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Cellulase and Xylanase Release from *Bacteroides succinogenes* and Its Importance in the Rumen Environment

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During growth of *Bacteroides succinogenes* in a liquid medium with cellulose as the source of carbohydrate, greater than 80% of the carboxymethylcellulase (endo-β-1,4-glucanase), xylanase, and aryl-β-xylosidase and 50% of the aryl-β-glucosidase released from cells into the culture fluid. Less than 25% of the cellobiase activity was detected in the culture fluid. Approximately 50% of each of the released enzymes measured was associated with sedimentable subcellular membrane vesicles. The vesicles appeared to be released from the outer membrane of intact cells by bleb formation, primarily in pockets between the cells and the cellulose, although a few unattached cells with blebs were seen. Many vesicles were seen adhering to cellulose, and they were also seen free in the culture fluid. These data suggest that *B. succinogenes* releases hydrolytic enzymes in nonsedimentable and particulate forms during growth by a mechanism which has until now received little attention. Cellulose incubated in a porous nylon bag in the rumen was colonized by bacteria resembling *B. succinogenes*, and subcellular vesicles were seen penetrating channels and fractures in the cellulose. On this basis, it is suggested that *B. succinogenes* cells in the rumen contribute to an extracellular population of subcellular vesicles that possess cellulolytic and hemicellulolytic activities which probably enhance polymer digestion and provide a source of sugars for microbes lacking polymer-degrading activity, thereby contributing to a stable heterogeneous microbial population.

Forages are readily degraded in the bovine and ovine rumens by cellulolytic bacteria and, to a lesser extent, by protozoa (5, 33) and anaerobic phycomycetous fungi (29). The major cellulolytic rumen bacteria include *Ruminococcus albus, Ruminococcus flavefaciens*, and *Bacteroides succinogenes* (22). Of several bacteria tested, *B. succinogenes* hydrolyzes undegraded cotton fibers and cellulose powder most effectively (19), its activity being similar to that of a mixture of rumen microorganisms (19, 23). *B. succinogenes* also digests significantly more cellulose from intact forages and causes greater weight loss from barley straw than do other cellulolytic rumen bacteria (13, 41). Examination of mixed cultures of rumen bacteria on straw revealed that *B. succinogenes*-like bacteria are more numerous than ruminococci (41). These data demonstrate that *B. succinogenes* is an important cellulolytic bacterium within tbe rumen; indeed, it is the major cellulolytic bacterium in the rumen of cattle fed monensin (W. J. Brulla and M. P. Bryant, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I121, p. 104). The recent development of a reproducible technique for the isolation of *B. succinogenes* will aid in the study of new isolates of this bacterium (42).

Cellulose hydrolysis by *B. succinogenes* has been studied by Groleau and Forsberg (18), who identified an endo-β-1,4-glucanase (carboxymethylcellulase) and a β-1,4-glucosidase-like (cellobiase) enzyme in cultures of this bacterium. The endoglucanase was partially released from cells in both a nonsedimentable form and a sedimentable form (100,000 × *g*), which was membranous. The membrane fragments were of particular interest, since they resemble membranous forms seen in close association with fibers when mixed cultures of rumen bacteria are grown on straw or cellulose (41).

Forages contain a variety of polymers including cellulose, hemicellulose, and pectin (12); consequently, a complex of enzymes is necessary for extensive polymer hydrolysis. Therefore, in this investigation, the release and distribution of cellulase and xylanase enzymes in cultures of *B. succinogenes* growing on cellulose was examined. A mechanism of vesicle release from cells

is described. Cellulose filter paper incubated in a nylon bag in the rumen was examined for colonization by the rumen microflora.

MATERIALS AND METHODS

Bacterium, medium, and cultural conditions. The source of *B. succinogenes* S85, the medium, and the cultural conditions have been described by Groleau and Forsberg (18). The medium used was that described by Scott and Dehority (38), except that ammonium sulfate served as the sole source of nitrogen, and ground Whatman No. 1 filter paper at a concentration of 0.2% (wt/vol) was the source of carbohydrate. Optical density measurements on cultures and cellulose disappearance were determined as described by Groleau and Forsberg (18).

Disruption of cells and concentration of extracellular enzymes. The cells were harvested and washed in 0.05 M sodium phosphate buffer (pH 6.8), made 1 mM with dithiothreitol, under a nitrogen atmosphere. The cells were disrupted by ultrasonication in the same buffer system while being gassed with nitrogen. These procedures and the concentration of extracellular enzymes from the culture fluid were described by Groleau and Forsberg (18). Anaerobic conditions were used throughout, since it has been observed that a loss in cellobiase activity can occur in the absence of either nitrogen or dithiothreitol if incubation takes place at 39°C (C. W. Forsberg and D. Groleau, Can. J. Microbiol., in press).

Enzyme assays. All assays were conducted in nitrogen-flushed tubes by using reagents saturated with nitrogen. The assay systems for endoglucanase, xylanase, and pebble-milled cellulose hydrolysis consisted of a 1-ml final volume containing 1% (wt/vol) of substrate in 0.05 M sodium phosphate buffer, pH 6.8, made 1.0 mM with dithiothreitol. The carboxymethylcellulose used was the sodium salt C-8758 (low viscosity; degree of polymerization, ca. 400; degree of substitution, ca. 0.7) from Sigma Chemical Co. Xylan prepared from larch wood was also obtained from Sigma. The pebble-milled cellulose was prepared from shredded Whatman no. 1 filter paper by making a 3% suspension in water and grinding it with flint pebbles in a roller mill (24 rpm) for 40 h.

