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ISOLATION AND CHARACTERIZATION OF *CLOSTRIDIUM HOBSONII* COMB. NOV.

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Abstract

A search for organisms yielding commercial products from bioresources resulted in a group of 37 isolates from a cattle-waste digester. Using strict anaerobic technique, the organisms were cultured by direct isolation and continuous perfusion on straw, ball-milled cellulose and dewaxed cotton as substrates. Gram-negatively staining rods (though showing a Gram-positive wall in electron micrographs) were straight to slightly-curved with tapering ends, 0·25–0·7 × 1·5–3·5 μm, with peritrichous flagellation. Cultures showed extensive degradation of filter paper; 27–42·7% and up to 31% solubilization of straw and cotton respectively. Cellobiose, -triose and -tetrose, but not glucose, resulted from cellulose hydrolysis. Fermentation products were ethanol, acetate, formate, lactate, succinate and, with some strains, propionate, with CO_2 and H_2 as fermentation gases. Though major tests were done on all, isolates U311, U191, T111, U62 and C400 were examined extensively. All the test strains showed strong to moderate fermentation of glucose, cellobiose, aesculin, maltose, fructose, lactose, mannose, melibiose, ribose, starch, xylose, xylan, raffinose and sucrose. The mol% G+C ratio of the DNA for three strains was 39·5–43·9 (T_m). Characteristics of the organisms isolated overlapped with those of Eubacterium cellulosolvens *NCDO 2433, 2430, ATCC 43171;* Clostridium cellobioparum *ATCC 15832 and* Clostridium thermocellum *ATCC 27405. In the present investigation* E. cellulosolvens *strains produced ethanol, spores in sporulation medium (*Lett. Appl. Microbiol., *1, 31, 1985) and a G+C ratio of 42·5 for strain 2433.* C. thermocellum *grew at 37°C. Under recommendations 3–4, rule 42 of the international code of nomenclature of bacteria, we propose the name* Clostridium hobsonii *for U311, a species of biotechnological importance, in honor of P. N. Hobson B.Sc., Ph.D., D.Sc., F.I.Biol., F.R.S.C., F.R.S.E.*

Key words: Anaerobic bacteria, taxonomy, *Clostridium hobsonii, Clostridium thermocellum, Clostridium cellobioparum, Eubacterium cellulosolvens*, silver staining, cellulose, hemicellulose, xylan, anaerobic digestion, SDS-PAGE, rumen bacteria.

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INTRODUCTION

The role played by anaerobic micro-organisms in the digestion of organic matter, and in particular cellulose and hemicellulose, is important and so investigations have been made into the exact role of these micro-organisms in the rumen, as well as in systems for the production of liquid and gaseous fuels with concomitant pollution control, and other products of industrial importance like acids, solvents and chemicals (Hobson *et al.*, 1984). The rumen has been the principal system investigated. In the rumen, two morphological types have been implicated as the main agents of cellulose breakdown: cocci (two species of *Ruminococcus*) and a rod (*Fibrobacter succinogens*, formerly known as *Bacteroides succinogenes*; Montgomery *et al.*, 1988) with a further rod type (*Butyrivibrio fibrisolvens*) playing a generally smaller role. Occasionally, representatives of other genera (e.g. *Clostridium*) or other unnamed morphological types have been isolated, but some of these have appeared to be fortuitous and have never been consistently found. For instance, *Clostridium cellobioparum* was isolated by Hungate (1944) and since then no report has appeared of its reisolation. There was a gap of more than a decade between the isolation of *Eubacterium cellulosolvens* by Bryant *et al.* (1958) and its reisolation by Gylswyk and Hoffman (1970) and by Prins *et al.* (1972). *Clostridium thermocellum*, initially isolated by Viljoen, Fred and Peterson in 1926, was next isolated by Ng, Weimer and Zeikus in 1977.

However, lack of continued isolation of a particular species from some environment should not be construed to mean that it does not play an important role. For instance, it was earlier suggested that as *Bacteroides succinogenes* has not been isolated on many occasions or in high numbers, then probably its role in the rumen was of little significance; however, today the concept has changed with its successful reisolation.

In the present report, a number of cellulolytic isolates obtained from a cattle-waste digester by direct

isolation and a continuous-culture technique as described previously (Sharma & Hobson, 1985*a*; Sharma, 1983) are compared with those species which had been classified as *Eubacterium cellulosolvens*, *Clostridium cellobioparum* and *Clostridium thermocellum*, and based on the results *Clostridium hobsonii* comb. nov. is suggested.

METHODS

Cultures

Isolates given in Tables 1 and 2 were from direct isolation and those in Tables 3–5 were from the continuous culture. Two strains of *Eubacterium cellulosolvens* were kindly given by Dr M. D. Collins, National Collection of Dairy Bacteria, National Institute for Research in Dairying, Shinfield, Reading, UK. *Eubacterium cellulosolvens* 2433 was one of the strains isolated by Prins *et al.* (1972) and the other strain 2430 was one of the strains isolated by Gylswyk and Hoffman (1970). *Eubacterium cellulosolvens* ATCC 43171, *Clostridium cellobioparum* ATCC 15832 and *Clostridium thermocellum* ATCC 27405 were procured from the American Type Culture Collection Center, Rockville, Maryland, USA.

Media

Peptone yeast extract (PY), peptone yeast extract glucose medium (PYG), PY-lactate and Py-pyruvate media were as described by Holdeman, Cato and Moore (1977). Medium 1 (Sharma & Hobson, 1985*a*) was used for the maintenance of cultures. ATTC *C. thermocellum* was initially grown in ATCC medium 1191, *C. cellobioparum* in ATCC medium 1015 and *E. cellulosolvens* in ATCC medium 1365. Detailed compositions of ATCC media were as given in *ATCC Catalogue of Bacterial Cultures and Phages*, 18th edn, ATCC, Rockville, Maryland, USA.

All cultures were incubated at 39°C unless stated otherwise. Optical densities were recorded at 640 nm in a Beckman DB spectrophotometer.

Purity of cultures

This was checked periodically both by microscopic examination and fermentation-product analysis over a period of 3 years and properties were found to be consistent.

Atmosphere in culture tubes for biochemical characterization

All the media for biochemical characterizations, unless stated otherwise, were prepared under oxygen-free carbon dioxide (20%) plus nitrogen (80%); this is similar to the procedure recommended by Holdeman, Cato and Moore (1977) for anaerobes. In the present work, the bacteria were isolated from a digester running at a pH of about 7·2 and might be expected to have optimum growth near neutral pH. Conversely, many

Table 1. Fermentation products on PYG medium (mmolar)

Product	Isolate and products												
	U62	C120	U311	T180	C400	T138	U191	T111	T211	C48	T83	U352	T113
Ethanol	89·6	7·6	96·3	70·2	4·1	4·8	92·7	89·9	71·1	70·4	16·1	78·8	86·5
Formate	P	P	P	P	P	P	P	P	P	P	P	P	P
Acetate	7·7	8·8	8·0	5·2	2·7	5·7	4·8	8·1	3·2	5·4	12·8	7·6	7·5
Propionate	0·5	—	—	—	1·1	2·7	—	0·7	0·3	0·8	2·1	0·6	—
Lactate	5·7	P	10·8	P	P	P	(P)	16·0	P	P	P	(P)	16·2
Succinate	1·8	P	7·9	P	(P)	P	7·5	5·2	P	P	P	P	10·4

Averages of duplicates.
P, (P); present but not quantified, present in trace amounts.
—; Nil.
Strain C120 formed some butyrate (5·0), no other n- or i-volatile fatty acids up to caproic were found.

