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Properties of a cellulolytic *Sporolactobacillus* and some non-sporing cellulolytic rods, presumptive clostridia, from an anaerobic digester

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Anaerobic digesters contain a wide variety of anaerobic cellulolytic bacteria. Many of these are sporing rods. Some isolates from a mesophilic cattle waste anaerobic digester were classified as *Sporolactobacillus* spp. A further group of bacteria could not be induced to sporulate. In some respects they resembled *Eubacterium*, but considering all the properties it is suggested that they should be classified as a species of *Clostridium*.

Cellulolytic bacteria are important constituents of many natural microbial systems, and the cellulolytic bacteria of the rumen have been particularly well characterized. Anaerobic, cellulolytic bacteria are also important in some present industrial fermentation processes, particularly anaerobic digesters, and they could have more importance in the future as they can directly convert cellulosic biomass to products which can be used as fuels or as feedstocks for further chemical processing.

The cellulolytic bacteria in anaerobic digesters have so far not been well characterized, although it is known that the digester flora contains more (and different) species than the rumen (Maki 1954; Hobson & Shaw 1974). Some 400 anaerobic, cellulolytic bacteria were more recently isolated from a mesophilic cattle waste anaerobic digester (Sharma 1983; Hobson *et al.* 1984; Sharma & Hobson 1985a) and these were found to contain at least 12 different species. The most numerous constituents of the cellulolytic population were sporing rods, some of which were classified as known species of *Clostridium* (Sharma & Hobson 1985a). Non-cellulolytic clostridia have also been shown to be present in large numbers in digesters (Siebert & Torien 1969; Hobson & Shaw 1974), and some previous cellulolytic isolates may have been clostridia (Maki 1954). However, non-sporing cellulolytic rods were also found in high numbers in the cattle waste digester and the properties of some are described here. Other sporing rods had properties which suggested that they were not clostridia but sporolactobacilli, and the properties of these species are described.

Materials and Methods

ISOLATION AND TESTING OF BACTERIA

Strictly anaerobic methods based on those of Hungate (1950) were used. Tests, except where specified, were done anaerobically and following, in general, the media and methods of Holdeman *et al.* (1977).

The strains were isolated from dilution cultures of samples from a 150 l, mesophilic, anaerobic digester which had been running for some years on dairy cattle waste, as described by Sharma & Hobson (1985a). The substrates used in the isolation cultures were either cellulose powder (C numbers), untreated (U numbers) or

alkali-treated (T numbers) barley straw. Fermentation and other tests were done as described by Sharma & Hobson (1985a). The PY basal medium and media based on this are the peptone-yeast media described by Holdeman *et al.* (1977). Growth with 0·3% and 0·7% methanol was tested as described by Genthner *et al.* (1981) except that medium 2 of Sharma & Hobson (1985a), based on that of Patel *et al.* (1980), was used with the vitamin concentration doubled.

All cultures, and particularly the sporulation medium developed during this work (Sharma & Hobson 1985b) were examined for spores.

G + C mol% ratios were determined as described previously (Sharma & Hobson 1985a) except that partially purified DNA was used as described by Hill (1968) and Gibson & Ogden (1979) employing *iso*propanol precipitation of DNA as one of the purifying steps. Owen & Lapage (1976) suggested that the assay should be done on partially purified DNA. G + C mol% were determined from Tm using DeLey's equation (1970).

Results

NON-SPORING RODS

Strains C375, C376, C378 and T266–T270 were obtained from the 4th to the 6th 10-fold dilution of digester contents on different days. Morphological and fermentation product tests were done on all isolates and strain C376 was characterized extensively.

All the isolates were actively cellulolytic. Cellulolysis on filter paper strips was 4 or 5 units except for strain T266 (3 units) in the tests previously described where maximum activity was 5 units. Degradation of untreated straw was 37%–45% except for strain T266 (31%), near the maximum found previously (Sharma & Hobson 1985a).

