cellulose of cellobiase released from glycogen granules by NaCl is shown in Table 5. Although some activity was lost during the rebinding process, 81.4% of added activity was found on the purified *F. succinogenes* glycogen after washing and no activity was detected in the supernatant, indicating that NaCl-released cellobiase still maintained the ability to bind to glycogen. Proteins released from glycogen granules by NaCl extraction and those able to rebind to the granules were examined by SDS-PAGE. Most proteins were efficiently eluted from the granules by NaCl. Proteins released exhibited an SDS-PAGE protein profile identical to those of proteins on untreated glycogen granules (not shown). SDS-PAGE protein profiles of cellular proteins, the glycogen granule-associated proteins, and proteins released from the granules by NaCl which are able to bind to SDS-purified *F. succinogenes* glycogen are shown in Fig. 7. Of the five major glycogen granule-associated proteins, four were able to rebind to the glycogen (Fig. 7B). In the assay of binding to cellulose, 17.5% of added activity was bound to cellulose after washing (Table 5). The specific activities of cellobiase in the supernatant and on cellulose were 56.7 and 66.1%, respectively, of that of NaCl-released cellobiase prior to the binding assay. This indicates that the enzyme was partially inactivated during the binding assay, and this is not surprising, since only bound cellobiase is highly resistant to inactivation during the extended time of incubation. Binding of the free form of cellobiase in a cell extract of glucose-grown cells to SDS-purified glycogen granules at a 60:1 (wt/wt) ratio of glycogen to protein was also examined. However, only 13% of the added activity remained bound after glycogen granules were washed in buffer, indicating that the free form of cellobiase may have lost much of its binding capacity.

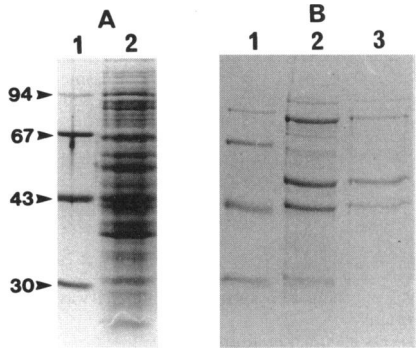


FIG. 7. SDS-PAGE protein profiles of S85 glucose-grown cells, glycogen granule-associated proteins, and the proteins rebound to the granules. (A) Lanes: 1, low-molecular-weight protein standards (Bio-Rad); 2, glucose-grown cells, 17 μg of protein (0.75-mm-thick SDS-polyacrylamide gel). (B) Lanes: 1, low-molecular-weight protein standards (Bio-Rad); 2, protein associated with glycogen granules prepared by method 2, 10.2 μg of protein; 3, proteins released from glycogen granules by NaCl and then rebound to glycogen prepared by method 3, 3.8 μg of protein (1.5-mm-thick SDS-polyacrylamide gel).

TABLE 5. Binding to SDS-purified *F. succinogenes* glycogen granules and Avicel crystalline cellulose of cellobiase released from glycogen granules by 1 M NaCl[a]

Polyglucose	Distribution of cellobiase after binding	
	Fraction	% Added activity in each fraction[b]
S85 glycogen[c]	Supernatant	0 (0)
	Glycogen	82.1 ± 0.4 (–)
	Wash	0 (0)
	Washed glycogen	81.4 ± 1.2 (3,615)
Avicel cellulose	Supernatant	38.5 ± 0.8 (2,282)
	Cellulose	21.7 ± 2.7 (–)
	Wash	0 (0)
	Washed cellulose	17.5 ± 1.0 (2,661)

[a] Cellobiase released by NaCl from *F. succinogenes* glycogen prepared by method 1 had a specific activity of 4,023 nmol of glucose min^{-1} mg of $protein^{-1}$.
[b] Expressed as means ± standard deviations. Values in parentheses are specific activities expressed as nanomoles of glucose $minute^{-1}$ milligram of $protein^{-1}$. –, not determined.
[c] Purified glycogen was prepared by method 3.