The enzyme reactions were started by the addition of enzyme and were incubated at 39°C for 30 min, with the exception of the pebble-milled cellulose hydrolysis reactions, which were incubated for 2 h. The reactions were stopped by heating in a boiling water bath for 10 min. The extent of hydrolysis was measured by determining the production of reducing sugar, using the Nelson-Somogi reagent (1). To correct for endogenous production of reducing power in the enzyme preparation, the same amounts of enzyme and substrate as were used in the assay were incubated separately for the duration of the assay, inactivated by boiling, and mixed before the determination of reducing sugar.

Cellobiase activity was determined as described by Groleau and Forsberg (18), except that the cellobiose was at a concentration of 1% (wt/vol) in the assay mixture, and the assays were conducted anaerobically in 0.05 M sodium phosphate buffer, pH 6.8, containing 1.0 mM dithiothreitol.

The aryl-β-glucosidase and aryl-β-xylosidase activities were assayed under a nitrogen atmosphere in 0.05 M sodium phosphate buffer, pH 6.8, containing 1.0 mM dithiothreitol. The substrates were *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside (Sigma), respectively, at 5 mM.

For all enzyme assays, 1 U of enzyme activity was defined as the amount of enzyme producing 1 nmol of product per min. The specific activity was expressed as the units of enzyme activity per milligram of protein.

Protein was determined by using the method described by Lowry et al. (25), with bovine serum albumin as the standard.

Electron microscopy. Membrane fragments were separated from the concentrated extracellular enzyme by centrifugation at 100,000 × *g* for 2 h, resuspended in the same buffer, made 4% (vol/vol) with glutaraldehyde in 0.05 M sodium phosphate buffer containing 1.0 mM dithiothreitol, and incubated for 1 h. The treated membrane fragments were sedimented by centrifugation at 100,000 × *g* for 2 h and enrobed in 2% (wt/vol) Difco purified agar in 0.067 M cacodylate buffer, pH 6.8.

To embed cells growing on cellulose, the fluid (9 ml) was removed from the culture tube, and the sediment was suspended in 10 ml of a mineral solution corresponding to that in the medium, but containing 0.2% glutaraldehyde. The mixture was incubated for 5 min and centrifuged at 4,500 × *g* for 10 min (4°C), and the sediment was suspended in 4% glutaraldehyde in 0.067 M cacodylate buffer, pH 6.8, for 1 h. The sample was centrifuged again, and the sediment was enrobed in 2% agar.

All agar-enrobed samples were washed three times in cacodylate buffer and postfixed in 1% (wt/vol) osmium tetroxide in the same buffer for 2 h at 22°C. The samples were subsequently washed in water, stained with uranyl acetate, and embedded in Epon as described by Beveridge et al. (4). Thin sections were cut, transferred to grids, stained with 2% uranyl acetate and lead citrate (35), and examined in a Philips EM300 electron microscope equipped with a goniometer stage.

Incubation of cellulose in the rumen. Ground Whatman no. 1 filter paper cellulose was added in 3-g quantities to bags constructed with nylon cloth (B. and S. H. Thompson, Scarborough, Ontario, Canada) that had a pore diameter of 0.05 mm. The bags were suspended for a 17-h period in the rumen of a 2-year-old Hereford steer that received a ration of 4 kg of alfalfa hay, 2.0 kg of ground corn, and 0.5 kg of soybean meal per day. The bags were then removed, the liquid was expressed, and the solids were sampled for electron microscopy.

RESULTS

Release of enzymes from *B. succinogenes*. It was reported by Groleau and Forsberg (18) that endoglucanase activity in *B. succinogenes* was partially released into the culture fluid during growth on cellulose and a portion of the

released activity was associated with membrane fragments. Since *B. succinogenes* was reported to partially degrade xylan, and since this hydrolytic activity was present in the culture fluid (11), it was undertaken to confirm the presence of xylanase activity and to determine its distribution within the culture. The culture was also tested for pectin degrading activity; however, no hydrolytic activity was detected.

Samples of a partial digest of xylan, prepared by using an extracellular enzyme preparation from *B. succinogenes* grown on cellulose, were chromatographed on paper by using the solvent system *n*-butanol–pyridine–water (60:40:30, vol/vol) and stained for pentoses and reducing sugar (39). This revealed the presence of spots corresponding to a xylose standard, but none corresponding to a glucose standard. No spots were detected on chromatograms when xylan was treated in a similar manner but with the enzyme preparation deleted from the reaction mixture. More slowly migrating saccharides, with migration rates correlating well with the expected values for xylobiose, xylotriose, and xylotetraose, were also detected (15). Xylose is reported to be one of the end products of xylanase activity of rumen bacteria; however, this does not exclude the presence of xylobiase activity in *B. succinogenes* (14, 45).

