Butyrivibrio hungatei sp. nov. and *Pseudobutyrvibrio xylanivorans* sp. nov., butyrate-producing bacteria from the rumen

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Two novel Gram-negative, anaerobic, non-spore-forming, butyrate-producing bacterial species, strains Mz 5^T and JK 615^T, were isolated from the rumen fluid of cow and sheep. Both strains were curved rods that were motile by means of single polar or subpolar flagellum and common in the rumen microbial ecosystem. Strain Mz 5^T produced high xylanase, proteinase, pectin hydrolase and DNase activities; 1,4- β -endoglucanase was also detected in the culture medium. The bacterium utilized a wide range of carbohydrates. Glucose was fermented to formate, butyrate, lactate, succinate and ethanol. The DNA G + C content was 42.1 mol%. The complete 16S rDNA sequence was obtained and phylogenetic relationships were determined. Strain Mz 5^T and related isolates were located in clostridial cluster XIVa and were closely related to *Pseudobutyrvibrio ruminis*, *Butyrivibrio crossotus*, *Roseburia cecicola* and *Eubacterium rectale*. The name proposed for this novel bacterium is *Pseudobutyrvibrio xylanivorans*; the type strain is Mz 5^T (= DSM 14809^T = ATCC BAA-455^T). Strain JK 615^T produced no fibrolytic activity, but utilized a wide range of carbohydrates. Glucose was fermented to formate, acetate, butyrate and ethanol. The DNA G + C content was 44.8 mol%. The complete 16S rDNA sequence was obtained and phylogenetic relationships were determined. Strain JK 615^T was located in clostridial cluster XIVa and was closely related to *Clostridium proteoclasticum*, *Butyrivibrio fibrisolvens* and *Eubacterium halii*. The name proposed for this novel bacterium is *Butyrivibrio hungatei*; the type strain is JK 615^T (= DSM 14810^T = ATCC BAA-456^T).

INTRODUCTION

Butyrivibrio isolates represent a significant proportion (10–30%) of culturable rumen bacteria in domestic and wild ruminants (Forster *et al.*, 1996, 1997; Stewart *et al.*, 1997) and have been observed in DNA samples of uncultivated rumen bacteria (Tajima *et al.*, 1999). A large number of *Butyrivibrio* strains contributes to fibre degradation in the rumen. Most isolates synthesize xylanase, amylase and cellodextrinase, but few strains produce 1,4- β -endoglucanase (Stewart *et al.*, 1997). *Butyrivibrio* isolates are usually able to utilize a wide spectrum of sugar monomers and oligomers.

Up to now, most butyrate-producing isolates have been assigned to *Butyrivibrio fibrisolvens* on the basis of common phenotypic and metabolic characteristics, despite their vast diversity and genetic unrelatedness. Great variation between *B. fibrisolvens* strains has been observed in reactions with polyclonal (Margherita *et al.*, 1964) and monoclonal (Hazlewood *et al.*, 1986) antibodies, fermentation products and utilization of sugars (Diez-Gonzalez *et al.*, 1999; Van der Toorn & Van Gylswyk, 1985), cell-wall composition (Hespell *et al.*, 1993), sugar composition of extracellular polysaccharides (Stack, 1988), and DNA–DNA hybridization (Hudman & Gregg, 1989; Mannarelli *et al.*, 1990). 16S rDNA sequencing has confirmed the vast genetic heterogeneity of and great phylogenetic distances between different isolates in the *Butyrivibrio* group (Forster *et al.*, 1996; Willems *et al.*, 1996; Kopečný *et al.*, 2001). Similar variations have been observed in butyrate-producing bacteria from the human gut (Barcenilla *et al.*, 2000).

Recently, three species have been described that are

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The EMBL accession numbers for the 16S rDNA sequences of *Pseudobutyrvibrio xylanivorans* Mz 5^T, *Pseudobutyrvibrio ruminis* JK 626, *Pseudobutyrvibrio xylanivorans* JK 23/2, *Pseudobutyrvibrio xylanivorans* JK 633, *Clostridium proteoclasticum* UC 142 and *Butyrivibrio hungatei* JK 615^T are AJ428548–AJ428553, respectively.

genetically related to *B. fibrisolvens*: lophotrichous *Butyrivibrio crossotus* from the human gut (Moore *et al.*, 1976); proteolytic *Clostridium proteoclasticum* (Attwood *et al.*, 1996); and lactate-producing *Pseudobutyrvibrio ruminis* (Van Gylswyk *et al.*, 1996) from the rumen. Descriptions of these species have significantly improved the taxonomy of butyrate-producing anaerobic bacteria from the digestive tract.