Release of cellobiase from cell envelopes of cellulose-grown cells. Glycogen granules could not be isolated from the cell envelope preparation of cellulose-grown cells by using the SDS extraction procedure (method 3) successfully applied to the isolation of glycogen granules from cells grown on glucose. Cellobiase was eluted from cell envelopes by NaCl and KCl but not by CHAPS and Triton X-100 (Table 6), although 73% of membrane proteins were released into the supernatant by CHAPS. Digestion with α-amylase released 33.1% of the cellobiase activity from the cell envelope preparation, and this was coincident with release of 36.6% of the carbohydrate into

TABLE 6. Elution of cellobiase from the cell envelope fraction of *F. succinogenes* S85 grown on Avicel crystalline cellulose[a]

Treatment[b]	Final concn	Enzyme released[c] (%)	Recovery (%)
α-Amylase	12,500 U/ml	33.1 ± 2.3	90.5
CHAPS	0.5% (wt/vol)	3.3 ± 2.7	114.4
Triton X-100	1.0% (vol/vol)	3.4 ± 2.1	129.5
NaCl	1.0 M	76.3 ± 3.6	114.9
KCl	1.0 M	80.9 ± 3.2	119.3

[a] Specific activity of cellobiase prior to the treatments was 440 nmol of glucose min^{-1} mg of protein $^{-1}$.
[b] DTT (2 mM) was included in all treatments.
[c] Expressed as means ± standard deviations.

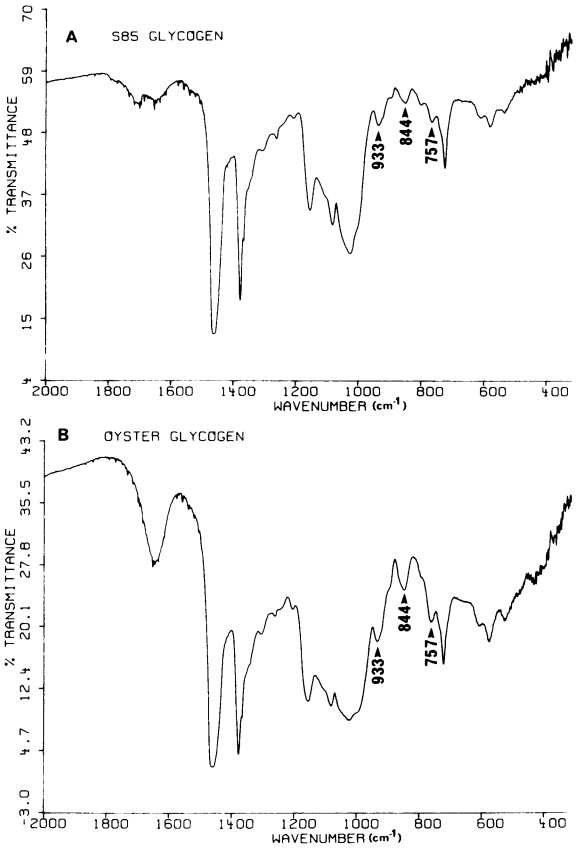
the supernatant. These data imply that cellobiase is bound to glycogen which is associated with the cell envelopes.

Nature of purified glycogen granules. Glycogen isolated from ultrasonically disrupted early-stationary-phase cells of *F. succinogenes* S85 grown on glucose by treatment with SDS (method 3) accounted for 13% of the dry weight of cells. Neither DNA nor proteins were detected in these preparations by scanning the UV $A_{240-300}$. Nor was protein detected by the Bradford assay technique (3). However, when the SDS-purified granules were hydrolyzed in 6 N HCl and analyzed for amino acids, a broad range was detected, but the total amount of the amino acids accounted for only 0.13% (wt/wt) of glycogen. Energy-dispersive X-ray analysis of the glycogen granules prepared without SDS treatment (method 2) exhibited no obvious energy-dispersive X-ray spectral peaks for metals. Phosphorus analyzed separately in the SDS-purified glycogen was less than 0.12 nmol/mg (dry weight) of granules, indicating that the phosphate content of the granules was very low.