The distribution of a number of hydrolytic enzymes between the cells and the cell-free culture fluid was determined at three sampling times during the growth of *B. succinogenes* with cellulose as the source of carbohydrate (Tables 1 and 2). The cellulose was not completely utilized until after at least 96 h of growth (Table 1). The distribution of protein between the cells and the cell-free culture fluid was similar at the three sampling times, with ca. 45% of the recoverable protein found in the cell-free culture fluid. Of the enzymes assayed (Table 2), the cellobiase was primarily cell associated, and the activity in the culture supernatant increased marginally at the later sampling times. The cellobiase was found to be an unstable enzyme (see above); thus its presence at a low concentration in the cell-free culture fluid does not necessarily infer that it is firmly bound to the cell. The aryl-β-glucosidase activity was lower than the cellobiase activity, but a greater proportion of its activity was in the culture fluid. By comparison with these enzymes, much more of the aryl-β-xylosidase, endoglucanase, xylanase, and cellulase activities were released into the culture fluid. Of this latter group, xylanase and cellulase partitioned into the culture fluid to the greatest extent. The specific activities of endoglucanase, xylanase, and cellulase were 5 to 10 times higher in the culture fluid than in extracts prepared from cells at the 96- and 168-h sampling times, indicating that their release was not simply a result of cell lysis. When the concentrated and dialyzed cell-free culture fluid was centrifuged at 100,000 × *g* for 2 h and the sedimentable and nonsedimentable fractions were assayed, ca. 50% each of aryl-β-glucosidase, aryl-β-xylosidase, endoglucanase, and xylanase activities were associated with the sedimentable materials (Table 3).

TABLE 1. *Characteristics of B. succinogenes grown with cellulose as the source of carbohydrate*

Incubation period (h)	Optical density[a]	% Cellulose (degraded)	Cellular protein (μg/ml)	Protein (μg/ml of cell-free supernatant)
48	0.23	13.5	63.4	46.7
72	0.28	—[b]	—	—
96	0.36	80.5	96.2	87.1
168	0.49	100.0	122.6	93.0

[a] Determined at 675 nm after the cellulose had settled by using matched test tubes (15 by 125 mm).
[b] —, Not determined.

Morphology of *B. succinogenes* growing on cellulose. When grown on cellulose, *B. succinogenes* adhered tightly to the cellulose particles, and some cells appeared to be pleomorphic, assuming the surface shape of the substrate, whereas others burrowed into the surface (18). Fibers were frequently seen linking the cells together and to the cellulose substrate (18, 24). As was observed in the present study, all cells that bound to cellulose, including those releasing vesicles, appeared to be intact and normal.

Figure 1 shows a pocket of vesicles characteristic of those frequently seen located between cells adhering to the cellulose and the cellulose fibers in the culture. Figure 2 is very suggestive of a bleb formed at the cell surface in the process of becoming a vesicle. Frequently vesicles were seen attached to the cellulose fibers (Fig. 3). The vesicles ranged from 0.05 to 0.1 μm in diameter, and the stained interiors of the vesicles indicated that they probably contained material. The suggestion that vesicles developed from blebs originating from the outer membrane is supported by the bleb shown in Fig. 4, which appeared to be an extension of the outer membrane of the cell, whereas the peptidoglycan layer and plasma membrane remained intact. The presence of blebs on cells free in the culture fluid was infrequent when compared with the pockets of vesicles produced by cells growing on cellulose, thus suggesting that the interaction of cells with cellulose predisposes them to vesicle release at the contact surface. The lack of blebs on free cells could perhaps also reflect the transient nature of blebs. After concentration of vesicles from the cell-free culture supernatant by ultrafiltration,

TABLE 2. *Distribution of hydrolytic enzyme activities between the cells and cell-free culture fluid of a B. succinogenes culture grown with cellulose as the source of carbohydrate*

Fraction	Sp act[a] at given incubation period (h):					
	48		96		168	
	Cells	Fluid	Cells	Fluid	Cells	Fluid
Cellobiase	27.0 (88)	5.0 (12)	33.7 (79)	10.0 (21)	27.8 (77)	11.0 (23)
Aryl-β-glucosidase	—[b]	—	6.2 (49)	3.6 (51)	3.3 (36)	8.2 (64)
Aryl-β-xylosidase	—	—	1.9 (12)	7.9 (88)	2.2 (18)	13.6 (82)
Endoglucanase	298.0 (39)	643.0 (61)	186.0 (18)	926.0 (82)	175.0 (20)	894.0 (80)
Xylanase	76.0 (18)	476.0 (82)	133.2 (13)	1,017.0 (87)	134.0 (11)	1,451.0 (89)
Cellulase[c]	2.4 (24)	10.3 (76)	3.0 (10)	31.1 (90)	5.1 (15)	35.7 (85)

[a] Expressed as nanomoles of product per minute per milligram of protein. Percent total enzyme activity is shown within parentheses.
[b] —, Not determined.
[c] From hydrolysis of pebble-milled cellulose.

TABLE 3. *Distribution of hydrolytic enzyme activities between sedimentable and nonsedimentable materials released into the culture fluid from B. succinogenes cells after 168 h of growth with cellulose as the source of carbohydrate*

Enzyme	Sp act[a]	
	Sedimentable fraction	Nonsedimentable fraction
Aryl-β-glucosidase	5.7 (49)	4.2 (51)
Aryl-β-xylosidase	7.4 (50)	27.4 (50)
Endoglucanase	1,000.0 (62)	450.0 (38)
Xylanase	1,798.0 (51)	1,256.0 (49)

[a] Expressed as nanomoles of product per minute per milligram of protein. Percent total enzyme activity is shown within parentheses. Fractionation at 100,000 × *g*.

followed by dialysis and sedimentation by ultracentrifugation, electron microscopy of the resuspended sediment revealed primarily small membrane sheets 0.1 to 0.5 μm in length which presumably represented membrane fragments from the vesicles (Fig. 5).

Old cultures (8 days) possessed few viable cells but contained high endoglucanase activity. Thin sections of these cultures revealed small cellulose particles which were coated only with membrane vesicles (Fig. 6 and 7). The surroundings revealed some lysed cells whose component parts were not adsorbed to the cellulose.