Strain C376 and the others were Gram-negative, anaerobic rods with some Gram-positive cells in young cultures. They were 0·5–0·8 × 2–6 μm, straight or slightly curved, with rounded ends, and with a few cells up to 10 μm and filaments over 50 μm. Strain T266 sometimes showed thinner rods. Growth was mucoid or gummy. Some swollen or bulbous forms could be seen in old cultures. Subculture with 0·3% methanol did not remove these forms, so it was concluded that they were not contaminants, and failure to survive 75° or 80°C for 10 min precluded them from being spores. Petrichous flagellation could be seen in electron micrographs, as could a Gram-positive cell wall.

Colonies in glucose roll-tubes were 1–3 mm in diameter, raised to convex, with undulate margin and yellow to light tan in colour. Colonies in cellulose roll-tubes were similar but with a distinct zone of clearing round the edge. This zone suggested that the cellulases were extracellular and diffusing or that motile bacteria were carrying cellulase activity beyond the edge

Table 1. Fermentation products of the non-sporing rods in PY and PYG media

	Concentration of product (mmol/l)				
	PY		PYG		
Product	C376	C378	T267	T268	C375
Ethanol	22·4	4·1	3·1	1·7	2·8
Formate	P	P	P	P	P
Acetate	4·8	6·1	11·7	7·8	11·7
Propionate	1·3	3·1	2·5	1·3	2·7
Butyrate	1·2	7·9	8·7	14·3	10·8
*iso*butyrate	0·3	0·0	0·0	0·0	0·0
*iso*valerate	0·7	0·0	0·0	0·0	0·0
Lactate	P	14·1	15·0	6·9	10·0
Succinate	P	20·2	4·6	2·5	8·8

P, Present but not quantified.

Means of three cultures. No *n* or *i* caproate or *n* valerate were detected.

PY, Peptone yeast medium; PYG, peptone yeast glucose.

Table 2. Analysis of head-space gases of non-sporing rods

Strain	Gases (%)			
	N_2	CO_2	H_2	CH_4
C376	40·6	17·8	41·6	—
C375	43·1	17·9	39·0	—
T268	45·2	17·3	37·5	—
Control*	90·0	10·0	—	—

* Initial gas, uninoculated medium.
—, Nil.

of the colony. Motility could be detected in vitally stained cells which had migrated from the edge of the mucoid growth. The bacteria produced a ropy, mucoid growth in peptone yeast glucose (PYG) liquid medium.

The isolates produced acetate, lactate, butyrate, propionate and succinate in PYG medium (Table 1) in excess of the concentration found in PY medium, as well as hydrogen (Table 2). Ethanol was produced in smaller amounts than in PY medium. In PY-pyruvate, acetate and butyrate production was higher than in PY medium, while ethanol was lower. In PY-lactate, ethanol production was almost twice that in PY medium.

Positive biochemical reactions are shown in Table 3. Sugars not fermented were adonitol, arabinose, dulcitol, inositol, lactose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, sorbitol and trehalose. Tests for meat digestion and production of hydrogen sulphide, acetyl-methyl-carbinol (AMC), catalase, lecithinase, lipase, urease and haemolysis were negative.

In the filter-strip test (Sharma & Hobson 1985a) the strip disintegrated to form a mass of paper particles and gelatinous growth on the bottom of the tube. Strains T266 and T270, which had least filter-strip activity, produced 31·2% and 37·2% loss in weight of untreated barley straw. With the other isolates hydrolysis of straw was from 38·0% to 45·7%. Spent cultures on ball-milled cellulose (paper) contained cellobiose, cellotriose and higher cellodextrins, with xylose.

Isolate C376 did not grow in the presence of 6·5% NaCl or 0·7% methanol, but it did grow in 0·3% methanol.

The optimum growth temperature was 35°–39°C, with some growth at 20°, 25° and 30°C. There was no growth at 45° or 50°C. Inoculated media held at 45° or 50°C for 20 h showed no growth on subsequent incubation at 39°C.

There was good growth at pH 6·5–8·0 with an optimum at 7·0–7·5, but no growth at pH 5·5 or 6·0. The viscosity of the cultures was greatest at pH 8. Growth in these tests was determined visually as the ropy growth upset instrumental measurements. The G + C ratio was 26%.

SPORING RODS

Strains U33, U353, C100, C236 and C432 were obtained from the 2nd to 4th 10-fold dilution cultures on different days. Strains U33, U353 and C432 were characterized extensively.