The aim of this study was to collect taxonomic data for the possible classification of novel species of butyrate-producing anaerobic bacteria from the animal digestive tract.

METHODS

Strains and culture conditions. Butyrate-producing strains were isolated from rumen fluid and faeces of domestic ruminants. Isolations were done under anaerobic conditions in medium M10 with 10% rumen fluid containing glucose or cellobiose (4 g l^{-1}) as a growth substrate. Reference strains were obtained from different culture collections (Table 1). We greatly appreciate the gifts of G. T. Attwood (Palmerston, New Zealand), K. Gregg (Perth, WA, Australia), N. O. van Gylswyk (Uppsala, Sweden) and C. S. Stewart (Aberdeen, UK). Bacterial cultures were kept in 15% (v/v) glycerol at -70°C . For further analyses, strains were grown in medium M10 with 10% rumen fluid and $4 \text{ g cellobiose l}^{-1}$ (Kopečný *et al.*, 2001) or in DSM medium no. 330 without rumen fluid.

Analyses of fermentation products. Short-chain fatty acids, ethanol, succinate and lactate were analysed by GLC following double ether extraction or methylation of culture supernatants (Holdeman *et al.*, 1977) on a Shimadzu GC-14A chromatograph equipped with an FID detector and Supelcowax 10 capillary column (Supelco).

Substrate utilization and degradation of biopolymers. Substrate utilization and enzyme activities were tested with kits for anaerobic bacteria: API 20 and ID 32 (bioMérieux). Separate analyses were carried out on extracellular activities of amylase, xylanase, laminarinase (1,3- β -glucanase), proteinase, pectin hydrolase and DNase (Kopečný & Bartoš, 1990; Avguštin *et al.*, 1997).

GC determination of cellular fatty acid composition. Cells cultured in DSM medium no. 330 without rumen fluid were harvested by centrifugation at 3000 r.p.m. at 4°C for 10 min. Pellets were washed twice in sodium phosphate buffer (50 mM, pH 6.5). Preparation of fatty acid methyl esters by saponification, methylation and extraction into hexane and sample clean-up were done following the procedure of Moore *et al.* (1994). Methyl esters were analysed on an HP fused silica capillary column Ultra2 (HP 19091B-102) using a Shimadzu GC-14 A chromatograph equipped with an FID detector and Chromatopac C-R6A for data processing. A calibration standard kit (fatty acid methyl esters in hexane, HP 19298-60500) was used for identification of fatty acids and equivalent chain length factors were calculated for unknown peaks (Moore *et al.*, 1994). Relative concentrations of individual fatty acids were calculated as percentages. Bacterial strains were grouped according to the similarity of their cellular fatty acid profiles.

Transmission electron microscopy. Bacterial colonies were resuspended in filtered water, transferred onto grids covered with Formvar support film (Robards & Wilson, 1993) and negatively stained with an equal volume of 0.5% (w/v) aqueous uranyl acetate (Robards & Wilson, 1993). Stained bacteria were air-dried at room

temperature and examined with a Philips CM 100 EM. The images were recorded by a Gatan Bioscan CCD camera using DIGITALMICROGRAPH software.

RFLP analyses. DNA of selected strains was isolated (Gregg *et al.*, 1994) and 16S rDNA regions (1490 bp) were amplified by PCR with the aid of primers fD1 and rP2 (Weisburg *et al.*, 1991; Barcenilla *et al.*, 2000). Obtained fragments were digested at 37°C for 2 h with restriction endonucleases *Hae*III and *Alu*I and then analysed by electrophoresis in 2% agarose gels (Sambrook *et al.*, 1989).