Table 2. Fermentation products on PY, PY-pyruvate and PY-lactate media (mmolar)

Product	Medium, isolate, products											
	PY				PY-pyruvate				PY-lactate			
	U311	T111	C400	U191	U311	T111	C400	U191	U311	T111	C400	U191
Ethanol	24·5	24·1	22·8	14·3	21·4	27·1	27·9	21·4	29·4	52·9	50·3	26·9
Formate	—	—	—	P	P	P	P	P	—	—	—	—
Acetate	13·5	13·6	14·3	18·0	27·5	34·0	31·3	30·0	16·5	14·3	15·0	13·0
Propionate	8·9	0·8	1·5	1·5	7·4	1·7	1·6	1·5	7·4	1·5	1·5	1·2
Lactate	P	P	(P)	P	P	P	P	P	P	P	P	P
Succinate	P	P	ND	ND	(P)	P	P	P	P	P	P	P

Isolate U311 formed n- and i-butyrate (7·1, 3·6) on PY medium and n-butyrate (2·5) on PY-lactate medium.
No other acids were formed.
Symbols as Table 1. ND; not determined. Averages of triplicate cultures.

Table 3. Fermentation properties of MC, GC and A isolates on PYG medium (mmolar)

Product	Isolate and products											
	MC							A			GC	
	1	2	3	7	8	9	10	7	9	10	11	23
Ethanol	44·4	105·1	58·6	25·0	25·0	14·0	30·1	12·2	44·9	87·3	3·8	5·8
Acetate	17·2	14·1	10·2	14·7	9·8	5·9	11·1	13·4	10·0	25·0	25·3	10·3
Propionate	—	0·1	—	0·4	—	1·0	0·1	0·6	—	—	0·6	0·3
Butyrate	—	0·7	—	—	—	—	—	1·7	—	0·5	—	—
Lactate	P	2·3	5·7	P	4·3	15·5	3·1	14·4	8·6	14·0	2·7	6·6
Succinate	P	1·6	4·2	P	9·5	2·8	1·3	1·6	5·2	0·9	5·4	4·6

Formate was produced by all isolates but not quantified.
P; present but not quantified.
—; Nil. i-Butyrate, n- and i-valerate and n- and i-caproate were not produced.
Incubated at 39° for 5 days, averages of 2 or 4 repeats of duplicate cultures.

Table 4. Fermentation properties of F isolates on PYG medium (mmolar)

Product	Isolate(s) and products											
	F 1	2	5	6	7	8	9	10	11	12	13	14
Ethanol	19·0	19·3	19·2	29·3	6·2	28·0	59·0	4·3	—	1·0	0·6	0·6
Acetate	13·1	7·9	15·1	28·4	5·9	13·6	7·7	7·5	7·5	14·0	10·0	9·0
Propionate	0·8	1·3	1·4	0·1	0·5	0·5	0·1	—	4·5	4·0	4·3	3·8
Lactate	2·8	P	10·6	5·4	25·4	12·3	5·7	P	4·2	0·9	8·7	P
Succinate	1·2	P	19·9	3·5	1·9	12·3	2·5	P	30·6	37·8	23·3	P

Formate was present but not quantified for all isolates. Isolate 2 produced 4·0 mM valerate.
All other acids as Table 1 were not detected.
For symbols see Table 1.

anaerobes require carbon dioxide. A gas phase of 100% carbon dioxide tends to give media of rather low pH after autoclaving, but with carbon dioxide and nitrogen mixtures a higher pH can be obtained, while keeping carbon dioxide and bicarbonate in the medium in equilibrium. The pH was measured with a meter with an electrode in the open culture tube, with the medium kept in equilibrium with bubbled carbon dioxide. Fermentation of a test compound was determined by comparing the pH of the culture, visual turbidity of growth and gas production, with an uninoculated culture.

Atmosphere in anaerobic jars

Cultures in anaerobic jars containing two sachets of cold palladium catalyst were under an atmosphere of 10% carbon dioxide, 90% hydrogen. Main characterization tests were done as recommended by Holdeman, Cato and Moore (1977), with a few modifications as described previously (Sharma, 1983).

Sporulation

This was tested by the methods described previously (Sharma & Hobson, 1985*b*).

Fermentation products

Acids, alcohols and gases were determined as described previously (Sharma & Hobson, 1985*a*).

Cellulolytic activities

Filter-strip disintegration, loss in weight of dewaxed cotton or of non-delignified barley straw were determined by the methods described previously (Sharma & Hobson, 1985*a*).

Qualitative analysis of sugars from cellulose hydrolysis

This was done to compare the new isolates with *E. cellulosolvens*, *C. cellobioparum* and *C. thermocellum*. The final method employed, after inadequate separation in our hands using the method of Vomhoff and Tucker (1965), was as follows:

The sugars were separated on TLC plates (20 × 20 cm) coated with homogeneous Kiesel Gel G60 (Merck). The slurry was prepared by homogenizing 25 g of Kiesel gel in 75 ml water for 2–3 min in a glass blender. Well-cleaned plates were rinsed with methanol and the slurry was spread with a Shandon apparatus. The plates were dried for 2–3 min in the air before drying for 5–10 min at 100°C. Plates were stored in a desiccator until used. The solvent mixture was *iso*-propanol:water:ethyl acetate (6·5:4·5:9·0).

Cultures with ball-milled cellulose were incubated for 3 months at 39°C and then 3 ml of supernatant was dried *in vacuo* in a desiccator and the solids taken up in the minimum amount of 50% *iso*-propanol. Standards were 1% solutions of glucose, cellobiose, xylose, cellotriose, cellotetrose and rhamnose in 50% *iso*-propanol.

Samples and standards were applied with a micropipette 2 cm above the bottom edge of a plate on which the gel film had been grooved 0·5 cm from each side to eliminate edge effects (Brown & Benjamin, 1965).

The plates were developed by allowing the solvent to ascend twice to 15 cm, with intermediate drying in the air current in a fume cupboard for 2 h (Thoma, 1964). The sugars were visualized by spraying with anisaldehyde (*p*-methoxy benzaldehyde: Stahl and Kaltenbach, 1961), and then drying at 100°C for 25 min. Only spots characterized by running with the standard sugars are mentioned in the results. In some cases, unidentified pink or blue spots were also present on the plates.

Desulfoviridin detection

This was done by the method of Postgate (1959) and also by a modified method (Sharma & Hobson, 1987).

Detection of dipicolinic acid (DPA)

The presence or absence of dipicolinic acid (pyridine-2,6-dicarboxylic) acid was detected by the method of Janssen, Lund and Anderson (1958).

Hibitane resistance tests

Since Hibitane (chlorohexidine gluconate; ICI Chemicals Ltd, UK) is autoclavable in low concentrations, it was sterilized in 7·5 ml of PYG broth in the following concentrations (μg/ml of the medium): 0·1, 1·0, 10·0 and 20·0.