Microflora growing on cellulose incubated in the rumen. Ground filter paper cellulose placed in a nylon bag with a 0.05-mm pore size and suspended in the rumen of a Hereford steer for 17 h was colonized by a variety of morphological types of bacteria, but those attached to the cellulose fibers had a very uniform morphology. They had typical gram-negative cell envelopes with thin peptidoglycan layers characteristic of *B. succinogenes*. Some adhered very closely to the cellulose, whereas others appeared separated from the substrate by 0.1 to 0.2 μm, perhaps suggesting the presence of a nonstaining outer coating. Figure 8 illustrates penetration of the cells between the cellulose fibers and the large numbers of subcellular vesicular structures apparently released from the cells. Figure 9 shows an enlargement of the vesicles at one end of a cell.

DISCUSSION

It was observed in this and in other studies that *B. succinogenes* cells grown with cellulose as the source of carbohydrate adhere tenaciously to the substrate (18, 21, 24). This characteristic has led to the belief that adhesion is necessary for efficient cellulose hydrolysis (21, 24). It was perhaps unexpected that greater than 80% of the endoglucanase (carboxymethylcellulase), cellulase, xylanase, and aryl-β-xylosidase activities was released into the culture fluid from *B. succinogenes* cells growing on cellulose, even though the cells appeared to be intact. The released enzymes had comparatively low cellulolytic activity, a fact documented previously (18), and this activity did not increase when the extracellular enzymes were isolated from the culture after all of the cellulose had been digested. The large amount of endoglucanase released from cells during growth perhaps indicates that it is not the limiting enzyme in cellulose digestion.

Endoglucanases have been found extracellularly in a variety of strains of *Bacillus* (32), whereas organisms such as *Cytophaga* possess only cytoplasmic, periplasmic, or membrane-bound endoglucanases (6). Between these extremes there is the gram-positive bacterium *Clostridium thermocellum*, in which 95% of the cellulase activity is extracellular (16), and the gram-negative bacteria, including *Acetivibrio cellulolyticus* (36), *Pseudomonas fluorescens* subsp. *cellulosa* (43, 46), *Cellulomonas* sp. (2,

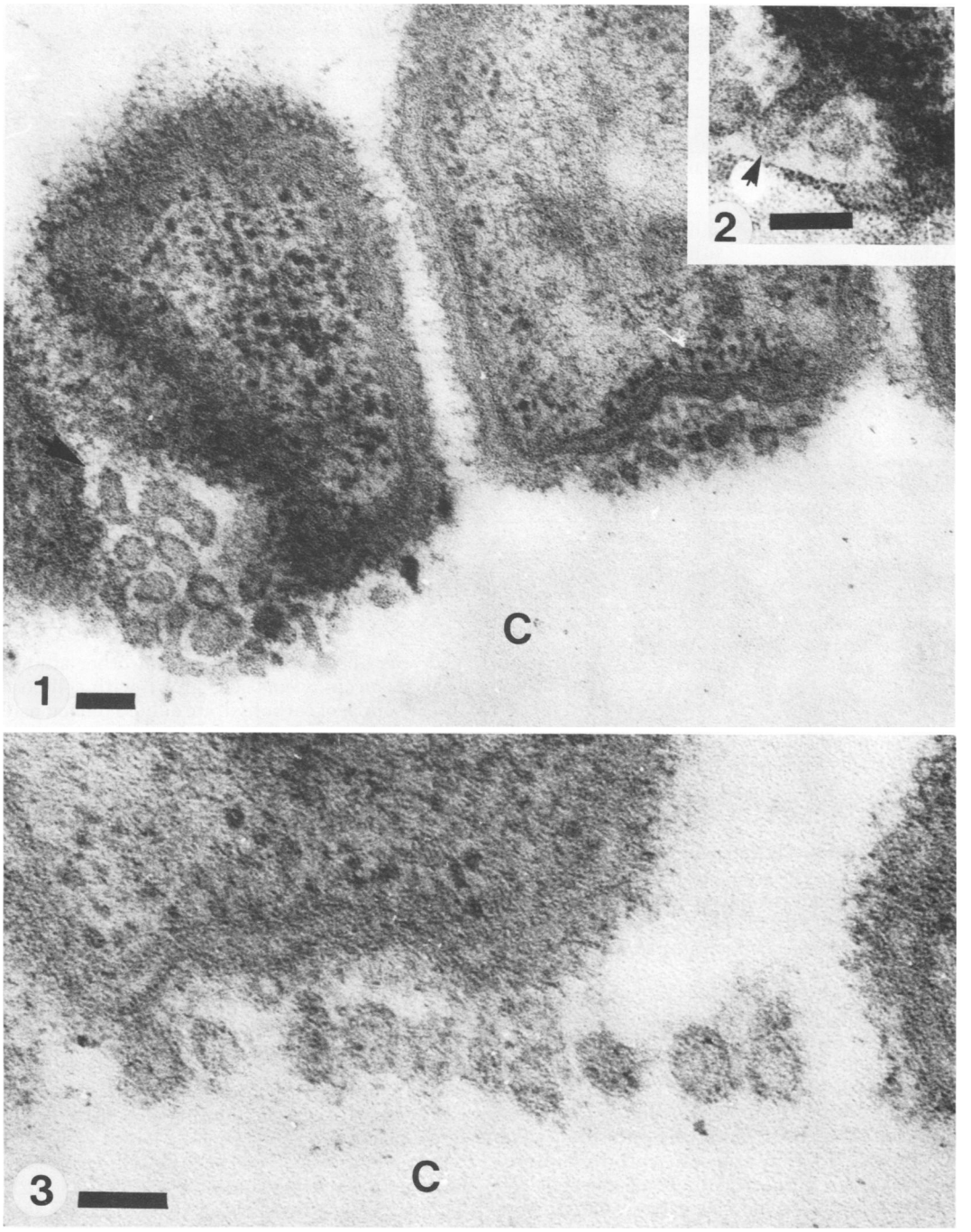
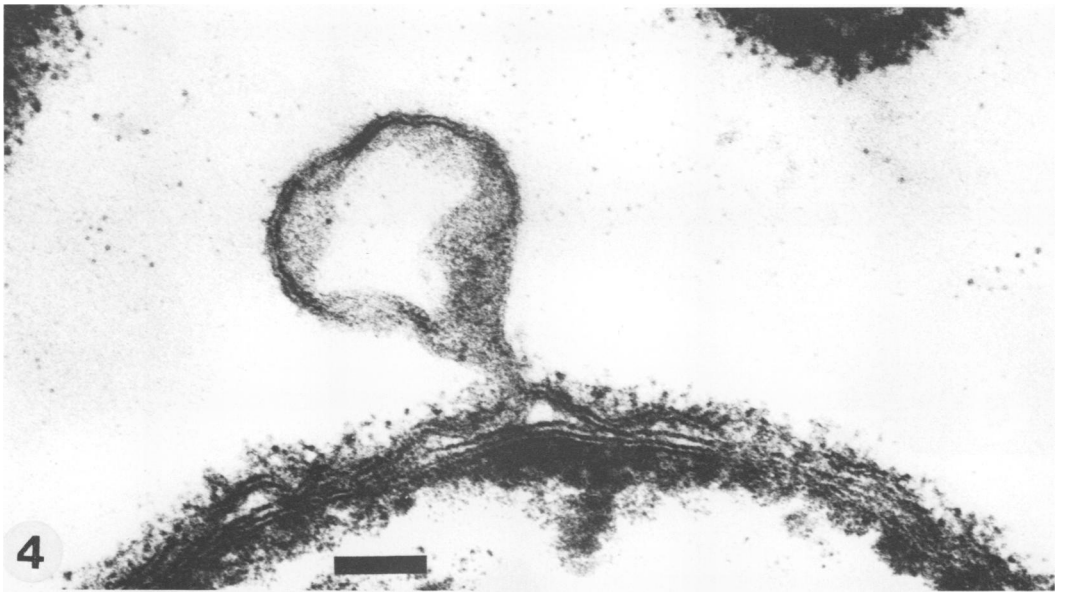