DNA sequencing and phylogenetic tree construction. 16S rDNA fragments were obtained after amplifying 500 bp regions of bacterial DNA with primers FP27/515R, FP3/RP16 and FP19/rP2 (Forster *et al.*, 1996) on a thermocycler. These fragments were sequenced with an ABI 310 capillary sequencer (Perkin-Elmer). After editing, full 16S rDNA sequences were compared with published sequences of related bacteria from the EMBL and GenBank nucleotide databases using BLAST and with data from the Ribosomal Database Project (Maidak *et al.*, 1994). The sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) in the BIOEDIT program (Hall, 1999) and a phylogenetic tree was generated with the PHYLIP package (Felsenstein, 1989). Sequence data for distance matrices and analysis were subjected to bootstrap resampling (resampled 1000 times) using the SEQBOOT program. The DNADIST program analysed distances using the Kimura-Nei correction (Kimura, 1980) trees produced from distance matrices that used the neighbour-joining method (Saitou & Nei, 1987). A consensus tree was generated by the CONSENSE program (Felsenstein, 1985).

DNA G + C content. Estimation of the G + C contents in bacterial chromosomal DNAs was done after extraction and enzymic hydrolysis by HPLC on a C18 reversed-phase column (Supelcosil LC18; Supelco) with the same guard column (Mesbah *et al.*, 1989). DNAs from *Escherichia coli* strain B (50 mol%) and calf thymus (42 mol%) were used as reference samples for G + C content.

Genera-specific PCR. Specific forward PCR primers developed previously for the genera *Butyrivibrio* (71f; 5'-CGG AGA ATT TAC GCT GAT GAA G) and *Pseudobutyrvibrio* (F2; 5'-AAT TTT CTA CGA TCC CTT CGG GG) were used with reverse primer 515R (Mrázek & Kopečný, 2001) to distinguish both genera.

RESULTS AND DISCUSSION

Genotypic variations

The high genetic diversity of *Butyrivibrio* isolates has been proven many times (Hudman & Gregg, 1989; Willems *et al.*, 1996; Forster *et al.*, 1996). To characterize our isolates, RFLP analysis (with restriction endonucleases *Alu*I and *Hae*III) of the 16S rDNA fragments from 42 *Butyrivibrio* isolates (Table 1), obtained from the digestive tract of ruminants, was used. According to variable fragment size in fingerprints, restriction patterns isolates clustered into five groups. Three of these groups corresponded with three previously described species: *B. fibrisolvens*, *P. ruminis* and *C. proteoclasticum*. Two other homogeneous groups of bacteria with different restriction profiles were observed (Table 2).

Representatives of each group (JK 615^T, UC 142, JK 626, Mz 5^T, JK 23/2 and JK 633) were selected and fragments of 1500 nt encoding the 5' end of the 16S DNA were

Table 1. Strains of *Butyrivibrio* used in the present study, their origin and DNA G + C content

Strain	Species*	G + C content (mol%)	Locality	Origin	Isolated by
ATCC 19171 ^T (=D1 ^T)	1	42·10	USA	Cow	M. P. Bryant
Bu 43	2	42·67	South Africa	Sheep	N. O. Van Gylswyk
CE 51	5	40·86	South Africa	Sheep	N. O. Van Gylswyk
JK 04	6	42·26	Czech Republic	Sheep	J. Kopečný
JK 08	6	47·90	Czech Republic	Sheep	J. Kopečný
JK 170	5	41·16	NSW, Australia	Sheep	J. Kopečný
JK 205	3	38·51	NSW, Australia	Sheep	J. Kopečný
JK 23/2	5	42·73	Czech Republic	Sheep	J. Kopečný
JK 611	4	41·8	Czech Republic	Sheep	J. Kopečný
JK 614	4	40·46	Czech Republic	Sheep	J. Kopečný
JK 615 ^T	4	44·88	Czech Republic	Sheep	J. Kopečný
JK 618	6	43·80	Czech Republic	Sheep	J. Kopečný
JK 623	5	40·92	Czech Republic	Sheep	J. Kopečný
JK 626	3	41·33	Czech Republic	Sheep	J. Kopečný
JK 633	6	48·39	Czech Republic	Sheep	J. Kopečný
JK 662	6	41·33	Czech Republic	Sheep	J. Kopečný
JK 663	5	ND	Czech Republic	Sheep	J. Kopečný
JK 668	5	ND	Czech Republic	Sheep	J. Kopečný
JK 669	2	39·79	Czech Republic	Sheep	J. Kopečný
JK 684	4	39·45	Czech Republic	Sheep	J. Kopečný
JK 689	4	41·26	Czech Republic	Sheep	J. Kopečný
JK 722	6	ND	Czech Republic	Sheep	J. Kopečný
JK 724	5	ND	Czech Republic	Sheep	J. Kopečný
JK 728	6	ND	Czech Republic	Sheep	J. Kopečný
JK 730	5	ND	Czech Republic	Sheep	J. Kopečný
Mz 10	6	52·98	Slovenia	Cow	M. Zorec
Mz 3	6	45·88	Slovenia	Cow	M. Zorec
Mz 4	5	ND	Slovenia	Cow	M. Zorec
Mz 5 ^T	5	42·12	Slovenia	Cow	M. Zorec
Mz 6	3	40·19	Slovenia	Cow	M. Zorec
Mz 7	5	ND	Slovenia	Cow	M. Zorec
Mz 8	5	40·05	Slovenia	Cow	M. Zorec
Mz 9	6	ND	Slovenia	Cow	M. Zorec
NCFB 2222	4	51·78	USA	Cow	M. P. Bryant
NCFB 2249	3	41·93	UK	Cow	M. J. Latham
NCFB 2397	5	38·71	USA	Unknown	J. M. Leatherwood
NCFB 2398	4	42·53	Scotland, UK	Cow	S. O. Mann
NCFB 2399	5	42·87	South Africa	Sheep	N. O. Van Gylswyk
NCFB 2432	6	50·09	South Africa	Sheep	N. O. Van Gylswyk
NCFB 2434	6	40·12	South Africa	Sheep	N. O. Van Gylswyk
NCFB 2435	6	39·72	South Africa	Sheep	N. O. Van Gylswyk
UC 142	2	41·06	Czech Republic	Sheep	J. Kopečný
X2D62	6	42·00	South Africa	Sheep	N. O. Van Gylswyk