SDS-polyacrylamide-gel electrophoresis

Sample preparation

Samples of centrifuged (10 000***g***, 30 min) and washed cells were lysed by sonication and subsequently spun to remove debris. Phenyl methyl sulfonyl fluoride (PMSF; 50 μl of 100 mM stock solution in isopropanol per ml of cell suspension) was added to inhibit serine proteases, and EDTA (1 μl/ml-final concentration) was added to inhibit metallo-proteases. PMSF and EDTA were added before lysis by sonication. Protein estimations, to quantitate equal amounts of protein load in each lane, were done by the use of the bicinchoninic acid (BCA-Pierce) method of Smith *et al.* (1985).

An equal amount of protein load (2 μg each) was used for the comparison of *C. hobsonii* with the other test cultures.

Electrophoresis

Gel preparations and running conditions were essentially as per the method of Laemmli (1970), with the following modifications:

- Bis-acrylamide was replaced by piperazine diacrylamide (BioRad) on a weight-to-weight basis, as developed and recommended by Hochstrasser *et al.* (1988*a*, *b*) to reduce background staining, increase physical strength of the gel and to reduce swelling of the gel as is normally seen with the gels made with bisacrylamide as the cross-linker.
- Acrylamide solution N (30%; 146 g acrylamide and 4·0 g piperazine diacrylamide dissolved in 500 ml deionized water) was mixed with two scoops of activated carbon and stirred for 2 h, left to stand for 1 h and then filtered through a 0·22 μm Millipore filter and stored at 4°C in an amber bottle. Charcoal treatment bleached away some impurities and color in the acrylamide.
- SDS was not used in the separating gel [12·5%, 20·8 ml of acrylamide solution N, 12·5 ml of 1·5 M Tris HCl, pH 8·8, and 16·7 ml deionized water] but its concentration was increased to 0·2% in the gel-running buffer, this was done to reduce the interference caused by SDS in silver staining the gel (Dunn & Burghes, 1983; Hochstrasser *et al.*, 1988*a*, *b*).

Gels with 12·5% T (1·5 mm thick) were run at 15 m amp gel stacking time, and 30 m amp/gel as the dye reached the separating gel and the current was kept at this value until the dye front reached the end of the gel. Gels were run in a Hoeffer gel chamber SE 600 wherein the gel-running buffer was cooled by circulating cold water (10°C). Generally, the following proteins (BioRad) were subjected to electrophoresis as described above for SDS-PAGE and served as molecular-weight standards: β-galactosidase, 116 250; phosphorylase b, 97 400; bovine serum albumin, 66 000; ovalbumin, 45 000; carbonic anhydrase (bovine), 31 000; soybean trypsin inhibitor, 21 500 and lysozyme, 14 400.

Silver staining of the gel

The method used was an adaptation from Oakley *et al.* (1980), Switzer *et al.* (1979), Adams and Sammons (1981), Huerkshoven and Dernick (1985) and Hochstrasser *et al.* (1988*a*). The protocol worked out by experimentation was as follows. The reagent volumes given below applied to two 1·5 cm thick, 16 × 20 cm gels per glass tray. Larger or smaller volumes might be appropriately made, depending on the gel thicknesses and/or numbers.

The gel was washed for 1 min in 400 ml deionized water to remove SDS.

Protein fixation: the gels were then soaked in 400 ml of ethanol:acetic acid:water (40:10:50) for 2 h, or in 20% TCA, and kept on an orbital shaker at 40 rpm. Methanol was not used at any step of the fixation, in order to avoid background staining. At times, the gel was left in this solution overnight and processed the next day.

The fixing solution was then replaced by 400 ml of ethanol:acetic acid:deionized water solution (10:5:85) and the gel washed in this solution for three changes of 30 min each.

The gel was further washed for two changes of 30 min each in 400 ml of deionized water.

The gel was placed in a staining solution (an

ammoniacal silver nitrate solution) for 10 min. The ammoniacal silver nitrate solution was prepared as follows. A 20% $AgNO_3$ solution (10 ml) was poured into 50 ml of deionized water in a 1 l beaker and put on the magnetic stirrer. Ten milliliters of concentrated ammonium hydroxide solution was slowly added so that the brown precipitate formed disappeared on further stirring. Then 15 ml of 1 M NaOH solution was added and well mixed. The solution was diluted to 1 l with deionized water and mixed well with the magnetic stirrer. The solution was crystal clear. This solution was not reusable.

The silver nitrate solution was decanted and the gel washed with three changes of 5 min each of 200–300 ml deionized water, followed by agitation for 2 h in fresh deionized water.

The deionized water was decanted and 300 ml of ammoniacal silver nitrate solution added and allowed to react with the gel for 90 min and the gel washed with four changes of 5 min each of 300–400 ml deionized water.

The water was decanted and 400 ml of reducing solution (0·5 ml of 37% formaldehyde and 5 ml of 1% citric acid in 1 l of deionized water) added. The usual development time was 15–20 min. The development was stopped by decanting the reducing solution and treating the gel for at least 1 min with 300 ml of 1% acetic acid solution (stop bath).

The stop bath was drained and the gel was given three washes (5 min each) with 300 ml deionized water.

Color enhancement to reinforce variegated color bands was achieved by agitating the gel for 2 h with 1% sodium carbonate solution, which was replaced with a fresh solution after an hour. Color enhancement given without the stop bath treatment or the subsequent washing could have led to a gel with a dark background.

Some gels were recycled to stain some weakly-staining bands by extensively washing the gel with the deionized water to remove the stop bath, or else omitting the stop-bath step, extensively washing the gel with deionized water to remove formaldehyde and starting the recycling with the ammoniacal silver nitrate solution treatment.

Gels which were overstained were destained by the use of Farmer's reducer (Kodak Photography product).

Gels from the pouring to the development stage were handled while wearing disposable gloves, to avoid fingerprinting the gel, to prevent the skin protein mixing with the sample because of the use of SDS and to avoid the toxic effect of acrylamide on the skin.

G+C ratio

Values were determined essentially by the procedure described by Gibson and Ogden (1979).

Optimum pH and temperature for growth

Optimum temperature for growth was monitored by growing test culture in 7·5 ml PYG broth at temperature intervals of 5°C from 20 to 50°C and 2°C from 35 to 39°C. Optical density and terminal pH was recorded after 20 h of growth. A control with the same inoculum (two drops) as the experimental cultures was prepared immediately before recording the optical density to account for background turbidity. In a few cases growth was also tested at 4 and 10°C. Media for optimum pH required some modifications as the pH tended to change after autoclaving. So, sodium bicarbonate was omitted from PYG medium and it was dispensed under 100% O_2-free nitrogen. The following pH values were tested: 4·0, 4·5, 5·0, 5·5, 6·0, 6·5, 7·0, 7·5 and 8·0. In a few cases, growth was also tested at pH 9·6. There was no caramelization of sugars at pH 4·0 and only a very slight browning occurred at pH 8·0, which was compensated for by using tubes of the same pH as controls against the test cultures. Optical density and pH were observed at 20 h intervals.

