APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1988, p. 1085–1090
0099-2240/88/051085-06$02.00/0

Vol. 54, No. 5

Isolation and Characterization of Xylan-Degrading Strains of *Butyrivibrio fibrisolvens* from a Napier Grass-Fed Anaerobic Digester†

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Received 7 December 1987/Accepted 18 February 1988

Six new xylanolytic bacterial strains have been isolated from a Napier grass-fed anaerobic digester. These strains were identified as *Butyrivibrio fibrisolvens* and were similar in many respects to ruminal isolates described previously. The new isolates exhibited a high degree of DNA homology with several ruminal strains of *B. fibrisolvens*. Xylan or xylose was required to induce the production of enzymes for xylan degradation, xylanase and xylosidase. Production of these enzymes was repressed in the presence of glucose. Xylanase activity was predominantly extracellular, while that of xylosidases was cell associated. The new isolates of *B. fibrisolvens* grew well in defined medium containing xylan as the sole carbon source and did not produce obvious slime or capsular layers. These strains may be useful for future genetic investigations.

The biological production of methane from plant residues and other waste materials has been done for many years in developing countries (28) and is of increasing interest as a partial solution to the energy needs of developed nations as well (15). Most of our knowledge about the microbial breakdown of plant biomass polymers comes from studies of microbial ecology in the rumen (19, 20, 23, 34). Thus, much of our conceptual understanding of biomass decomposition in anaerobic digesters has been extrapolated from research investigations of the ruminal ecosystem (32). Anaerobic digestion of complex organic matter to methane consists of a cascade of biochemical conversions catalyzed by different physiological groups of interacting microorganisms (35). The hydrolysis of plant structural polymers to smaller units represents the initial step in biological methane production.

Xylan is a major plant structural polymer, second only to cellulose in natural abundance (10). This polymer is efficiently degraded anaerobically by members of several bacterial genera including *Bacteroides* (14, 29, 30), *Ruminococcus* (9, 23, 34), *Clostridium* (23), and *Butyrivibrio* (5, 16–18, 34). The importance of these organisms has been recognized during investigations of ruminal and soil isolates. Among these, *Butyrivibrio fibrisolvens* is reported to be one of the most numerous and important organisms for the degradation of xylan in the rumen (16, 18, 23). Recent studies of xylanolytic species of *Clostridium* indicate that this group may also be of particular importance in soil (25).

Anaerobic digesters maintained on dried grass or similar feeds represent long-term enrichment cultures for organisms which are capable of efficiently degrading structural plant polymers. The purpose of this study was to isolate representative xylanolytic bacteria from an anaerobic digester which can be cultivated on defined medium and grow rapidly with xylan as the sole fermentable carbon source. In this study, we have characterized six new xylanolytic isolates, which were identified as *B. fibrisolvens*.

MATERIALS AND METHODS

Anaerobic digester. The strains used in this study were isolated from the mixed liquid of an anaerobic tank reactor (35°C), which has been described previously (35). This reactor was fed mature, hot-air-dried Napier grass (*Pennisetum purpureum* Schum.; USDA PI 300086) supplemented with trace elements and had been in operation for 16 months at the time of sampling. The daily volatile solids loading rate was 3.7 g/liter, with 47% conversion to mixed gases. The pH was maintained between 6.8 and 7.0.

Medium and growth conditions. The GS strains of *B. fibrisolvens* were isolated and grown in a defined mineral salts medium with vitamin and organic acid supplements as described by Stevenson (33). Oat xylan (5 g/liter) was added as the sole carbon source. Sodium carbonate was added at either 0.3 or 0.6 g/liter to accommodate carbon dioxide levels of 5 and 10%, respectively. Basal medium PY (21) containing glucose was used in experiments to analyze fermentation products. Anaerobic conditions were maintained in a Coy model AALC-S anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) with a mixture of hydrogen, carbon dioxide, and nitrogen (either 10:5:85 or 7.5:10:82.5).