FIG. 1. *Membrane vesicles in a pocket between a cell and the cellulose (C) substrate (observed at 96 h). Bar, 0.1 μm.*

FIG. 2. *A membrane vesicle apparently in the process of being formed from a bleb on the cell surface, in a pocket between a cell and the cellulose. Bar, 0.1 μm.*

FIG. 3. *Membrane vesicles released from a B. succinogenes cell and bound to cellulose (C). This perhaps illustrates the gradual release of vesicles from pockets between cells and the cellulose, and the origin of vesicles coating fragments of cellulose in old cultures (see Fig. 6). Bar, 0.1 μm.*



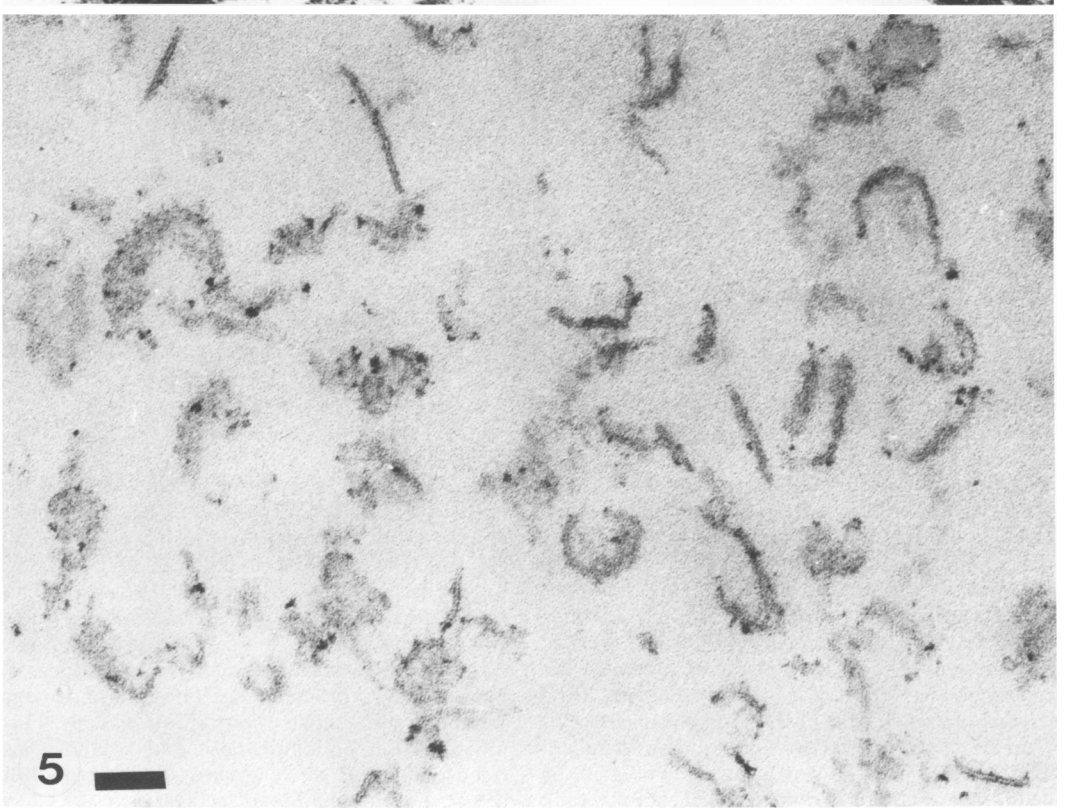
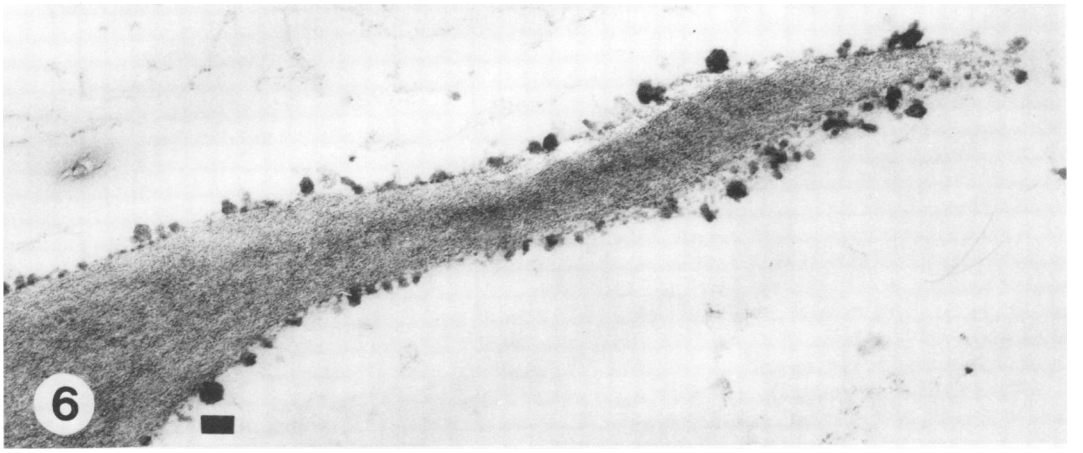


FIG. 4. *A bleb formed from the outer membrane of a B. succinogenes cell in the liquid phase of a culture with cellulose as the source of carbohydrate. Bar, 0.1 μm.*

FIG. 5. *Membrane fragments isolated from the cell-free culture fluid of B. succinogenes cells grown on cellulose. The concentrated materials were dialyzed by concentration