Adaptation tests

Adaptation experiments for growth at higher temperature were conducted for cultures U311, T111, U62 and C120. The adaptation experiments were first conducted in PYG medium and then in the following three media, developed during the course of the investigation: L-3, L-3-1 and L-3-2; the latter two were variations on the L-3 medium. L-3 medium had the following composition (g/100 ml): Bacto-casitone, 0·3; glucose, 0·5; cellobiose, 0·6; K_2HPO_4, 0·03; KH_2PO_4, 0·2; sucrose, 6·844; trisodium citrate, 0·3; $MgSO_4$, 0·3; sodium lactate, 0·5; NaCl, 0·01; $CaCl_2 . 2H_2O$, cysteine HCl 0·1 and volatile fatty acids, vitamins and trace elements as used for medium 2 (Sharma & Hobson, 1985*a*). L-3-1 and L-3-2 media were as for the above medium except that L-3-1 contained only glucose while L-3-2 had only cellobiose; this was done on the assumption that some strains might show a preference for one of the two carbon-sources and the presence of the other might interfere with the utilization of the substrate of choice.

Inocula were prepared from the cells by centrifugation and by then carefully transferring the sediment to a fresh medium. A large inoculum was used as it seemed very likely that only a small number of the cells growing in a culture would be adapting relatively rapidly, and the usual procedure of transferring small inocula proved inadequate.

Increase in temperature of incubation was gradual (1°C per sub-culture) after transfers (usually 10) at 45°C onwards.

RESULTS AND DISCUSSION

For the amended description of *Clostridium hobsonii* comb. nov., the present isolates were compared with reported properties of *E. cellulosolvens*, *C. cellobioparum* and *C. thermocellum*, except for a few properties which were tested in the present work. The general properties of present isolates are shown in Tables 1–5.

Table 5. Gas composition in the head-spaces of PYG cultures

Isolate	Gas (%)			
	N_2	CO_2	CH_4	H_2
U311	36·2	58·3	—	5·4
U191	35·6	56·3	—	8·1
T111	36·4	59·0	—	4·6
Control	90·0	10·0	—	—

Average of triplicates. —; Nil.

Colony form

E. cellulosolvens
Surface colonies 3–5 mm, circular, entire, flat, slightly translucent; deep colonies lenticular (Bergey, 1974). Deep colonies in cellulose rumen-fluid agar have sharp edging and are light tan in color. Some colonies show branching which gives them a star-like appearance; one strain produces woolly colonies (Prins *et al.*, 1972).

C. cellobioparum
Surface colonies 0·5–1·5 mm, circular, entire, convex, opaque, creamy white to yellowish, glossy (Bergey, 1974). Young colonies become more complex in shape, often by growth of a daughter disc at right angles to the original one (Hungate, 1944).

C. thermocellum
Surface colonies watery, slightly convex, frequently with an insoluble yellow pigment (Bergey, 1974). Deep colonies of three strains in CM 3 agar (Ng, Weimer & Zeikus, 1977) were tannish yellow, roughly round but filamentous. All strains produced a yellow pigment on cellulose which was not evident on cellobiose.

Present isolates
Surface colonies on cellobiose agar (medium 2; Sharma & Hobson, 1985*a*) were watery, slightly convex, with an insoluble pigment and a light tan color which became pronounced in transmitted light. Colonies in cellulose roll tubes (medium 2) were mainly circular, although some were lenticular. In some colonies, the margin gave off shoots and one isolate produced woolly colonies. The description shows that with the named genera and present isolates colonies are similar in shape, size and color.

Cell morphology

E. cellulosolvens
In young cultures cells are Gram-positive and show long chains with a peculiar zig-zag pattern. In older cultures, cells become Gram-negative and the chains break up to singles and pairs. The cells are 0·7 and 3 μm (typically 1–2 μm) in length by 0·5–1·2 μm in width (typically 0·6–0·8 μm) (Prins *et al.*, 1972) with peritrichous flagellation.

C. cellobioparum
Two cells may remain attached and on one occasion in a colony growing in cellulose agar a long spiral of cells was seen. Cells are Gram-negative rods 3–5 μm long and 0·3–0·4 μm wide, appearing slightly curved (Hungate, 1944) with peritrichous flagellation. The cells decolorize easily (Holdeman, Cato & Moore, 1977). This could be taken as saying that the cells are Gram-positive.

C. thermocellum
These have been described as Gram-negative rods, 0·5–0·6 × 4–5 μm (Ng, Weimer & Zeikus, 1977) and Gram-negative rods 0·6–0·7 × 2·5–3·5 μm (Bergey, 1974, ATCC 27405, NCIMB 10682), with peritrichous flagellation.

Present isolates
Long filamentous chains, which broke mainly into pairs, could be seen in young cultures. The cells were Gram-negative but had a Gram-positive wall structure in electron micrographs (EM — Fig. 1). It has been reported that Gram negative staining by cells having Gram positive wall is often seen (Sharma & Hobson, 1986). Cells were straight to slightly-curved rods, with tapering ends, 0·25–0·45 × 1·5–3·5 μm, though longer cells up to 4–5 μm could be seen in the same isolates and wider cells 0·65–0·75 μm, were seen in some isolates. One isolate was shown to be motile with peritrichous flagellation (Fig. 2). All others with vitally-stained cells appeared motile in wet mounts.

Spores

E. cellulosolvens
Spores have not been reported. However, in the present investigation, NCDO strains 2433 and 2430 and ATCC 43171 survived heat at 80°C for 10 min after sporulation in the medium described previously (Sharma & Hobson, 1985*b*), although it took more than a week of subsequent incubation for these strains

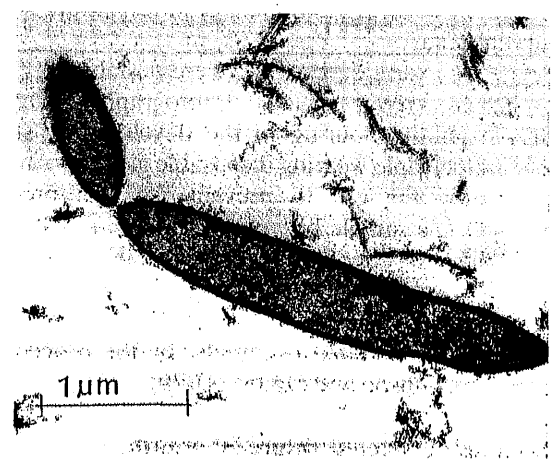

Fig. 1. Electron micrograph of a transverse section of *C. hobsonii* (culture number U311). Note Gm+ve cell-wall structure.

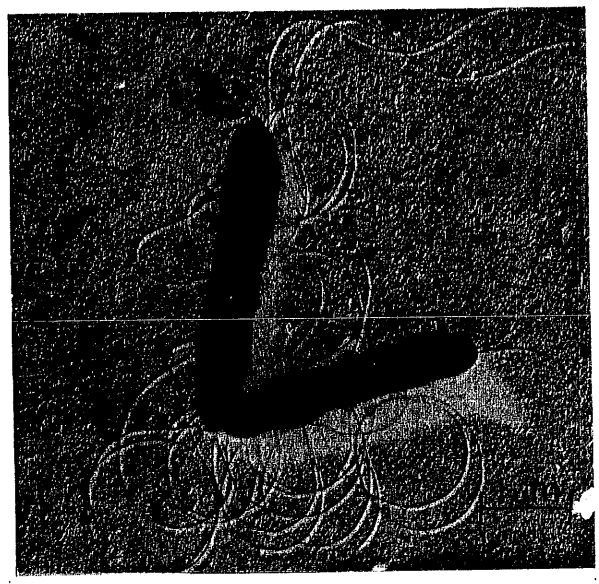

Fig. 2. Shadow casting of *C. hobsonii* comb. nov. (isolate number U311) with gold–palladium on formvar-coated grid, 30°.

and the present isolates to give turbidity. Strain 2433 showed better growth than strain 2430. Microscopic examinations, and in a few cases fermentation products also, were used to rule out the possibility of contaminants.