ND, Not determined.

*Numbers refer to the following species: 1, *B. fibrisolvens*; 2, *C. proteoclasticum*; 3, *P. ruminis*; 4, *B. hungatei*; 5, *P. xylanivorans*; 6, *Butyrivibrio* spp.

sequenced. Neighbour-joining analysis of a distance matrix obtained from a multiple-sequence alignment resulted in an unrooted phylogenetic tree of selected strains with *Clostridium roticum* as an outgroup (Fig. 1). All strains tested were located in the XIVa cluster of the Gram-positive bacteria (Collins *et al.*, 1994). For two groups of isolates, the assignment of two novel species, *Butyrivibrio*

hungatei and *Pseudobutyrvibrio xylanivorans*, is proposed. The proposed novel species were significantly different from previously described species. All *Butyrivibrio* isolates formed two large groups.

The first group included isolates related to the type strain, D1^T, of *B. fibrisolvens*, a cluster of *C. proteoclasticum* and

Table 2. RFLPs obtained with *AluI* and *HaeIII* with 16S rDNA fragments from *Butyrivibrio* isolates

Group of isolates*	n†	Restriction endonuclease	Fragment size (bp)
<i>B. fibrisolvens</i>	2	<i>AluI</i>	950, 420
		<i>HaeIII</i>	560, 270
<i>P. ruminis</i>	8	<i>AluI</i>	445, 420, 220
		<i>HaeIII</i>	1100, 270, 200
<i>C. proteoclasticum</i>	7	<i>AluI</i>	950, 380
		<i>HaeIII</i>	300, 250
<i>B. hungatei</i>	8	<i>AluI</i>	570, 300
		<i>HaeIII</i>	900, 300, 250
<i>P. xylanivorans</i>	17	<i>AluI</i>	880, 420, 220
		<i>HaeIII</i>	1100, 320, 270

**B. fibrisolvens* strains were D1^T and Mz10. *P. ruminis* strains were NCFB 2249, JK 08, JK 205, JK 618, JK 626, JK 662, JK 728 and Mz 6. *C. proteoclasticum* strains were NCFB 2432, NCFB 2435, Bu 43, JK 669, JK 722, UC 142 and X2D62. *B. hungatei* strains were NCFB 2398, NCFB 2434, JK 611, JK 614, JK 615^T, JK 684, JK 689 and Mz 9. *P. xylanivorans* strains were CE 51, NCFB 2397, NCFB 2399, JK 04, JK 23/2, JK 170, JK 623, JK 633, JK 663, JK 668, JK 724, JK 730, Mz 3, Mz 4, Mz 5^T, Mz 7 and Mz 8.