with a PM-10 (Amicon Canada) membrane and dilution with 0.05 M sodium phosphate buffer, pH 6.8, before embedding. Note the sheet-like structure of the membrane fragments. Bar, 0.1 μm.*



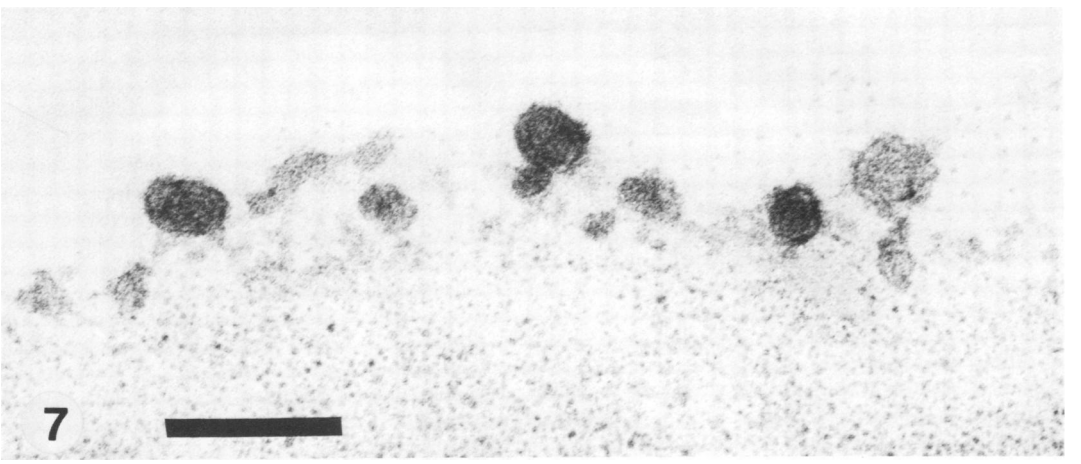


FIG. 6. *An 8-day-old culture of B. succinogenes grown on cellulose. Note the membrane vesicles coating the cellulose particle. Bar, 0.1 μm.*

FIG. 7. *A high-magnification photomicrograph of a section of the cellulose shown in Fig. 6, illustrating the membranous nature of the vesicles. Bar, 0.1 μm.*

9), *Cellvibrio* sp. (3, 28, 44), *Ruminococcus flavefaciens* (30), and *B. succinogenes* (18), in which the glucanase activity is often more firmly bound, depending upon the organism and its stage of growth. In those bacteria with demonstrated xylanase activity, for example, *C. thermocellum* (16), *R. flavefaciens* (30, 31), and *B. succinogenes*, the distribution of xylanase between the cells and the cell-free culture fluid corresponded to that of the glucanase activity.