C. cellobioparum
Spores rarely remain attached to cells. Although spores were originally described as spherical (0·9 μm) by Hungate (1944), the same strain has been reported as forming spherical to oval terminal spores (Bergey, 1974).

C. thermocellum
Spores are terminal, ovoidal (Ng, Weimer & Zeikus, 1977), but size has not been reported. According to Avegerinos and Wang (1980) the type strain, ATCC 27405, produces spherical spores on solid media but not in broth.

Present isolates
In liquid media these had terminal, spherical spores rarely attached to cells, about 0·9–1·2 μm, the larger size occurring rarely and only in cultures with the larger cell-size. Only one isolate produced spores (1 per 10 fields) on solid media.

The above results suggest, again, that all these genera are the same. *Eubacterium* either does not sporulate easily or produces detached spores which could have escaped earlier detection. A number (U62, 191, 313, 316, 352; T111, 179; C121, 122) of the present isolates showed some terminal regions which did not take up the spore stain. Ellner's medium (1956), which will sporulate the poorly sporulating *C. welchii*, provided heat resistance only up to 70°C for 10 min for 10 of the present isolates, thus confirming the finding of Duncan and Strong (1968) that Ellner's medium does not provide heat-resistant spores. However, all these cultures sporulated well in the sporulation medium described previously (Sharma & Hobson, 1985*b*), and produced heat-resistant spores (80°C).

Sugars liberated from cellulose

E. cellulosolvens
No glucose detected, but it can use glucose (Prins *et al.*, 1972).

C. cellobioparum
No glucose detected in 3 month-old spent cultures, but it can use glucose (Hungate, 1944).

C. thermocellum
No glucose detected (Ng, Weimer & Zeikus, 1977), but it metabolizes glucose, although it has some preference for cellobiose (Ng & Zeikus, 1982); this contradicts the earlier reports that glucose was not metabolized.

Present isolates
Glucose was not detected in spent culture liquids (concentrated or otherwise), though cellobiose, -triose and-tetrose were found.

Fermentation products

E. cellulosolvens
The type strain $B_3$48 forms primarily lactate with small amounts of acetate and formate but no butyrate, propionate, succinate or hydrogen (Bryant *et al.*, 1958). Strains isolated by van Gylswyk and Hoffman (1970) produce small amounts of butyrate, valerate and hydrogen.

Holdeman, Cato and Moore (1977) reported the production of lactate, formate, butyrate and succinate, but it may or may not produce propionate. Prins *et al.* (1972) found two groups, one conforming to the type culture (Bryant's $B_3$48), the other producing substantial amounts of hydrogen, formate and butyrate in addition to lactate and taking up acetate from the medium. Small amounts of propionate were also produced. However, Prins *et al.* (1972) thought that, by looking at the carbon recoveries, one compound was lacking in fermentation products and this was presumed to be ethanol. One strain isolated by them was shown to produce 0·5 mM ethanol in the present investigation. Uptake or production of acetate by *Butyrivibrio fibrisolvens* depends on cultural conditions (Bergey, 1974) and even Prins *et al.* (1972) found one of their strains took up, rather than produced, propionate. So the uptake of acetate by their second group, apart from strain variation, could also be a reflection of cultural conditions.

C. cellobioparum
It produces ethanol, acetate, formate, lactate, succinate, CO_2 and H_2 (Hungate, 1944).

C. thermocellum
It produces ethanol, formate, acetate, lactate, succinate, CO_2 and H_2 (Bergey's Manual, 1974). However, for the same type strain (ATCC 27405), formate production is not reported in the 1984 edition. Viljoen *et al.* (1926) reported the production of formate and also some butyrate with some strains. McBee (1950) also detected formate with *C. thermocellum* strains.

Present isolates
These produced ethanol, acetate, formate, lactate and succinate, with some propionate; U311 produced some butyrate on PY medium and C120 some on PYG medium. Only one isolate, F-2, out of 37 tested produced small amounts of valerate in addition to other products. Ethanol production was quite variable (Tables 1–4). The lack of ethanol detection with *E. cellulosolvens* could be because of a lack of sufficiently sensitive analytical methods. Prins *et al.* (1972), using an enzyme assay, did not find it, but in the present work it was found with one of the strains. The *Anaerobe Manual* does not report ethanol for *C. cellobioparum* but Hungate (1944) detected it: CO_2 and H_2 were also produced by the isolates tested.

The fermentation products are similar in the named genera and the present isolates. The amount of a particular product formed can vary from strain to strain of a species and even for the same strain under different conditions of growth.

Optimum temperature and pH of the present isolates
Isolate U311 was examined in detail, with results being determined after 20 h of growth. Growth was nil at pH 5·0 and 8·0 and there was a sharp optimum at pH 6·5. A little growth was apparent at 20°C, but none at 45 and 50°C and the optimum was 35–37°C. Optical density measurement to observe bacterial growth is a widely-followed method (Stabholz *et al.*, 1993*a*, *b*; Lyte & Ernst, 1992; Li *et al.*, 1993; Newbold & Rust, 1992) and compares favourably to the plate count method (Li *et al.*, 1993). Meng *et al.* (1991) have also shown that bacterial growth measurements by optical density or by colony counts do not show any statistically significant differences; this means optical density measurement can reflect the specific growth-rate of a given organism under the test parameters.

Other biochemical properties of the present isolates with those of the test cultures are listed in Table 6.

Cellulolytic activity
Only two isolates obtained in the present work by direct isolation showed filter-strip disintegration of three units (an arbitrary scale described previously; Sharma & Hobson, 1985*a*) and loss in weight of ground, untreated straw of 25·8–27%. For the rest of the isolates, loss in weight of straw varied and was up to 42·7%, and filter-strip disintegration was 4–5 units. As shown in Table 8, this activity stayed more or less the same, unlike the cellulolytic *Sporolactabacillus* species (Sharma & Hobson, 1986) which lost much of its activity. McBee (1950) found that the *C. thermocellum* strain isolated by Viljoen *et al.* (1926) had lost cellulolytic activity. Dewaxed cotton solubilization by the present group of isolates from continuous culture was up to 31% (data not presented).

Sulfate reduction
Some of the present isolates reduced sulfate and formed sulfides from cysteine. Cultures kept at 50°C overnight reduced sulfate on further incubation at 39°C, but cultures kept at 60°C did not, although the cells remained viable; this could suggest that in these isolates sulfate reduction is plasmid mediated and is 'cured' at elevated temperature (60°C), unlike reduction in *Desulfotomaculum nigrificans*.