Analysis of glycogen isolated from *F. succinogenes* S85 with SDS (method 3) or without SDS extraction (method 1) after acid hydrolysis in either 2 or 4 N HCl by HPLC with either the Aminex HPX-42A column or the Dionex CarboPac PA1 anion-exchange column showed that D-glucose was the only sugar present, indicating that glycogen of *F. succinogenes* S85 was a homopolymer of glucose. The *Fibrobacter* glycogen granules exhibited an infrared spectrum very similar to that of oyster glycogen with diagnostic absorbance peaks at 757, 845, and 933 cm^{-1} (Fig. 8A and B). Absorbance peaks at 758 ± 2 and 930 ± 4 cm^{-1} are indicative of α-(1,4)-linked polyglucosans, while absorbance at 844 cm^{-1} is characteristic of α-glucosidic linkages (2). Examination of *Fibrobacter* glycogen by transmission electron microscopy with negative staining showed that it was present in the form of granules which were present mostly in pairs (Fig. 8C). The granules had a uniform shape and a size ranging from 32 to 45 nm in diameter.

DISCUSSION

In this study the OM from glucose- and cellulose-grown cells of *F. succinogenes* was isolated by using a NaCl and sucrose wash procedure previously developed for the isolation of cell envelope fractions from the marine bacterium *Alteromonas haloplanktis* (19). Successful application of the method to *F. succinogenes* cells is based on the observation that the bacterium has a comparatively high salt requirement for optimal growth (4), a characteristic in common with marine bacteria (40). The release of membrane fragments into the wash solutions was coincident with the loss of OM from *F. succinogenes* cells. The membrane fragments had a density similar to



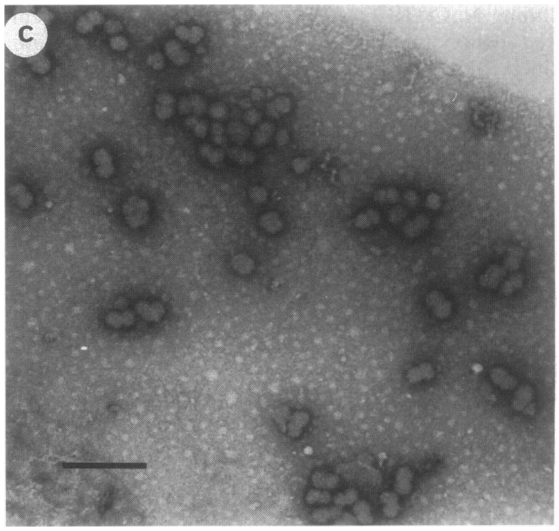


FIG. 8. Infrared spectra of glycogen prepared by method 3 from *F. succinogenes* (A) and from oysters (B). (C) Morphology of glycogen granules from *F. succinogenes* S85 prepared by method 3 and negatively stained with 1% (wt/vol) ammonium molybdate (pH 7). Bar, 0.2 μm.

that of the OM from *Salmonella typhimurium* (52) and a low SDH activity. Membranes with similar properties were isolated both from cellulose-grown cells and from the extracellular culture fluid of these cells. The SDS-PAGE profile of separated proteins from the OM of glucose-grown cells corresponded closely to that of proteins from both OM of cellulose-grown cells and extracellular membrane vesicles and was different from that of the CM. These data demonstrate that the OM has been separated from the CM of *F. succinogenes*. Furthermore, the data confirm the OM origin of the extracellular membrane vesicles from cellulose-grown cells.

The highest activities of endoglucanase, xylanase, and acetylxylanesterase were in the OM, although the greater proportion of these enzymes was in the CM and cytoplasm. Monitoring of the fractionation of the cell envelopes by electron microscopy showed that a small portion of the OM remained on the cells after the washing procedure, and this was also suggested by detection of the two OM proteins of approx 94 kDa in the CM preparation. Thus, the high concentration of the glucanases in the CM could be at least partially due to OM contamination. The reason that a large proportion of the glucanases are present in the cytoplasm is unclear. It is known that some proteins are posttranslationally exported (54), and this may account for the large proportion of the cytoplasmic fibrolytic enzymes. However, they may also be loosely bound and released from membranes by the French press treatment used to disrupt the cells. Glucanases usually perform their hydrolytic function either on the cell surface or extracellularly. In this regard, we speculate that the glucanases are exported posttranslationally in *F. succinogenes*, and the cytoplasm serves as a reservoir of the glucanases for secretion.