In *A. cellulolyticus*, the exo- and endoglucanases are released during growth, whereas the cellobiase remains primarily cell bound (36). Similarly, in *Cellulomonas* sp. strain CS1-1, *Cellvibrio vulgaris*, and *P. fluorescens* subsp. *cellulosa*, the endoglucanase is released from cells during active growth on a cellulosic substrate (9, 28, 43). Oberkotter and Rosenberg (28) interpreted the endoglucanase release from *C. vulgaris* as active secretion. In *B. succinogenes* cultures during growth on cellulose, the endoglucanase- and xylanase-specific activities in the cell-free culture fluid were ca. 5 and 10 times higher, respectively, than the cell-associated activities. Furthermore, little cellobiase activity was detected in the cell-free culture fluid, and no cell lysis was observed in young cultures or in cells grown on cellulose. These data, in conjunction with those reported previously (18), indicate that endoglucanase and xylanase are released from *B. succinogenes* during growth in the absence of significant cell lysis.

The extracellular cellulases have been partially characterized in a *Cellulomonas* sp. and *P. fluorescens* (2, 46). In both cases, multiple forms of the endoglucanase were observed, and the largest one had a molecular weight of approximately 118,000 (2). In comparison with these

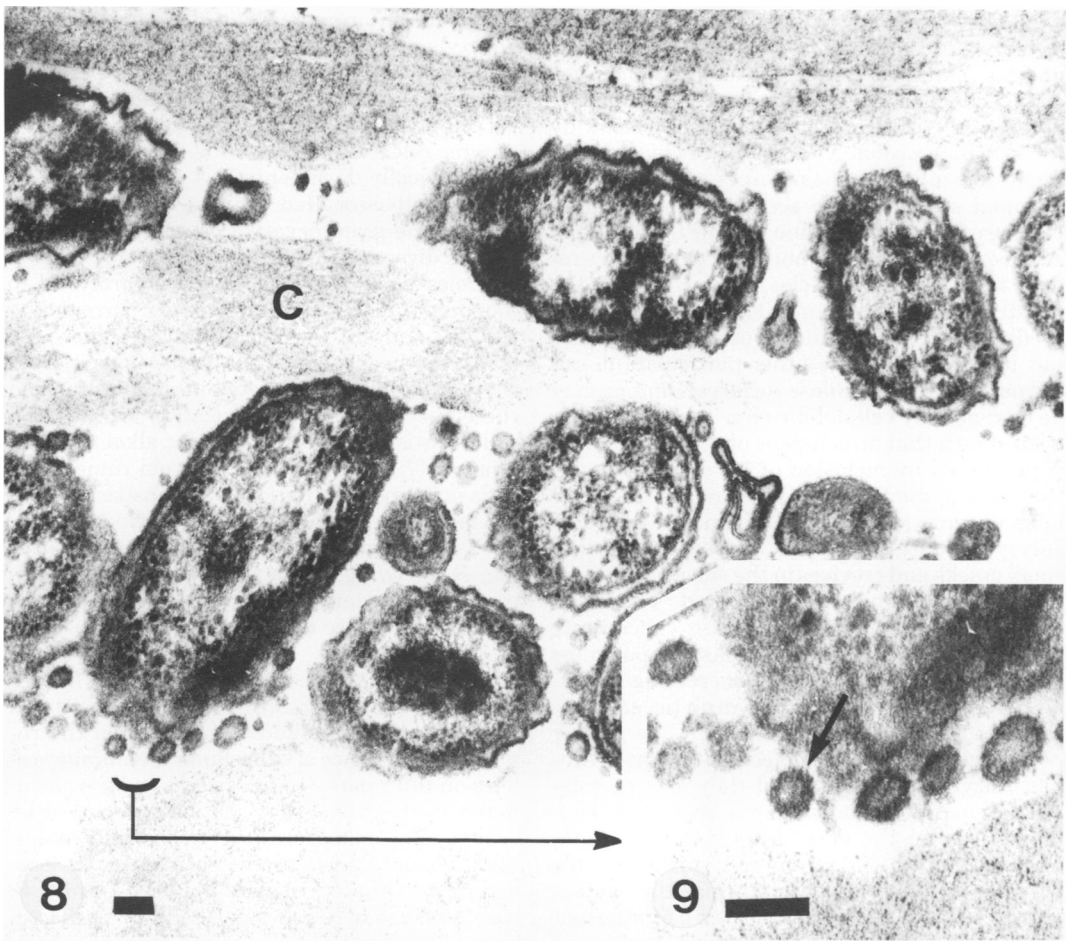


FIG. 8. *Bacterial cells and membrane vesicles associated with ground filter paper cellulose (C) incubated in a nylon bag in the rumen for 17 h. Bar, 0.1 μm.*

FIG. 9. *An enlargement of vesicles in close proximity to the end of a cell shown in Fig. 8. Bar, 0.1 μm.*

results, the endoglucanase and xylanase activities released from *B. succinogenes* were distributed almost equally between a nonsedimentable fraction and a sedimentable fraction obtained by centrifugation at 100,000 × *g*. The nonsedimentable endoglucanase has since been separated into two fractions; one with a molecular weight of 45,000, and the other with a molecular weight in excess of 4×10^6 (D. Groleau, personal communication). In view of the size heterogeneity of the glucanase and xylanase activities, a large proportion of each of these enzymes undoubtedly is associated with membrane fragments. Consequently, release of these enzymes occurs by a mechanism that has hitherto been inadequately described and is more complex than the present models for secretion of extracellular enzymes (34). More work is needed to thoroughly characterize this secretion mechanism since it perhaps is widespread. For example, *R. flavefaciens* cells, during growth in pure culture, release cellulase, endoglucanase, and xylanase into the culture fluid, which, on the basis of molecular-sieve chromatography, has a molecular weight in excess of 800,000 (30). Other rumen bacteria that release enzymes associated with membrane fragments or other proteins are *Anaerovibrio lipolytica* (20) and *Bacteroides amylophilus* 70 (26).