The present isolates were found to grow in the presence of 0·1–1·0 μg/ml of Hibitane, but not in the presence of 10–20 μg/ml. As sulfate reduction seemed to be plasmid mediated and 'curable', it is not a constant taxonomic character which can be used in definition of a species. Prevot (1948) also observed that clostridia lose and acquire the property of sulfate reduction.

Adaptation tests
It is well known that high-temperature adaptation requires that cells show some structural and functional adaptation in their enzymes and proteins (Zuber, 1979). However, no unequivocal reason (Lungdahl, 1979; Zuber, 1981) for the exact basis of thermophily is known which would provide a basis for producing an environment fostering quick adaptation of the culture to higher temperature, though transfer of thermal-resistance factors has been suggested as a possibility in some cases (Castenholz, 1979).

The formulation of the media was to meet some of these changes produced in the cell at high temperature. For instance, a higher sucrose concentration was used as an osmotic stabilizer to eliminate the pleomorphic forms observed in PYG medium at higher temperature. Divalent cations can stabilize some protein molecules (Langworthy *et al.*, 1979) so $MgSO_4$ and $CaCl_2$ were included in higher concentrations than normally used in medium 2. The initial pH was 6·9 and the final pH values of cultures were 6·4–6·5.

L-3 medium, with its variants L-3-1 and L-3-2, was found to eliminate the pleomorphic forms found in PYG medium at elevated temperature. However, growth could not be induced beyond 50°C. So after a few unsuccessful attempts at higher temperatures the experiment was abandoned. It was concluded that although growth at higher temperature through adaptation seemed possible, it would require a much longer time than that available. Though the adaptation of the present isolates to higher temperature growth was successful up to 50°C in the time available, the adaptation of cells growing at higher temperature to lower temperature is more convenient. It was reported earlier (Sharma & Hobson, 1985*a*; Sharma, 1983) that *Desulfotomaculum nigrificans* NCIMB 8395 (T-range

Table 6. General properties of the present isolates and named species

Test	U311	U191	T111	U62	C400	EC1*	EC2	EC3	EC4	CC1	CC2	CT
AMC	—	—	—	—	—	N	N	N	—	—	—	
Arabinose	—	—	—		(+)	1	—	—	—	+	+	+
Adonitol	(+)	—				N	N	N	—	N	—	
Cellobiose	+	+	+	+	+	+	+	+	+(+)	+	+	+
Dulcitol	(+)	—				N	N	N	—	N	+	
Dextrin	(+)	(+)	(+)	—		+	—	—	N	(+)	N	
Aesculin pH	—	—	—		(+)	N	N	N	—	(+)	N	—
Aesculin hyd	+	+	+		+	4(2)	+	+	+	N	+	
Fructose	+	(+)	+	+	+	+	+	5	—	+ –	+ –	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	(+)	(+)				N	N	N	—	N	—	
Inulin	—	(+)	—		(+)	5(1)	+	+	—	—	—	
Lactose	(+)	(+)	(+)	(+)	(+)	4(1)	—	+	+ –	(+)	(+)	+
Maltose	(+)	(+)	(+)	(+)	+	+	+	+	+(+)	+	+	+
Mannitol	—	—	—		(+)	—	—	—	—	(+)	(+)	
Mannose	(+)	+	(+)		(+)	1(1)	N	—	—	+	+	+
Melezitose	(+)	(+)	—		(+)	—	N	N	—	—	—	
Melibiose	(+)	(+)	(+)		+	3(2)	N	N	—	+	+	
Raffinose	(+)	(+)	(+)	+	+	+	N	7	–(+)	(+)	(+)	+
Galactose					+	N	+	(+)	(+)	+	+	
Rhamnose	—	—	—		(+)	—	N	—	—	—	(+)	
Ribose	(+)	(+)	+		(+)	N	N	N	—	N	+	
Salicin	(+)	—	—		(+)	+	+	+	–(+)	—	(+)	
Sorbitol	(+)	—	—			N	N	N	—	N	—	
Starch hyd	+	+	+	+	+6	N	—	—	N	—	+	
Sucrose	+	+	+	+	+	+	+	+	+(+)	(+)	—	+
Trehalose	(+)	—	—		(+)	1	N	—	+ –	—	—	
Xylose	(+)	+			+	5	—	—	—	+	+	+
Xylan	(+)	(+)	(+)	(+)	(+)	5	—	4	N	+	N	+
Gelatin	+	+	+	+	+	N	N	N	(+)	N	—	
Milk	RCD	RCD	RCD	RCD	RCD	N	N	N	C–	N	a	
Meat	—	—	—	—	—	N	N	N	—	N	—	
Indole	—	—	—	—	—	N	N	N	—	N	—	
Nitrate red	—	—	—	—	—	N	N	N	—	N	—	
Catalase	—	—	—	—	—	N	N	N	—	N	—	
Bile	—	—	—	—	—				+		—	
Erythritol	—	—	—	—	—	N	N	N	—	N	—	
Lecithinase	—	—	—	—	—	N	N	N	—	N	—	
Lipase	—	—	—	—	—	N	N	N	—	N	—	
Haemolysis						N	N	N	N	N	—	
Motility	+	+	+	+	+	+	+	+	+	+		
Spore	+	+	+	+	+	—	—	—	—	+	+	+
Urease	—	—	—	—	—	N	N	N	—	N	N	
Lactate	E	E	E	N	E	—	N	—	—	N	—	
Pyruvate	A	A	A	N	A	N	N	N	—	N	A	
G+C	N	39·5	43·8	N	43·9	N 42·5*	N	N	N	N	28, 25	39·1 38·1

*EC1, 2, 3, 4. *E. cellulosolvens*: Prins *et al.* (1972); Bryant *et al.* (1958); van Glyswyk and Hoffman (1970); Holdeman, Cato and Moore (1977).
CC1, 2. *Cl. cellobioparum*: Hungate (1944); Holdeman, Cato and Moore (1977).
CT. *Cl. thermocellum*: Viljoen *et al.* (1962); Ng, Weimer and Zeikus (1977).
(+), Weakly positive.
N, Not reported. 4(2) etc., number of strains positive (weakly) out of those tested; 7 for EC1, 9 for EC 3. E, Ethanol. A, Acetic acid.
Blanks under the present strains show tests not done.
No other properties than those shown have been recorded for CT and some of the properties described by Viljoen *et al.* do not now seem to be accepted by Bergey.
RCD, rennet clot digested; C, clot; a, acid.
*Determined in present investigation.
With *E. cellulosolvens* spores were not detected by the authors reported but heat resistance and spores were shown in the present experiments.
In the present fermentation tests a difference of 0·2 pH unit, from the control was taken as weakly positive, 0·4 pH unit, or more as positive.
Production of ethanol would not change the pH, so a smaller pH change than usual was taken as positive. Visual turbidity and gas in Durham tubes (compared with control) were also used as an indicator of fermentation.

Table 7. Filter strip disintegration and loss in weight of water insoluble fraction of straw by some *C. hobsonii* comb. nov. isolates

Isolate number	Filter strip disintegration	Loss in wt. of straw (%)*	Final pH
U62	4	39·6	6·5
U191	5	35·3	6·4
U311	4	41·8	6·5
U352	5	33·8	6·6
T83	3	27·0	6·4
T111	4	37·3	6·1
T113	5	32·2	6·3
T138	3	25·8	6·4
T180	4	36·9	6·4
T211	4	34·6	6·4
C48	5	39·1	6·4
C120	5	42·7	6·0
C400	5	36·2	6·2

Filter strip disintegration showed the maximum disintegration unit 5. Duplicate tubes showed the same units of disintegration.
*Averages of triplicates.