The concentration of the 118-kDa EG3 was substantially increased in the OM. This is consistent with its hydrolytic role in cellulose digestion. The 118-kDa EG2 does not have high affinity for the OM, as demonstrated by its release from cells by NaCl. However, the membrane vesicles from the extracellular culture fluid lacked EG2 even though they were not treated with NaCl, indicating that the enzyme was never tightly associated with this membrane or with the OM. Previous immunoelectron microscopy showed that EG2 was associated with protrusions or disturbances of the OM at a few localized sites on the cell surface (48). Since EG2 was present in the nonsedimentable fraction of the extracellular culture fluid, this suggests that the released EG2 is probably associated with very small fragments that are no longer membranous in nature, or at least not sedimentable. The suggestion by McGavin et al. (48) that release of the enzyme from cells was triggered by contact with cellulose is still plausible. Since the enzyme has both catalytic and binding domains (47), it is very important to know whether one or both of these sites are exposed at the surface to reveal whether the cell-associated enzyme has a role in adhesion as well as cellulose hydrolysis.

The chloride-stimulated cellobiosidase was mainly found in membrane vesicles and the nonsedimentable fraction of the extracellular culture fluid from cellulose-grown cultures, but little was found in the OM fraction from cells, which suggests a preferential association with membrane fragments released. A patchy distribution of chloride-stimulated cellobiosidase and/or the antigenic related components on membranous, protuberant structures of the outer surface was observed by Huang and Forsberg (30) by immunogold labelling of cells with polyclonal antibodies against the enzyme. In addition, production of vesicles by membrane blebbing during growth has also been suggested by Forsberg et al. (17) on the basis of the observation by electron microscopy. Thus, it appears that the chloride-stimulated cellobiosidase binds to less stable areas of the OM that form membranous protrusions which tend to bleb off from cells to produce the membrane vesicles during growth. The absence of chloride stimulation of cellobiosidase activity in glucose-grown cells supports the contention that the enzyme is induced by growth on cellulose.

Gaudet and Gaillard (23) and Stewart et al. (56) reported production of glycogen by both glucose- and cellulose-grown cells of *F. succinogenes*. Our observation that most cellobiase was bound to glycogen granules in glucose-grown cells was unexpected, since only those enzymes involved in either synthesis or hydrolysis of the glucan had previously been reported to be bound to glycogen granules (53). In addition, we found that several other proteins efficiently bound to the glycogen granules as well, and the function of these proteins is unclear at the present. The granules were not digested by the bound cellobiase, and the enzyme was not displaced by the substrate cellobiose or analogs of glycogen, maltose, and maltotriose. This excludes the expected substrate-enzyme interaction. The inability of detergents to elute cellobiase from the glycogen granules indicates the lack of hydrophobic interactions between them. The release of cellobiase from glycogen by high salt is suggestive of a polar effect on the substrate enzyme interaction (57) rather than an ionic interaction, since glycogen is uncharged. The site of interaction of cellobiase with glycogen does not appear to be the catalytic site, because the activity of the cellobiase bound to glycogen was similar to that of the released enzyme. In this respect the cellobiase-glycogen association is similar to the interaction of the *Trichoderma reesei* β-glucosidase with polysaccharides in the fungus cell wall, which was to function as an "anchor glycan" (50). The fact that the bound cellobiase was stable in the presence of air, whereas the unbound enzyme required the presence of a reducing agent, a characteristic previously noted by Forsberg and Groleau (20), demonstrates a unique functional aspect of binding which involves protection of a reduced sulfhydryl group. The enzyme may have a unique glycogen-binding domain reminiscent of a glycogen storage site in glycogen phosphorylase (55) or a starch binding domain (58) separated from the catalytic domain. Substrate binding domains are a common feature of glucanases (24), although in β-glucosidases, with the exception of a recent report by Gräbnitz et al. (25), these domains have not been recognized. Binding of cellobiase to cellulose was much lower than to glycogen granules. Whether the low binding is due to nonspecific binding is unclear, since some cellular proteins of *F. succinogenes* S85 and BSA also bind to cellulose (unpublished data).