These strains were Gram-variable, straight to slightly curved rods, 0·5 × 1–1·5 μm. The terminal portions were rather more darkly stained than the rest and were slightly swollen. Spores were difficult to demonstrate but occurred most frequently in older cultures and were spherical to elliptical in shape and terminal in position. Cells from aerobic plates were slightly smaller than those in anaerobic media, being 0·2–0·3 μm wide.

Anaerobic colonies in glucose roll-tubes were cream, circular, entire with raised centre, 0·5–1 mm diameter with a 1–2 mm diameter

Table 3. Positive biochemical reactions of strain C376

Test	Reaction	Test	Reaction
Cellobiose	+	Salicin	(+)
Dextrin	+	Starch (hyd)	+
Erythritol	(+)	Sucrose	(+)
Aesculin (hyd)	+	Xylose	(+)
Fructose	+	Xylan	(+)
Glucose	+	Gelatin	+
Glycerol	(+)	Milk	p, d
Maltose	+	NO_3 reduction	+

(+), Weak positive; p, d, peptonization, digestion.
For negative tests see text.

mucoid zone around them. On blood agar, colonies were flat, mucoid, transparent, 1–3 mm diameter with umbonate centre and undulate margin. Strain C432 gave little growth on egg-yolk agar, but the other isolates gave colonies of 0·5–2 mm diameter with umbonate centre and entire or slightly undulate margin. During 3 years of laboratory transfers the cultures developed the ability to grow aerobically; initial visible growth on nutrient agar showed as pin-head colonies which later became light tan, 1–2 mm diameter, umbonate with slightly erose margins. Anaerobic culture in PYG medium gave a uniform turbidity with 'silky' waves in a creamy to light tan growth. The cells eventually sedimented with some clumping.

Strain C432 did not grow at 20° or 50°C but grew between 30° and 45°C with a sharp optimum at 39°C. Strains U353 and U33 showed a little growth at 20°C, none at 50°C and a broad optimum between 27° and 35°C. All three strains showed a little growth between pH 4 and 5. Growth increased from pH 5 to pH 8 with strain C432, but strain U353 showed an optimum at pH 7·5 and strain U33 had optimum growth between pH 7·0 and 7·5. Final pH values in fermentation tests were about 3·8–4·0.

Table 4. Biochemical reactions of the sporing rods

Test	Reaction		
	U33	U353	C432
Acetyl-methyl-carbinol	(+)	–	–
Arabinose	+	+	+
Cellobiose	+	+	+
Dextrin	+	(+)	(+)
Erythritol	(+)	(+)	–
Aesculin hydrolysis	+	+	+
Fructose	+	+	+
Glucose	+	+	+
Inositol	–	–	–
Inulin	+	+	–
Lactose	+	+	+
Maltose	+	+	+
Mannitol	–	(+)	+
Mannose	+	+	(+)
Melezitose	+	+	+
Melibiose	–	–	+
Raffinose	(+)	+	(+)
Rhamnose	+	+	+
Ribose	+	+	+
Salicin	+	+	+
Sorbitol	+	+	–
Starch	–	–	–
Sucrose	+	+	+
Trehalose	+	+	+
Xylan	+	(+)	(+)
Xylose	+	+	+
Gelatin	(+)	(+)	(+)
Bile growth	+	+	+
Milk	ac	acd	ac

(+), Slight positive; a, c, d, acid, clot, digestion.
Meat, indole, nitrate, catalase, bile, lecithin, lipase and urease tests were all negative. There was slight α haemolysis.

Table 5. Fermentation products of the sporing rods in PYG medium

Product	Concentration of product (mmol/l)		
	U33	U353	C432
Ethanol	9·7	3·3	5·0
Acetate	6·5	5·8	6·0
Propionate	1·8	2·4	1·0
Lactate	78·8	114·0	75·0
Succinate	ND	T	ND

T, Trace; ND, not determined; PYG, peptone yeast glucose.
No other volatile fatty acids up to C6 were produced. Means of three cultures.