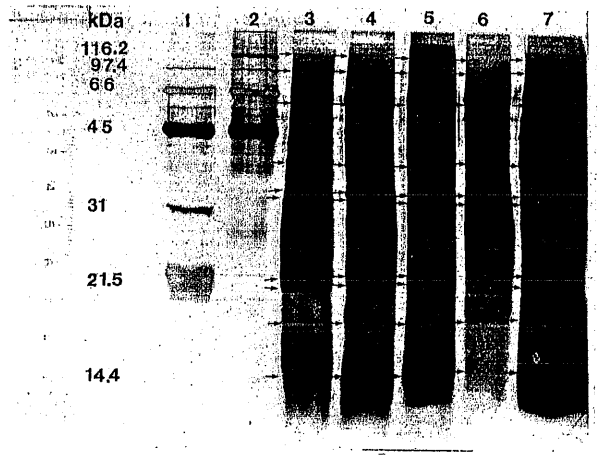

Fig. 3. SDS-PAGE comparison of *C. hobsonii* U 311 whole cell lysates with the whole cell lysates of other cultures. Lanes 1, 2: molecular mass markers; lane 3: *C. hobsonii*; lane 4: *C. cellobioparum*; lane 5: *E. cellulosolvens*; lane 6: *C. thermocellum* growth at 55°C; lane 7: *C. thermocellum* growth at 37°C. Arrows are drawn for only a few polypeptides showing similar positioning, though there are far more which share a similar position; for details see text.

45–70°C, and T_{opt} 55°C) and *Clostridium thermosaccharolyticum*. NCIMB 9385 (T_{opt} 55°C and T_{min} 45°C) could be gradually adapted to growth at 39°C. Likewise, *C. thermocellum* ATCC 27405 could be adapted to show some growth at 37°C. However, it is also well documented that thermophily is a secondary, rather than primary, character (Langworthy *et al.*, 1979) and Egorova (1938) has isolated many thermophiles from Arctic regions. Mesophiles having a growth span extending well into the normally considered thermophilic range have been well documented; for instance, *Bacillus stearothermophilus* strains can grow from 37 to 70°C (*Bergey's Manual*) and *Clostridium thermohydrosulfuricum* and *Thermoanaerobacter ethanolicus* have growth spans over 40°C, covering the usual mesophilic and thermophilic range. Other examples of bacteria growing from 37 to 70°C include, though are not limited to, *Bacillus schlegelii* (Schenk & Aragno, 1979) and *Chloroflexus auranticus* (Pierson & Castenholz, 1974). Other common mesophiles, such as *Bacillus subtilis* (SW-25) and *Bacillus coagulans* (43P) have maximal growth temperatures of 50 and 60°C, respectively (Pace & Campbell, 1967). *Streptomyces albus* can readily grow from 25 to 60°C (Waksman & Henrici, 1943). From the point of view of morphological and biochemical tests described above, the present isolates are very similar to *E. cellulosolvens*, *C. cellobioparum* and *C. thermocellum* and so this suggests that these latter organisms are the same.

Polypeptide profiles (SDS-PAGE)

As is evident from Fig. 3, the polypeptides produced by all cultures generally ranged from lower than 14 kDa to over 116 kDa. *C. hobsonii*, *C. cellobiparum* and *E. cellulosolvens* produced 54, 52 and 54 clearly discernible bands in the molecular range from over 116 kDa to lower than 14·4 kDa. *Clostridium thermocellum* (grown at 55°C) produced 48 polypeptides, mainly in the range from 21·5 to over 116 kDa, whereas growth at 37°C of the same organism produced 51 polypeptides in the molecular range shown by other organisms (mainly 14 kDa to over 116 kDa). This phenomenon of having relatively low concentrations of lower molecular weight polypeptides (usually below 31 kDa) has already been observed with high temperature growth of the one organism: *Methanobacterium thermoautotrophicum*, where growth at 72°C revealed relatively fewer bands below 31 kDa than did growth at 55 or 48°C (Sharma, 1988; other unpublished observations). Apart from this, it is evident that the polypeptide profiles of the cultures tested appear similar, as is shown by the arrows drawn (Fig. 3). Based on polypeptide positioning, intensity and pattern, percentage similarity of *C. hobsonii* and *C. cellobioparum*, *E. cellulosolvens*, *C. thermocellum* (growth at 55°C) and *C. thermocellum* (growth at 37°C) is 87, 92·6, 81·5 and 94·4% respectively.

G+C ratio

Dr Holdeman reported (private communication) that G+C values were not available for *E. cellulosolvens*, so a value was determined here and it was found to be very close to the present isolates. The values for *C. cellobioparum* are rather different. However, the two values reported for the same strain differ in themselves (Hungate, 1944; Holdeman, Cato & Moore, 1977) and variation in G+C values for different strains and species of genera seems to be common. Reported values for *Pseudomonas* vary from 35·1 to 45% and up to 55 and 70%; 29–35% and 37–50% and as high as 62–67% have been reported for *Vibrio* spp. For *Ruminococcus flavefaciens* strains, 39·8–43·5% have been reported (Sharma, 1968), and for 19 strains of

Bacillus stearothermophilus 44–53%. Likewise, species having similar or nearly similar G + C values may not be the same organism; for instance, *Bacillus megaterium* has a G+C ratio of 36–38% which is close to the upper limits of 39·7 for *Staphylococcus aureus*, 39% for *Streptococcus pneumoniae* and 38·4–38·6 for *Streptococcus faecium*. The importance of a particular G + C value as a taxonomic criterion may thus seem unclear. However, Krieg (1968) and Sharpe (1979) suggested that overall morphological, biochemical and physiological characters, as well as G + C ratios, should be taken into account for identification and taxonomic positioning.

Further evidence

Various methods, morphological, biochemical, physiological properties, G + C ratio (Kaneuchi *et al.*, 1976; Sly, 1985; Urakami & Komagata, 1986; Murray, 1986), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Moore *et al.*, 1980; Corbel *et al.*, 1982; Jackman, 1985; Jackman & Pelczynska, 1986; Clink & Pennington, 1987; Barsotti *et al.*, 1988; Slayne *et al.*, 1990) or thin-layer isoelectrofocusing (Gjerde, 1982), immunological studies (Postgate & Campbell, 1963), DNA–DNA hybridization studies (Holdeman & Johnson, 1982; Cummins & Johnson, 1971) or DNA–rRNA hybridization studies (Tanner *et al.*, 1981), aliphatic linear polyamines (Hamana & Matsuzaki, 1992), membrane fatty acids and their derivatives (Brondz & Olsen, 1986; Guckert *et al.*, 1991; DeLuca *et al.*, 1990; Asselineau & Asselineau, 1990), have been used to prove similarities of various taxonomically different species or for simple taxonomic positioning of some unknown species using some reference strains. In the present work, the first approach mentioned, combined with the use of polypeptide profiles (SDS-PAGE) has been used, this is widely followed, time tested and quite comprehensive in itself. Some variation in the polypeptide profile of the same strain is acceptable because of growth conditions or as a result of strain variation. For instance, Bekker, Hogh and Jenssen (1990) have noted variations in the polypeptide profiles of *Fusobacterium nucleatum* as a reflection of growth conditions, and Abath, Almeido and Ferreira (1990) noted variations in polypeptide profiles of *Yersinia pestis* as a result of strain variations. Yotis *et al.* (1991) have also reported some slight variation in polypeptide profiles of serovars a, b and c of *Treponema denticola*, although the banding pattern was reproducible with each serovar on SDS-PAGE analysis. All the characters studied and compared for the proposed species *C. hobsonii* are reflections of the genetic makeup of the strains and show strong similarity. The approach followed in the present work suggests characteristics which are similar and thus prove the similarities of the species.