In cellulose-grown cells in which glycogen could not be separated from membranes by differential centrifugation, 67.2% of the cellobiase was present in the CM fraction. Gaudet and Gaillard (23) demonstrated by electron microscopy that cellulose-grown cells of *F. succinogenes* contained glycogen granules. However, we failed to isolate the granules from cellulose-grown cells by SDS extraction. Digestion of the cell envelope preparation with α-amylase released cellobiase with corresponding loss of carbohydrate, indicating the presence of glycogen in cellulose-grown cells. Furthermore, the fact that nondenaturing detergents are unable to elute cellobiase from the cell envelope even though CHAPS solubilized at least 73% of the membrane protein, while salts did release enzyme, supports the contention that the membrane-associated cellobiase of cellulose-grown cells is really bound to glycogen present in the membrane preparation. The form of glycogen in cellulose-grown cells is unknown, but since it could not be isolated by either differential centrifugation or SDS extraction, it is possible that the glycogen exists in a nongranule form, as previously observed with mouse liver tissue (12), and

that it is readily solubilized by SDS extraction. On reexamination of published work by König et al. (36), Dawes (11) noted the attachment of glycogen to the cell envelope in lysed cells of *Methanococcus vannieli*. It is unclear whether glycogen in the cell envelope preparation of cellulose-grown cells of *F. succinogenes* is mixed with, or indeed is attached to, the CM. In glucose-grown cells, only 12.4% of the cellobiase was associated with the CM fraction, which suggests that the interaction of the glycogen granules with the CM was minimal in these cells. Glycogen usually accumulates when growth is limited by the supply of utilizable nitrogen in the presence of excess exogenous carbon (11). However, exceptions are known, as exemplified by the glycogen synthesis which occurs during carbon-limited continuous culture of *Streptococcus sanguis* (34). In this regard, synthesis of glycogen in the cellulose-grown cells of *F. succinogenes* does not appear to be unique.

Recently, Gaudet et al. (22) reported that synthesis and turnover of glycogen occurred simultaneously in *F. succinogenes* S85 when cells were grown with glucose as the carbon source, implying a continuous involvement of glycogen in the carbon metabolism. In other bacteria, such as *Methylococcus* NCIB 11083, stored glycogen has been reported to serve as a carbon and energy source to maintain viability during carbon starvation (42, 53). Cheng et al. (7) have described a similar phenomenon in rumen bacteria in which a large proportion of the bacterial population was packed with cytoplasmic inclusions containing polysaccharide when cows were fed with a high-energy diet of fine-size particles. However, the population possessing the polysaccharide inclusions was lower in cows fed with a diet of coarse-size particles. This suggests that the glycogen granules are a storage product accumulated during growth in an energy-rich environment and are used under energy-poor conditions. In this regard, it will be of value to study the nature of the dynamic changes in glycogen content and the corresponding regulation of enzymes involved in glycogen metabolism of ruminal microorganisms grown in energy-poor environments, such as cellulose or high-fiber forage, and during transitions to high-energy environments. The ability of *F. succinogenes* S85 to synthesize glycogen may serve as a stabilizing influence for the bacterium in the rumen by providing an alternate source of carbon and energy between meals by the host animal. How the glycogen has assumed a secondary role in the binding of cellobiase, and why this binding occurs, present new phenomena not yet recognized for other organisms. Insight into this unique relationship and the physiological significance of the binding will be provided once the fine structure of the cellobiase has been determined and site-directed mutations in the gene have been created to produce cells with a cellobiase lacking glycogen-binding activity.