The GS strains of *B. fibrisolvens* were grown in screw-cap tubes (150 by 16 mm) at 37°C without agitation for the analysis of fermentation products. Cultures for the analysis of xylan-degrading enzymes were grown in minimal medium containing xylan (50 ml in 125-ml serum bottles) with gyratary agitation (125 rpm) at 37°C for 48 h. Taxonomic tests were performed as described by Holdeman et al. (21).

Twenty-two isolates of *B. fibrisolvens* were grown in complex medium (17) and used as sources of DNA for hybridization studies. These strains have been described previously (8, 17) from an author's (R.B.H.) stock collection or were generously provided by M. P. Bryant (University of Illinois, Urbana), B. A. Dehority (Wooster, Ohio), N. O. van

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† Florida Agricultural Experiment Station publication number 8537.

Glyswyk (Pretoria, South Africa), or R. A. Mackie (Pretoria, South Africa).

Analysis of fermentation products. Organic acids and alcohols were analyzed in the broth from 48-h cultures in a Tracor model 560 gas chromatograph (Tracor Instruments, Austin, Tex.) as described (21).

DNA base composition and hybridization. DNA was isolated by a modification of the method of Berns and Thomas (2). Cell lysates were gently extracted four times with Tris-phenol. DNA was precipitated with 2 volumes of ethanol, suspended in TE buffer (100 mM Tris hydrochloride, 1 mM EDTA, pH 8.0), and treated with DNAse-free RNase A (20 μg/ml) and proteinase type XIV (100 μg/ml) for 1 h at 37°C. After four additional extractions with phenol-chloroform, the DNA was precipitated with 0.6 volume of isopropanol, dissolved in TE buffer, and extensively dialyzed in TE buffer. DNA concentrations were estimated spectrophotometrically at 260 nm. DNA purity was checked by scanning the absorbance of DNA samples between 200 and 400 nm and monitoring the absorbancy ratios at 260/280 nm and at 260/230 nm. DNA preparations were also subjected to electrophoresis in 1% agarose gels to check for shearing and degradation.

DNA base composition was determined from the midpoints of thermal melting profiles by the equation of Mandel et al. (26). Homology between DNA from different isolates was determined spectrophotometrically from the kinetics of DNA reassociation by the method described by DeLey et al. (11). Melting and renaturation profiles were measured with a Response II thermal programming system (Gilford Instruments, Middleton, Wis.). The reactions were performed as described by Nakamura and Sweezey (27).

Electron microscopy. Overnight cultures were prepared for ultrastructural examination by growth in stationary tubes. For whole-mount examination, cell suspensions were placed on Formvar-coated 200-mesh copper grids which had been coated with 0.1% bacitracin to improve wetting. Cells were allowed to settle for 3 to 5 min. Excess liquid was drawn off, and the grids were air dried. Grids were shadowed at a 30° angle with a carbon-platinum source in an electron gun with a turbomolecular-pumped vacuum evaporator (Balzers model MED 010).

Cells were fixed for transmission electron microscopy by a modification of the procedure described by Boyles (4). Actively growing cells were briefly fixed in growth medium by the addition of 0.5 volume of buffered 2% glutaraldehyde (50 mM lysine, 50 mM sodium cacodylate, pH 7.2). Cells were immediately harvested by centrifugation (5,000 × *g*, 5 min). The supernatant was replaced with freshly prepared 2% glutaraldehyde in cacodylate-lysine buffer, and fixation was continued for 30 min at room temperature. After a second centrifugation, the supernatant was replaced with 2% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) and incubated for 50 min at room temperature, followed by a 15-min postfixation in ice-cold 1% osmium tetroxide in 50 mM cacodylate buffer (pH 7.2). Cells were washed in ice-cold deionized water, held in 1% uranyl acetate at room temperature for 2.5 h, dehydrated, and embedded in Spurr low-viscosity resin. Sections were cut with diamond knives on an LKB Ultrotome III microtome, poststained with uranyl acetate and lead citrate, and examined with a Philips EM-301 transmission electron microscope.