Taking numerical taxonomy and unweighted group pair arrangements into consideration (this leads to thermophily being only one characteristic), then using 75 operational taxonomy units (morphological, biochemical, physiological and G + C ratios) the isolates of the present group show among themselves a similarity coefficient (S_{sm}) of 92·2%. When the present isolates as a group are compared with *E. cellulosolvens*, *C. cellobioparum* and *C. thermocellum* combined as a group, they show a homology of 93·3%, suggesting that the species under consideration are the same. Since they all fall into one group, a simple dendrogram is not drawn.

CONCLUSIONS

It can be deduced from the foregoing that the present isolates, with isolate U311 suggested as a species type of *C. hobsonii*, share similar morphological characters with *C. cellobioparum*, *E. cellulosolvens* and *C. thermocellum*. Sporulation by some strains of *E. cellulosolvens* tested in this work, including ATCC 43171, is confirmed. The present isolates stain Gram-negative, but have a Gram-positive cell wall, as was made evident by electron micrographs. Likewise, *C. hobsonii* produced similar major fermentation products (ethanol, acetate, formate, lactate, succinate, with some strain propionate) to those produced by other test cultures. Production of hydrogen and carbon dioxide as the fermentation gases released by the present isolates is similar to other test cultures.

Other similarities of the present isolates to the other test cultures are as follows:

- No glucose liberated as a result of cellulose hydrolysis, other sugars released are similar.
- A variety of similar biochemical characteristics.
- G + C ratios falling within the range for a given species.
- Partial adaptation of growth of *C. hobsonii* to higher temperature and adaptation of the growth of *C. thermocellum* to 37°C.
- Similar polypeptide profile shown by SDS-PAGE analysis. According to Jackman (1987), a comparison of polypeptide profiles of whole cells provides the same level of differentiation as is achieved by DNA–DNA hybridizations.
- In stating the above similarities, some slight variation shown by these cultures has also been taken into consideration, but then such variation is normally encountered with different strains of the same species.

It is concluded from the above evidence that the *named* genera and species are similar and that they and the present isolates should be included in a new speices — *Clostridium hobsonii* comb. nov. As the temperature ranges for growth of thermophilic bacteria and the possibilities of gradual adaptation or mutation of mesophilic to thermophilic strains are not at present fully investigated, the exact relationship of mesophilic to thermophilic bacteria can be a matter of debate.

Since the present isolates were consistently found in the digester and have high cellulolytic and hemicellulolytic activity (non-delignified barley straw contained both cellulose and hemicellulose), it suggests

they were playing a significant role in anaerobic digestion. Since the isolates are similar to *E. cellulosolvens* and *C. cellobioparum*, it can be safely assumed that this species — *C. hobsonii* (comb. nov.) — is also playing a role in the digestion of cellulosic and hemicellulosic materials in the rumen; this suggests the present species is the more widely distributed in nature than had been thought previously because of its sporadic isolation.

Emended description of *Clostridium hobsonii* comb. nov.

Surface colonies on cellobiose agar (medium 2, Sharma & Hobson, 1985*a*) are 0·5–1·5 mm, watery, slightly convex, with an insoluble yellow pigment and light tan color, more pronounced in transmitted light.

Colonies up to 3 mm can be found if the colonies are well dispersed. Colonies in cellulose roll tubes (medium 2) are mainly circular, although some are lenticular. In some colonies, some off-shoots may be present and a few isolates may produce woolly colonies.

Cell morphology

Long filamentous chains are seen in young cultures and the chains break mainly into pairs. Cells stain Gram-negatively but have a Gram-positive wall structure in EM, and are straight to slightly-curved rods with tapering ends, 0·25–0·7 (typically 0·25–0·45) × 1·5–3·5 μm. Some cells may show some beaded structure.

Spores

In liquid media, spores are terminal, spherical, rarely attached to cells and about 0·9–1·2 μm, the larger size occurring rarely and only in cultures with the larger cell size. Spore formation is unlikely on solid media. Sporulation was found in the medium of Sharma and Hobson (1985*b*).

Fermentation products

Fermentation products include ethanol, acetate, formate, lactate and succinate, with, in some cases, propionate. Some isolates may produce butyrate on PY or PYG medium, but butyrate is generally not produced. Exceptionally, some valerate may be produced in small amounts. Ethanol production is variable (Tables 1–5). No glucose, but cellobiose, -triose and -tetrose, is found in spent cellulose cultures.

Head space gases include carbon dioxide and hydrogen, though the latter may be absent in a few cases (Table 6). Major characterization tests are as follows: acetyl methyl carbinol is generally not produced. Isolates may weakly, or not at all, ferment the following: adonitol, inositol, mannitol, melezitose, rhamnose, sorbitol and trehalose. There is a small fall in aesculin pH. Glucose is strongly fermented by all strains, which may ferment to a varying extent the following: cellobiose, aesculin, maltose, dulcitol, fructose, insulin, lactose mannose, melibiose, ribose, salicin, starch, xylose, raffinose and sucrose; raffinose and sucrose may occasionally not be fermented. Erythritol is usually not fermented. Gelatin is hydrolyzed. Milk is clotted and also digested in some cases. Meat is not digested. Lecithinase and lipase are not produced. Nitrate is not reduced. Colonies are non-hemolytic. Urease, as tested for by the current methods, is not produced. Ethanol is produced from lactate and acetate from pyruvate (Table 2). G+C mol % ratio is from 38·1 to 43·9 and, in one exceptional circumstance, has been reported to be 28; 25 for the same strain (Hungate, 1944; Holdeman, Cato & Moore, 1977).

Optimum pH and temperature

Cultures show no growth at pH 5·0 and 8·0 and there is a sharp optimum at pH 6·5. A little growth is apparent at 20°C but generally none at 45 and 50°C and the optimum usually is 35–37°C. Some strains may show optimum growth at 60–65°C. Since thermophily is a secondary, rather than a primary, character, as suggested earlier (Langworthy *et al.*, 1979; Zuber, 1981), there appears to be no need to separate strains with high temperature optima into biotypes. Gradual adaptation to higher temperature and vice versa may be possible under suitable environments.

Habitat

Isolated from an anaerobic cattle-waste digester. Other similar sources, including the rumen, can be considered as possible habitats.

Strain deposition

The type strain *C. hobsonii* is strain U311. This strain has been deposited with NCIMB, Torry Research Station, Aberdeen, UK.

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