†Number of strains representing each species.

a cluster of *B. hungatei*. These two clusters were closely related to *B. crossotus*. The second group included two clusters containing *P. ruminis* and *P. xylanivorans*, respectively. Both groups were related to butyrate-producing bacteria from the human colon (UnId L1-83, L1-810 and T1-815), which were similar to *Roseburia cecicola* and *Eubacterium rectale*.

16S rDNA sequence similarities of *B. hungatei* with *B. fibrisolvens* (93.7%), *C. proteoclasticum* (95.7%) and *B. crossotus* (90.6%) were estimated. Similarly, sequence similarities of *P. xylanivorans* with *P. ruminis* (96.4%), *B. fibrisolvens* (89.5%) and *B. crossotus* (89.6%) were also determined.

DNA G+C content data are summarized in Table 3. The mean values for most isolates are in the range 40–44 mol%. This range is in agreement with values described for the genera *Butyrivibrio* and *Pseudobutyrvibrio* (Stewart *et al.*, 1997; Van Gylswyk *et al.*, 1996). The G+C content data of novel strains resembling *C. proteoclasticum* were in the same range, contrary to the published value (28 mol%) for the type strain of *C. proteoclasticum*, which was significantly lower (Attwood *et al.*, 1996). The mean values for G+C contents of the novel species were 41.6 mol% for *B. hungatei* and 42.7 mol% for *P. xylanivorans*.

Fermentation end products

All strains under analysis fermented glucose to butyrate, hydrogen, ethanol, carbon dioxide and formate. Isolates

Fig. 1. Phylogenetic tree showing relationships of 16S rDNA sequences of different isolates of *Butyrivibrio*. Bar, 5 substitutions per 100 nt. The tree was constructed using neighbour-joining analysis of a distance matrix obtained from a multiple-sequence alignment. *Clostridium oreticum* was used as an outgroup sequence.

producing lactate were mainly *P. ruminis* strains. In fact, lactate production is one of the main characteristics for the identification of *P. ruminis* (Van Gylswyk *et al.*, 1996). High production of lactate by *Butyrivibrio* isolates was connected with the presence of butyryl-CoA/acetate CoA transferase (Diez-Gonzalez *et al.*, 1999). In agreement with that, in our *P. ruminis* strains, lactate production was associated with acetate utilization. Roche *et al.* (1973) described these isolates as acetate-utilizing butyrvibrios. Lactate was also produced by *B. fibrisolvens* D1^T.

Table 3. Some characteristics that distinguish different butyrate-producing bacteria from the digestive tract of ruminants

Species: 1, *B. fibrisolvens* (strain D1^T); 2, *P. ruminis* (strains NCFB 2249, JK 205, JK 626 and Mz 6); 3, *C. proteoclasticum* (strains Bu 43, JK 669 and UC 142); 4, *B. hungatei* (strains NCFB 2398, NCFB 2434, JK 611, JK 614, JK 615^T, JK 684, JK 689 and Mz 9); 5, *P. xylanivorans* (strains CE 51, NCFB 2397, NCFB 2399, JK 04, JK 23/2, JK 170, JK 623, JK 633, JK 663, JK 668, JK 724, JK 730, Mz 3, Mz 4, Mz 5^T, Mz 7 and Mz 8). With the exception of DNA G+C content, results are expressed as percentage of positive results.

Characteristic	1	2	3	4	5
DNA G+C content (mol%)*	41.2 (1)	41.1 ± 1.3 (4)	40.2 ± 0.6 (3)	41.6 ± 1.1 (6)	42.7 ± 1.4 (14)
Hydrolase activity:					
Amylase	0	0	0	0	57
Xylanase	100	0	33	33	100
Proteinase	0	0	100	0	86
Laminarinase	0	0	33	33	36
DNase	0	75	0	33	64
Carboxymethyl cellulase	100	25	0	0	43
Pectinase	0	0	67	17	36
Fermentation products:					
Acetate production	0	0	100	100	0
Lactate production	100	100	0	0	10
Ethanol production	0	25	0	67	57
Utilization (API) tests:					
Rhamnose	100	0	100	50	36
Activity (ID 32) tests:					
Leucyl-glycine arylamidase	100	0	0	0	21
Histidine arylamidase	100	0	0	33	29
α -Fucosidase	100	0	100	67	7
Glycine arylamidase	100	0	0	17	29
PCR primers†					
<i>Pseudobutyrvibrio</i>	0	100	0	0	100
<i>Butyrvibrio</i>	100	0	100	100	0

*The numbers in parentheses refer to the numbers of strains examined.