Xylanase and xylosidase activity. Cells were harvested by centrifugation in a Fisher microcentrifuge (10,000 × *g*, 2 min), washed by suspension in 50 mM sodium phosphate buffer (pH 6.8), and harvested again by centrifugation. Both the original culture broth and the cell pellet from each sample were stored at −20°C until analyzed. The xylanase and xylosidase activities remained stable for at least 1 week under these storage conditions.

Cells were disrupted in the above buffer by the addition of lysozyme and chloroform as described previously (7). Xylanase activity was measured by determining the extent of dye solubilization with Remazol brilliant blue-xylan as the substrate as described (3), except that assays were carried out at 37°C in 50 mM sodium phosphate buffer (pH 6.8). Xylan-blue units are reported as the increase in absorbance (595 nm) of solubilized Remazol brilliant blue-xylan per minute per milligram of cell protein or per milliliter of culture broth. Xylanase activity was also measured as an increase in reducing sugar with the Nelson-Somogi reagent (1). Under the conditions of our assays, solubilization of 1 absorbance unit of dye corresponded to the production of 0.7 μM reducing sugar.

Xylosidase activity was measured by determining the rate of hydrolysis of *p*-nitrophenol-β-D-xylopyranoside (1.1 mM final concentration) in 50 mM sodium phosphate buffer (pH 6.8). Assays were carried out at 37°C and terminated by the addition of 2 volumes of 500 mM sodium carbonate. Absorbance was measured at 405 nm, and activity is expressed as micromoles hydrolyzed per minute. Under these assay conditions, 1 μmol of hydrolysis corresponds to an increase in absorbance of 0.070.

Assays and sample preparation were routinely carried out in the laboratory without provision for maintaining anaerobiosis. Preliminary experiments were carried out in the anaerobic chamber and in a glove bag under nitrogen. Both xylosidase and xylanase activities were stable during exposure to air.

Activities in broth and in pellets are expressed per milligram of cell protein in the culture. Protein was measured with the Folin reagent as described by Layne (24).

Chemicals and reagents. All biochemicals used in this study were obtained from Sigma Chemical Co., St. Louis, Mo. Mixed gases were purchased from Airco Industrial Products, Murray Hill, N.J. Inorganic salts were purchased from Fisher Scientific Company, Orlando, Fla.

RESULTS

Occurrence and isolation of xylanolytic strains. Xylanolytic bacteria were isolated from an anaerobic digester by direct plating of serially diluted samples on mineral salts medium containing xylan as the sole carbon source. Dilutions, plating, and incubations were carried out under anaerobic conditions. Digester fluid contained approximately 10^6 CFU/ml that were capable of forming colonies on this medium after 48 h. Although most of these colonies were presumed to be capable of using xylan or its degradation products, only 10% produced obvious halos of clearing, indicative of xylanase secretion (Fig. 1). Of those which formed halos, half formed opaque colonies. Upon microscopic examination, these opaque isolates were found to be gram-positive rods containing endospores and were presumed to be species of *Clostridium*. The translucent colonies forming halos were more variable in appearance, although most were gram-negative, curved rods.

Representative gram-negative isolates (140 strains) were further examined for their ability to be serially transferred on xylan plates, for growth on complex medium, for growth under aerobic conditions, and for obvious polysaccharide production during growth on glucose. Most grew well on

both xylan and complex medium and were obligately anaerobic. Six isolates which did not produce obvious capsular material were selected for further study. Isolates which did not produce slime were chosen to avoid potential problems in future genetic studies involving DNA purification, conjugation, and transformation. These isolates all grew as curved rods which tended to form chains, lacked spores, contained motile cells, produced butyrate as the principal fermentation product from glucose on complex medium, required carbohydrate for growth, were indole negative and bile sensitive, and did not have an absolute requirement for either hemin or organic acids (although both stimulated growth). By these criteria, the isolates were assigned as *B. fibrisolvens* (5, 17–19, 21). These isolates were assigned as different strains primarily by carbohydrate utilization. Strains GS110 and GS111 were quite similar except for differences in colony size. Strain 110 typically formed much larger colonies on solid media.