The biochemical reactions and fermentation products from growth in PYG medium were as shown in Tables 4 and 5. Carbon dioxide was the only fermentation gas detected. All the strains survived heating at 80°C for 10 min. The G + C mol% value for strain U353 was 42·5.

The filter-strip activity was 3 units. This activity survived on subculture for some years, but the ability to degrade straw decreased to almost zero after subculture for a year or so. The sugars remaining in spent cellulose (ball-milled filter paper) medium were glucose, cellobiose, cellodextrins and xylose.

Discussion

NON-SPORING RODS

A number of the cellulolytic rod-shaped bacteria from this digester were assigned to the genus *Clostridium* (Sharma & Hobson 1985a). These bacteria were spore-forming, although sporulation was difficult to produce with some isolates. Sporulation could not be induced in the present isolates even in a sporulation medium used for other poorly sporulating cultures (Sharma & Hobson 1985b). The genus *Eubacterium* seemed suitable for the isolates as they are Gram-positive (although weakly staining), anaerobic, non-spore-forming rods fermenting sugars to a mixture of acids and not forming

propionic acid as a major end product or lactic acid as the sole major end product (Holdeman & Moore 1974).

Eubacterium limosum has been isolated from intestinal contents, mud, the rumen and digested sewage sludge (Genthner *et al.* 1981). These authors suggested that methanol utilization, also shown for the type strain of *E. limosum*, was a definite property of the species. Strain C376 grew very poorly on methanol in a medium very similar to that of Genthner *et al.* (1981). Strain C376 also differed from *E. limosum* in some other properties and no cellulolytic strains of this species have been recorded. Cellulolytic bacteria classified as *E. cellulosolvens* (formerly *Cillobacterium cellulosolvens*) have been isolated from the rumen. The properties of *E. cellulosolvens*, particularly fermentation products, reported by van Gylswyck & Hoffman (1970) and Prins *et al.* (1972), vary but ethanol production was not reported. The present isolates, while broadly similar to the strains of *E. cellulosolvens* in sugars fermented, do not correspond in sucrose and fructose fermentations which are given as a diagnostic test for *E. cellulosolvens* (Holdeman *et al.* 1974).

The present isolates are thus similar to *Eubacterium* spp., but the G + C ratio differs considerably from the one value reported for *Eubacterium* of 49% (*E. limosum*, Genthner *et al.* 1981). The G + C ratio of C376 was 26%. This is more in accord with the most frequent values for *Clostridium* (25%–30%, Gottschalk *et al.* 1981). The production by strain C376 of butyrate and ethanol amongst other fermentation products also links the isolates to the motile, saccharolytic clostridia, and cellulolysis is a common property of this genus. The present status of *Eubacterium* is also uncertain so it seems best to suggest that the isolates are non-sporing members of the genus *Clostridium*, but at the moment not to give a species name.

SPORING RODS

The general properties of the isolates, and especially the almost homolactic fermentation, suggest a classification as *Sporolactobacillus*. *Sporolactobacillus inulinus* is the only recognized species of the genus at present (Kitahara 1974). The strains under discussion differ from *S. inulinus* in strongly fermenting some of the pentoses and lactose, cellobiose, melezitose, salicin and aesculin. They also ferment xylan and dextrin, and weakly hydrolyse gelatin. The reaction with milk also differs. Kitahara & Suzuki (1963) and Kitahara & Lai (1967) said that *S. inulinus* did not produce fermentation gas. Our strains produced a very small amount of gas in Durham tubes which was found to be CO_2.

Doores & Westoff (1983) looked for sporolactobacilli in environmental samples using enrichment and heat shock as selective methods. They concluded that sporolactobacilli were not prevalent in the environment, but they did isolate two strains from soil. Soil could be a general source of these bacteria in cattle faeces, and the anaerobic digester may provide a suitable enrichment system.

There are some variations between the isolates, but these are possible within a species. It was proposed that these isolates should be classified as a new species of *Sporolactobacillus* and given the species name *cellulosolvens* with U353 as the type strain. However, the cultures, both those preserved by lyophilization after some time in subcultures and those kept by subculture, had lost cellulolytic activity by the time they were deposited with a culture collection. It seemed best, therefore, to refrain from naming the species until more strains have been isolated, possibly from other digesters.

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