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REFERENCES

1. **Ames, B. N., and D. T. Dubin.** 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. **235:**769–775.
2. **Barker, S. A., E. J. Bourne, and D. H. Whiffen.** 1956. Use of infra-red analysis in the determination of carbohydrate structure. Methods Biochem. Anal. **3:**213–245.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Bryant, M. P., I. M. Robinson, and H. Chu.** 1959. Observations on the nutrition of *Bacteroides succinogenes*. J. Dairy Sci. **42:**1831–1847.
5. **Buchanan, C. J., and W. J. Mitchell.** 1992. Two β-glucosidase activities in *Fibrobacter succinogenes* S85. J. Appl. Bacteriol. **73:**243–250.
6. **Chart, H., and B. Rowe.** 1992. A simple procedure for the preparation of lipopolysaccharide for the production of antisera. Lett. Appl. Microbiol. **14:**263–265.
7. **Cheng, K.-J., R. Hironaka, D. W. A. Roberts, and J. W. Costerton.** 1973. Cytoplasmic glycogen inclusions in cells of anaerobic gram-negative rumen bacteria. Can. J. Microbiol. **19:**1501–1506.
8. **Clarke, A. J.** 1990. Chemical modification of β-glucosidase from *Schizophyllum commune*: evidence for essential carboxyl groups. Biochim. Biophys. Acta **1040:**145–152.
9. **Clarke, A. J., V. Sarabia, W. Keenleyside, P. R. MacLachlan, and C. Whitfield.** 1991. The compositional analysis of bacterial polysaccharides by high-performance anion-exchange chromatography. Anal. Biochem. **199:**68–74.
10. **Darveau, R. P., and R. E. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella* strains. J. Bacteriol. **155:**831–838.
11. **Dawes, E. A.** 1991. Storage polymers in prokaryotes, p. 81–122. *In* S. Mohan, C. Dow, and J. A. Coles (ed.), Prokaryotic structure and fashion. Cambridge University Press, Cambridge.
12. **de Bruijn, W. C.** 1973. Glycogen, its chemistry and morphologic appearance in the electron microscope. Ultrastruct. Res. **42:**29–50.
13. **deDuve, C., J. Berthet, and H. Beaufay.** 1959. Gradient centrifugation of cell particles, theory and applications. Prog. Biophys. Biophys. Chem. **9:**325–369.
14. **Dickie, P., and J. H. Weiner.** 1979. Purification and characterization of membrane-bound fumarate reductase from anaerobically grown *Escherichia coli*. Can. J. Biochem. **57:**813–821.
15. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
16. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1977. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115:**717–722.
17. **Forsberg, C. W., T. J. Beveridge, and A. Hellstrom.** 1981. Cellulase and xylanase release from *Bacteroides succinogenes* and its importance in the rumen environment. Appl. Environ. Microbiol. **42:**886–896.
18. **Forsberg, C. W., and K.-J. Cheng.** 1990. Integration of rumen microorganisms and their hydrolytic enzymes during the digestion of lignocellulosic materials, p. 411–423. *In* D. E. Akin, L. G. Ljungdahl, J. R. Wilson, and P. J. Harris (ed.), Microbial and plant opportunities to improve lignocellulose utilization by ruminants. Elsevier, New York.
19. **Forsberg, C. W., J. W. Costerton, and R. A. Macleod.** 1970. Separation and localization of cell wall layers of a gram-negative bacterium. J. Bacteriol. **104:**1338–1353.
20. **Forsberg, C. W., and D. Groleau.** 1982. Stability of the endo-β-1,4-glucanase and β-1,4-glucosidase from *Bacteroides succinogenes*. Can. J. Microbiol. **28:**144–148.
21. **Forsberg, C. W., and K. Lam.** 1977. Use of adenosine-5′-triphosphate as an indicator of the microbiota biomass in rumen contents. Appl. Environ. Microbiol. **33:**528–537.
22. **Gaudet, G., E. Forano, G. Dauphin, and A.-M. Delort.** 1992. Futile cycling of glycogen in *Fibrobacter succinogenes* as shown by *in situ* ^{1}H-NMR and ^{13}C-NMR investigation. Eur. J. Biochem. **207:**155–162.
23. **Gaudet, G., and B. Gaillard.** 1987. Vesicle formation and cellulose degradation in *Bacteroides succinogenes* cultures: ultrastructural aspects. Arch. Microbiol. **148:**150–154.