Table 1 summarizes the range of carbohydrates utilized for growth by these isolates in mineral medium. None of these isolates utilized starch or carboxymethyl cellulose, although all grew well on xylan and on cellobiose. All isolates utilized xylose, glucose, and arabinose, and none grew on mannitol. Other sugars were somewhat variable. During growth with glucose, small amounts of pyruvate, oxaloacetate, succinate, and propionate were produced in addition to the major products, butyrate and lactate.

Ultrastructural examination. The six isolates of *B. fibrisolvens* appeared to be essentially identical in ultrastructure. Whole-mounted cells possessed a single subapical flagellum, one to two times the length of the cell body (Fig. 2A). Cells were curved and slightly tapered at the ends, with a typical central nucleoid and dense peripheral cytoplasm (Fig. 2B and C). Diameters near the middle of the cells ranged from 0.21 to 0.32 μm. Lengths of individual cells varied from 2.0 to 4.0 μm. Cells tended to remain attached after cross wall formation, creating filaments several micrometers in length. Electron-lucent inclusions were often observed in cells grown on glucose as a carbon source (Fig. 2C) and were less abundant in cells grown with xylan. These inclusions were aggregates of 25- to 35-nm-diameter spherical particles. Efforts to remove these particles from sections with amylase or to stain them with polysaccharide-specific silver stains (13) were unsuccessful, suggesting that the particles were not glycogen.

The cell envelope structure appeared somewhat unusual (Fig. 2D). It contained two membranes separated by a dense central layer which was presumed to represent the peptidoglycan. Each of the two membranes contained a more densely staining layer facing the central peptidoglycan layer.

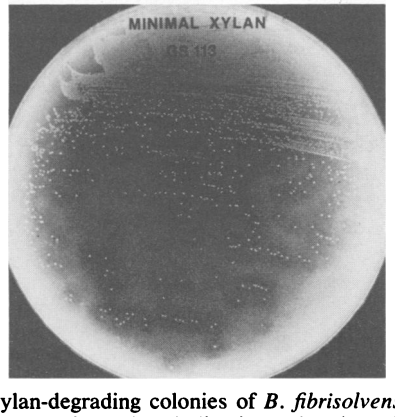

FIG. 1. Xylan-degrading colonies of *B. fibrisolvens* GS113 surrounded by zones of clearing, indicating xylan degradation. Plates were incubated for 48 h at 37°C.

TABLE 1. Carbohydrate utilization

Carbohydrate	Growth[a]					
	GS110	GS111	GS112	GS113	GS116	GS117
Glucose	+	+	+	+	+	+
Xylose	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+
Xylan (oat)	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+
Lactose	+	+	+	−	+	+
Maltose	+	+	s	−	+	+
Sucrose	+	+	−	−	+	+
Fructose	+	+	−	−	+	−
Galactose	s	+	−	−	+	+
Raffinose	s	s	s	s	−	+
Mannitol	−	−	−	−	−	−
Carboxymethyl cellulose	−	−	−	−	−	−
Starch (corn)	−	−	−	−	−	−

[a] Symbols: +, vigorous growth; −, no growth; s, slight growth.

DNA composition and hybridization to other isolates of *B. fibrisolvens*. The DNA base composition of the two most active xylanolytic isolates, strains GS112 and GS113, was found to be 41.6 and 41.2 mol% guanine plus cytosine (G+C), respectively. The DNA from these two strains was greater than 80% homologous, based on the kinetics of thermal reassociation (Table 2). In comparison with 22 other isolates identified as *B. fibrisolvens*, strains GS112 and GS113 exhibited strong homology, ranging from 71.1 to 80.2%, with five strains, E21c, 1L6-31, NOR-37, B835, and E9a. These strains ranged in base composition from 41.2 to 41.9% G+C. The lack of homology to many of the other isolates of *B. fibrisolvens* suggests that the taxonomy of this group may not be adequately defined.