†See Mrázek & Kopečný (2001).

Isolates of *C. proteoclasticum* and *B. hungatei* did not produce lactate at all or did so in small quantities (less than 0.5 mmol l⁻¹). Production of acetate was observed in most cultures of *C. proteoclasticum* and *B. hungatei*. Ethanol was produced by most strains of *P. xylanivorans* and *B. hungatei*.

Hungate (1966) preliminarily classified isolates producing butyrate and acetate but not producing lactate and ethanol as '*Butyrvibrio alactacidigens*'.

Substrate utilization

The substrate utilization of our 42 *Butyrvibrio* and *Pseudobutyrvibrio* strains was tested with API 20A test kits. No strains produced catalase or indole. All strains used glucose, cellobiose, maltose and aesculin. Only strains JK 633 and JK 730 did not use sucrose and mannose. Results obtained with all other substrates tested were variable. These facts are in agreement with previous attempts to reclassify *Butyrvibrio* isolates (Hungate, 1966; Hudman & Gregg, 1989).

Polysaccharidase activities

Amylase production was observed in many *Butyrvibrio* isolates (McAllister *et al.*, 1990). This activity was characteristic for most of the *P. xylanivorans* isolates (Table 3). Other existing and proposed species did not produce any significant amylase activity at all. Xylanase activity was typical of *B. fibrisolvens* D1^T and isolates of *P. xylanivorans*. *P. ruminis* did not produce any xylanase activity. In *C. proteoclasticum* and *B. hungatei*, the xylanase activity varied (Table 3). Laminarinase activity was observed in several isolates of *C. proteoclasticum*, *B. hungatei* and *P. xylanivorans* only; *B. fibrisolvens* and *P. ruminis* isolates did not produce this enzyme activity at all.

β -Endoglucanase was characteristic of *B. fibrisolvens* D1^T and several isolates of *P. ruminis* and *P. xylanivorans*. No activity was observed in strains of *C. proteoclasticum* and *B. hungatei*. Pectin hydrolase was not produced by strains of *B. fibrisolvens*, *P. ruminis* or *B. hungatei*. In the rest of the tested species, the activity differed. The presence of pectin hydrolase was observed only in *C. proteoclasticum*.

isolates. Activity of α -fucosidase was characteristic of *B. fibrisolvens* D1^T and *C. proteoclasticum*. α -Fucosidase was not detected in *P. ruminis* or in most of the *P. xylanivorans* isolates. α -Fucosidase activity was not observed in all isolates of *B. hungatei*.

Proteinase and peptidase activities

It has been accepted that *Butyrivibrio* strains have an important role in protein degradation in the rumen (Attwood & Reilly, 1995). Proteinase activity (Table 3) was characteristic for all strains of *C. proteoclasticum* and most isolates (12 out of 14 strains tested) of *P. xylanivorans*. Azocasein was not hydrolysed by all strains of *B. hungatei*, *P. ruminis* or *B. fibrisolvens* D1^T. The distribution of proteolytic activity estimated with azocasein did not fully correlate with the gelatin hydrolysing activity observed in API 20 tests. Glycine arylamidase and leucyl-glycine arylamidase activity was observed in *B. fibrisolvens* D1^T and in a few isolates of *P. xylanivorans*. Similar distribution of enzyme activity was observed in the case of histidine arylamidase; this activity was also observed in one isolate of *B. hungatei*.

Deoxyribonuclease (DNase) activity

Nuclease activity (Table 3) was observed in most strains of *P. xylanivorans* and *P. ruminis*, and also in some strains of *B. hungatei*. No DNase activity was detected in the culture media of *B. fibrisolvens* or *C. proteoclasticum* strains.