24. **Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren.** 1991. Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. Microbiol. Rev. **55:**303–315.
25. **Gräbnitz, F., M. Seiss, K. P. Rücknagel, and W. L. Staudenbauer.** 1991. Structure of the β-glucosidase gene *bglA* of *Clostridium thermocellum*: sequence analysis reveals a superfamily of cellulases and β-glycosidases including human lactase/phlorizin hydrolase. Eur. J. Biochem. **200:**301–309.
26. **Groleau, D., and C. W. Forsberg.** 1981. Cellulolytic activity of the rumen bacterium *Bacteroides succinogenes*. Can. J. Microbiol. **27:**517–530.
27. **Harvey, M., C. W. Forsberg, T. J. Beveridge, J. Pos, and J. R. Ogilvie.** 1984. Methanogenic activity and structural characteristics of the microbial biofilm on a needle-punched polyester support. Appl. Environ. Microbiol. **48:**633–638.
28. **Huang, L., and C. W. Forsberg.** 1987. Isolation of a cellodextrinase from *Bacteroides succinogenes*. Appl. Environ. Microbiol. **53:**1034–1041.
29. **Huang, L., and C. W. Forsberg.** 1988. Purification and comparison of the periplasmic and extracellular forms of the cellodextrinase from *Bacteroides succinogenes*. Appl. Environ. Microbiol. **54:**1488–1493.
30. **Huang, L., and C. W. Forsberg.** 1990. Cellulose digestion and cellulase regulation and distribution in *Fibrobacter succinogenes* subsp. *succinogenes* S85. Appl. Environ. Microbiol. **56:**1221–1228.
31. **Huang, L., C. W. Forsberg, and D. Y. Thomas.** 1988. Purification and characterization of a chloride-stimulated cellobiosidase from *Bacteroides succinogenes* S85. J. Bacteriol. **170:**2923–2932.
32. **Kamio, Y., and H. Takahashi.** 1980. Isolation and characterization of outer and inner membranes of *Selenomonas ruminantium*: lipid compositions. J. Bacteriol. **141:**888–898.
33. **Karkhanis, Y. D., J. Y. Zeltner, J. Jackson, and D. J. Carlo.** 1978. A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram-negative bacteria. Anal. Biochem. **85:**595–601.
34. **Keevil, C. W., P. D. Marsh, and D. C. Ellwood.** 1984. Regulation of glucose metabolism in oral streptococci through independent pathways of glucose 6-phosphate and glucose 1-phosphate formation. J. Bacteriol. **157:**560–567.
35. **Kohring, S., J. Wiegel, and F. Mayer.** 1990. Subunit composition and glycosidic activities of the cellulase complex from *Clostridium thermocellum* JW 20. Appl. Environ. Microbiol. **56:**3798–3804.
36. **König, H., E. Nusser, and K. O. Stetter.** 1985. Glycogen in *Methanolobus* and *Methanococcus*. FEMS Microbiol. Lett. **28:**265–269.
37. **Kornberg, A.** 1955. Lactic dehydrogenase of muscle. Methods Enzymol. **1:**441–443.
38. **Kotarski, S., and A. A. Salyers.** 1984. Isolation and characterization of outer membranes *Bacteroides thetaiotaomicron* grown on different carbohydrates. J. Bacteriol. **158:**102–109.
39. **Kudo, H., K.-J. Cheng, and J. W. Costerton.** 1987. Electron microscopic study of the methylcellulose-mediated detachment of cellulolytic rumen bacteria from cellulose fibers. Can. J. Microbiol. **33:**267–272.
40. **Laddaga, R. A., and R. A. Macleod.** 1982. Effects of wash treatments on the ultrastructure and lysozyme penetrability of the outer membrane of various marine and two terrestrial gram-negative bacteria. Can. J. Microbiol. **28:**318–324.
41. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