Xylanolytic activity. Both xylosidase and xylanase activities were readily detected in liquid assays with all six isolates during growth on xylan (Tables 3 and 4). Both activities are expressed as per milligram of cell protein to correct for differences in extent of growth, although xylanase activity was primarily extracellular. The actual protein content in the broth containing xylanase was very low and represented only a small percentage of the protein in the cell pellet.

Xylosidase activity was observed only in disrupted cell pellets; no activity was detected on the cell surface or in the broth. During growth on xylan as the carbon source, strains GS110 and GS113 exhibited the highest level of activity (Table 3). Lower levels of xylosidase activity were found in all strains grown with xylose, and only trace amounts of activity were found during growth on glucose. Inclusion of glucose with xylan resulted in xylosidase activities similar to those in cells grown with glucose alone. Xylosidase activity was not strongly repressed by the inclusion of xylose with xylan (not shown).

Table 4 summarizes the activities of secreted xylanases from the six isolates. Xylanase activities were highest in strains GS113 and GS112 during growth on xylan. Very low levels of activity were detected during growth on glucose, and intermediate levels were detected during growth on xylose. As with xylosidase, the inclusion of glucose with xylan strongly repressed xylanase production, while inclusion of xylose had less repressive action (not shown).

Additional experiments were conducted to examine the

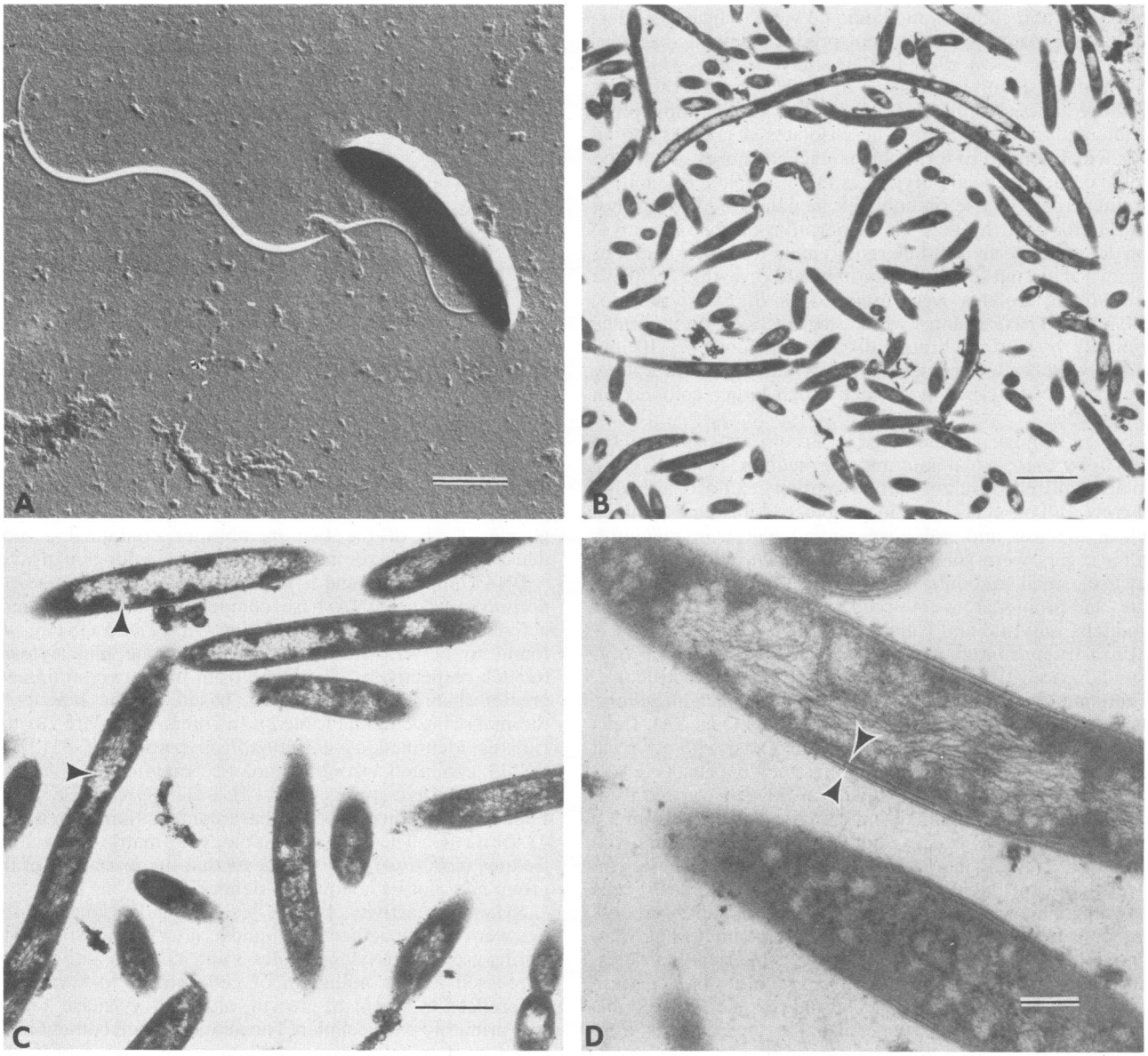

FIG. 2. Electron micrographs of *B. fibrisolvens* GS113. (A) Platinum-shadowed whole-mounted cell illustrating the single, subapically attached flagellum, two to three times the length of the cell body. Bar, 0.5 μm. (B) Thin section of cells illustrating the filamentous nature of cells which remain attached after cross wall formation. Bar, 1.0 μm. (C) Aggregates of electron-lucent spherical inclusions 25 to 35 nm in diameter (arrowheads) in glucose-grown cells. Bar, 0.5 μm. (D) High-magnification micrograph illustrating cell envelope structure. The envelope consists of two membranes, separated by a thicker, more densely staining layer (arrowheads). Bar, 0.1 μm.