Detailed ultrastructural examination of *B. succinogenes* grown on cellulose revealed that the outer membrane was pliable, since it conformed to the contours of the substrate, and tended to undulate in the absence of cellulose (Fig. 4). The absence of strong adhesion of the outer membrane to the underlying peptidogly-

can layer, in conjunction with the observed vesicularization induced in pockets between cells and the cellulose substrate, clearly demonstrates the mechanism of vesicle production in *B. succinogenes*. Vesicles are formed from blebs by forces which probably include Brownian movement and inherent shear forces of a streaming colloidal system which act by literally tearing cellulose-bound membrane regions from the cell surface. Once free, membrane fragments reanneal, forming small vesicles which adhere to the substrate for some time. The exact mode of binding of the cell wall to cellulose is unclear, but it is specific, since other particulate debris did not adsorb to cellulose surfaces. Binding may be mediated by cellulolytic enzymes, since it has been shown that attachment of cells to cellulose is prevented by inclusion of 1% of cellobiose in the binding medium (27). The released subcellular enzymatically active fragments undoubtedly potentiate hydrolysis by penetrating into small cracks and crevices in the substratum from which cells are normally excluded. The small vesicular packets of enzymes also concentrate and localize cellulase and xylanase on polymers away from the cells, thereby increasing the effective area of contact of hydrolytic enzymes with the particular substrate.

The origin of the low-molecular-weight hydrolytic enzymes is unclear, but they may be fragmentation products arising from the outer membrane or from the periplasmic space, or both. The reorganization at the cell surface during growth on cellulose must maximize the contact of the cell with cellulose and may be important in providing high concentrations of hydrolytic enzyme(s) favorably aligned for efficient cellulose hydrolysis (44).

Suzuki et al. (43) observed that when *P. fluorescens* subsp. *cellulosa* is grown in a chemostat with cellobiose as the limiting nutrient, the extracellular endoglucanase activity is equal to that of a culture with cellulose as the source of carbohydrate. This supports the contention that derepression of cellulase synthesis, rather than interaction with cellulose, is the primary factor in cellulase secretion. Endoglucanase production and release by *B. succinogenes* is higher when cells are grown on cellulose rather than on the soluble substrate glucose (18); however, the effect of carbon-limited growth on the production of glucanase activity has not been tested. The endoglucanases of *P. fluorescens* subsp. *cellulosa* have low molecular weights compared with the bulk of the endoglucanase activity in *B. succinogenes*, which is associated with membrane fragments; consequently, the response of *B. succinogenes* to a limiting nutrient may be different. In *R. flavefaciens*, cellobiose limitation leads to an increase in the endoglucanase and xylanase activities released into the culture fluid (31). The comparative effects of carbon-limited slow growth rates versus affinity binding to cellulose on the stimulation of hydrolytic enzyme release by *B. succinogenes* remain to be unequivocally documented.

Cell wall-associated enzymes are thought to provide the gram-negative bacterial cell with a unique digestive capability in that complex food particles such as cellulose are hydrolyzed to monomers in a zone immediately surrounding the cell and are readily available to the cells' transport mechanisms (10). It has been reported that most bacteria isolated from nature hold these enzymes in close association with the cell wall; this is true for example, for alkaline phosphatase (7), which is released from rumen bacteria only under conditions of extensive cell lysis in animals in which bloat has been induced (8). Perhaps in the case of some bacteria; for example, *B. succinogenes*, a sufficient excess of glucanase and xylanase is produced so that their loss to the culture fluid does not jeopardize cell growth.

The membrane vesicles released from *B. succinogenes* cells resembled those seen associated with filter paper cellulose incubated in the rumen. The presence of subcellular membrane vesicles on filter paper cellulose and on straw incubated in the rumen has also been reported by others (41). In all cases, rod-shaped cells resembling *B. succinogenes* have been observed associated with the particulate materials. Since *B. succinogenes* is a prevalent cellulolytic bacterium in the rumen, it is conceivable that subcellular membrane vesicles released from this bacterium contribute significantly to polymer degradation in the rumen. In fact, Gawthorne (17) has isolated from a rumen sample of partially digested plant material a membrane-like fraction which has cellulase, xylanase, endoglucanase, and cellobiase activity.

The release of vesicular packets of hydrolases from *B. succinogenes* during growth on cellulose helps account for its ability to support the growth of *Selenomonas ruminantium* (37) and *Treponema bryantii* (40) when grown in coculture with each of them. Since much of the cellulase is released from *B. succinogenes* cells during growth, the extracellular enzymes provide a ready source of cellobiose or glucose for the associated bacteria, even though they lack cellulolytic enzymes. Similarly, this principle applies to hemicellulose digestion. In terms of the rumen ecosystem, the release of low-molecular-weight and outer-membrane fragments with polysaccharidase activity from *B. succinogenes* and other polysaccharide-degrading bacteria may be

an important mechanism for providing mono- and disaccharides to organisms lacking cellulases and hemicellulases, thereby enhancing microbial diversity.

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