42. **Linton, J. D., and R. E. Cripps.** 1978. The occurrence and identification of intracellular polyglucose storage granules in *Methylococcus* NCIB 11083 grown in chemostat culture on methane. Arch. Microbiol. **117:**41–48.
43. **MacKenzie, S. L.** 1987. Gas chromatographic analysis of amino acids as the N-heptafluorobutyryl isobutyl esters. J. Assoc. Off. Anal. Chem. **70:**151–160.
44. **Malburg, L. M., Jr., J. M. Tamblyn Lee, and C. W. Forsberg.** 1992. Degradation of cellulose and hemicelluloses by rumen microorganisms, p. 127–159. *In* G. Winkleman (ed.), Microbial degradation of cellulose and hemicellulose by rumen microorganisms. VCH Verlagsgesellschaft mbH, Weinheim, Germany.
45. **McDermid, K. P., C. R. MacKenzie, and C. W. Forsberg.** 1990. Esterase activities of *Fibrobacter succinogenes* S85. Appl. Environ. Microbiol. **56:**127–132.
46. **McGavin, M., and C. W. Forsberg.** 1988. Isolation and characterization of endoglucanase 1 and 2 from *Bacteroides succinogenes* S85. J. Bacteriol. **170:**2914–2922.
47. **McGavin, M., and C. W. Forsberg.** 1989. Catalytic and substrate-binding domains of endoglucanase 2 from *Bacteroides succinogenes*. J. Bacteriol. **171:**3310–3315.
48. **McGavin, M., J. Lam, and C. W. Forsberg.** 1990. Regulation and distribution of *Fibrobacter succinogenes* subsp. *succinogenes* S85 endoglucanases. Appl. Environ. Microbiol. **56:**1235–1244.
49. **McGavin, M. J., C. W. Forsberg, B. Crosby, A. W. Bell, D. Dignard, and D. Y. Thomas.** 1989. Structure of the *cel-3* gene from *Fibrobacter succinogenes* S85 and characteristics of the encoded gene product, endoglucanase 3. J. Bacteriol. **171:**5587–5595.
50. **Messner, R., K. Hagspiel, and C. P. Kubicek.** 1990. Isolation of a β-glucosidase binding and activating polysaccharide from cell walls of *Trichoderma reesei*. Arch. Microbiol. **154:**150–155.
51. **Montgomery, L., L. B. Flesher, and D. Stahl.** 1988. Transfer of *Bacteroides succinogenes* (Hungate) to *Fibrobacter* gen. nov. as *Fibrobacter succinogenes*. J. Bacteriol. **171:**3310–3315.
52. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. J. Biol. Chem. **247:**3962–3972.
53. **Preiss, J.** 1984. Bacterial glycogen synthesis and its regulation. Annu. Rev. Microbiol. **38:**419–548.
54. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
55. **Quiocho, F. A.** 1986. Carbohydrate-binding proteins: tertiary structures and protein-sugar interactions. Annu. Rev. Biochem. **55:**287–315.
56. **Stewart, C. S., C. Paniagua, D. Dinsdale, K. J. Cheng, and S. H. Garrow.** 1981. Selective isolation and characteristics of *Bacteroides succinogenes* from the rumen of a cow. Appl. Environ. Microbiol. **41:**504–510.
57. **Suelter, C. H.** 1985. A practical guide to enzymology, p. 4–6. John Wiley & Sons, Inc., New York.
58. **Svensson, B., H. Jespersen, M. R. Sierks, and E. A. MacGregor.** 1989. Sequence homology between putative raw-starch binding domains from different starch-degrading enzymes. Biochem. J. **264:**309–311.
59. **Towbin, M., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
60. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
61. **Zamze, S. E., M. A. J. Ferguson, E. R. Moxon, R. A. Dwek, and T. W. Rademacher.** 1987. Identification of a phosphorylated 3-deoxy-manno-octulosonic acid as a component of *Haemophilus influenzae* lipopolysaccharide. Biochem. J. **245:**583–587.