localization of xylanase activity during growth on xylan alone. Washed cell pellets of all strains contained appreciable levels of xylanase activity. Cell pellets of strains GS110, GS111, GS112, and GS113 contained xylanase activity equivalent to 30 to 40% of that secreted. The cell pellet from strain GS117 contained the highest level of cell-associated xylanase activity, equivalent to 70% of that secreted, while that from strain GS116 contained the least, equivalent to 10% of secreted activity. These results indicate that a significant fraction of xylanase activity either remains cell associated or tends to remain bound to undigested xylan, which is also present in the pellet. Attempts to cleanly separate cells from undigested xylan on sucrose gradients have met with limited success.

DISCUSSION

Xylanolytic bacteria were readily obtained from an anaerobic digester being maintained on Napier grass. These organisms were capable of growth on xylan as the sole carbon source in a mineral-based, defined medium. *Clostridium* spp. and *B. fibrisolvens* appeared to be the most abundant xylan-degrading organisms present in our Napier grass-fed digester that were capable of forming colonies on defined medium. Other more fastidious xylan-degrading bacteria may be as or more numerous but unable to grow under our conditions of selection. *Butyrivibrio* spp. have also been reported to be among the most numerous ruminal bacteria capable of xylan degradation (17, 18). The specific strains

TABLE 2. DNA hybridization between two mesophilic digester isolates and previous isolates of *B. fibrisolvens*[a]

Test strain	% DNA reassociation with DNA from:	
	GS112	GS113
GS112	100	83.0
GS113	83.0	100
E21c	71.1	71.5
1L6-31	78.4	80.2
NOR-37	74.6	77.3
B 835	74.5	74.5
E9a	72.4	72.8
DF16f	17.4	21.6
49	22.4	26.5
CF4c	21.1	19.2
R-28	26.3	26.8
LM8/1B	22.3	20.1
C3	24.7	25.6
Pi-7	25.4	28.0
S-2	30.9	31.4
AcTF2	18.4	20.2
D1	16.8	20.9
ARD-22a	25.6	24.7
ARD-23c	24.2	23.8
Pi-26	18.4	19.7
787	18.9	22.7
E46a	20.2	20.6
H4a	25.4	20.8
H13b	18.4	19.9

[a] Each value represents the mean of three determinations. The standard deviation was <5%.

described in this study produced no obvious slime or capsular material under any of our growth conditions and may be particularly useful for future genetic studies.

The ultrastructure of the six isolates of *B. fibrisolvens* from our anaerobic digester was quite similar to that of other species of this genus examined previously. Cheng and Costerton (6) found a wall consisting of two trilaminar structures after ruthenium red staining of one of their isolates. Sharpe et al. (31) and Dibbayawan et al. (12) demonstrated paired membranes in *B. fibrisolvens* similar to those which we observed. The identity of the electron-lucent peripheral granules remains a mystery. Under heavy lead citrate staining, they appeared to have fibrous contents (not shown). These granules are presumed to represent some form of storage product, since they were particularly abundant during growth with glucose as the carbon source. However, these granules did not appear to react histochemically as would be expected for starch or glycogen.

Both xylan and, to a lesser extent, xylose appeared to induce the production of both xylanase and xylosidase activities in our isolates of *B. fibrisolvens*, consistent with

TABLE 3. Cell-associated xylosidase activity during growth with different carbohydrates

Isolate	Xylosidase sp act (μmol hydrolyzed/min per mg)			
	Xylan	Xylose	Glucose	Xylan + glucose
GS110	0.06	0.02	0.005	0.005
GS111	0.04	0.02	0.01	0.001
GS112	0.02	0.01	0.001	0.003
GS113	0.10	0.01	0.001	0.002
GS116	0.014	0.01	0.003	0.007
GS117	0.043	0.01	0.001	0.001

TABLE 4. Extracellular xylanase activity produced during growth with different carbohydrates

Isolate	Xylanase activity[a] (increase in A_{595})			
	Xylan	Xylose	Glucose	Xylan + glucose
GS110	2.0	0.45	0.001	0.001
GS111	1.2	0.15	0.006	0.001
GS112	2.3	0.19	0.006	0.013
GS113	4.9	0.14	0.008	0.010
GS116	0.6	0.09	0.02	0.005
GS117	0.4	0.01	0.02	0.001

[a] Activity is expressed as increase in absorbance due to the solubilization of Remazol brilliant blue-xylan per milligram of total cell protein. Values represent averages from two separate experiments.

earlier observations of xylanolytic *Butyrivibrio* spp. isolated from ruminal samples (22). Both of these enzymes were subject to glucose repression but were not as strongly repressed as xylose. This is particularly interesting, since it is likely that both glucose and xylose are metabolized via many common enzymatic steps. Within an anaerobic digester, these organisms are exposed to a mixture of sugar monomers and oligomers which may alter the level of enzyme production. Strains which have been genetically modified to eliminate repression by glucose and other carbohydrates or to serve as hyperproducers may allow more rapid breakdown of biomass.

Appreciable xylanase activity sedimented with the cell pellet and undegraded xylan. Ultrastructural examination showed no evidence of secretory vesicles, although these have been reported for some other microbial glycosidases (14). The cell envelope of *B. fibrisolvens* is quite complex, with double membranes separated by a thickened, darkly staining region which is presumed to include the peptidoglycan. It is not unlikely that specialized secretion systems are required for the efficient export of xylanases by these organisms.

Although our two most active xylan-degrading isolates (GS112 and GS113) showed considerable DNA homology with some of the previously identified ruminal isolates of *B. fibrisolvens*, the lack of significant homology to many of the prior isolates suggests that this species is not a homogeneous group. Further studies are needed to improve the taxonomy of this abundant group of anaerobic bacteria.

ACKNOWLEDGMENTS

These studies were supported by the Gas Research Institute/University of Florida Cooperative Research Program, by the Florida Agricultural Experiment Station, and by the U.S. Department of Agriculture.

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