Cellular fatty acids

Cellular fatty acid profiles have been used to establish taxonomic relationships among anaerobic bacteria (Holdeman *et al.*, 1977; Mayberry *et al.*, 1982; Moore *et al.*, 1994) and have also proven to be useful in the classification of anaerobic Gram-negative bacteria (Stokes *et al.*, 1991). The most extensive work on 21 different *Butyrivibrio* strains was done by Miyagawa (1982). Moore *et al.* (1994) determined the fatty acid composition only in *B. fibrisolvens* D1^T (=ATCC 19171^T). Miyagawa (1982) found that the cellular fatty acid composition varied considerably among strains and divided the *Butyrivibrio* isolates into two major groups based on fatty acid and fatty aldehyde composition: Group 1 contained branched-chain fatty acids as major components, whereas Group 2 contained only straight-chain fatty acids as major components. Although it is difficult to compare directly our results with those of other authors (different growth media and analyses conditions used), our data (Table 4) in general confirm those of Miyagawa (1982). The fatty acids of *P. xylanivorans* and *B. fibrisolvens* contained a low proportion of branched-chain fatty acids and the other three groups of isolates contained the same branched-chain fatty acids as the major ones previously detected by Miyagawa (1982): iso-C_{14:0} and anteiso-C_{15:0}, which are the discriminating fatty acids of *C. proteoclasticum* isolates; iso-C_{15:1}, a major, characteristic fatty acid of isolates of

Table 4. Cellular fatty acid composition of five species of butyrate-producing bacteria

Species: 1, *B. fibrisolvens* (n=2); 2, *P. ruminis* (n=8); 3, *C. proteoclasticum* (n=7); 4, *B. hungatei* (n=8); 5, *P. xylanivorans* (n=17). Strain numbers of isolates of each species are given in the legend to Table 2. —, Not present or less than 0.5% of total acids. Mean percentage of the total acids is given for each fatty acid of a set of strains of a species.

Fatty acid	1	2	3	4	5
C _{12:1}	—	1.5	1.5	1.3	1.2
C _{12:0}	0.9	1.4	—	—	—
C _{13:1}	—	4.0	0.8	0.5	0.8
iso-C _{14:0}	—	0.6	8.2	2.1	—
C _{14:0}	2.8	14.2	3.8	2.6	3.8
iso-C _{13:0} 3-OH	—	—	2.7	1.4	—
iso-C _{15:1} /C _{13:0} 3-OH*	0.7	12.7	—	1.5	3.1
anteiso-C _{15:1}	—	—	1.5	2.3	—
iso-C _{15:0}	—	3.0	—	—	0.9
anteiso-C _{15:0}	—	0.7	6.3	1.7	—
C _{15:1} c10	1.5	2.2	—	0.9	1.8
C _{15:0}	0.8	5.5	3.1	2.7	2.2
iso-C _{14:0} 3-OH	—	5.4	—	—	2.4
C _{14:0} 2-OH	—	—	15.6	4.4	1.2
C _{14:0} 3-OH/iso-C _{16:1} *	—	1.6	0.8	1.7	—
C _{16:1} c9	1.2	—	—	—	0.7
iso-C _{16:0}	—	—	16.5	15.0	—
C _{16:1}	0.6	—	—	12.8	—
C _{16:0}	41.3	27.6	7.8	9.1	38.2
C _{15:0} 2-OH	1.7	—	1.4	—	3.9
iso-C _{17:1}	—	1.0	—	3.5	1.2
anteiso-C _{17:1}	7.5	5.2	—	—	6.6
iso-C _{17:0}	0.8	—	—	—	1.8
anteiso-C _{17:0}	3.9	—	14.6	17.2	2.6
C _{17:1}	1.3	—	—	—	0.7
C _{17:0}	1.6	—	—	1.9	2.0
iso-C _{16:0} 3-OH	—	—	—	—	1.0
C _{16:0} 2-OH	—	—	2.3	6.9	1.1
C _{16:0} 3-OH	0.6	—	—	—	—
iso-C _{18:1}	—	—	—	0.9	—
C _{18:1} c9	2.8	—	—	—	—
C _{18:1} c11/t9/t6	6.4	—	—	—	—
C _{18:1} t11	—	—	—	—	1.1
C _{18:1}	1.3	—	—	—	1.4
C _{18:0}	5.7	1.2	2.0	1.5	1.6
iso-C _{17:0} 3-OH	0.7	—	—	—	4.0
C _{17:0} 2-OH	12.7	—	—	—	—
iso-C _{19:1}	1.9	0.8	1.1	1.4	1.2

*The two fatty acids are identified together as one peak by GLC.

P. ruminis; and anteiso-C_{17:0}, found in *C. proteoclasticum*, *B. hungatei* and *B. fibrisolvens* D1^T. Our results also confirmed that among straight-chain fatty acids, C_{16:0} was generally the major fatty acid in *Butyrivibrio* and *Butyrivibrio*-like isolates. The two *Pseudobutyrvibrio* species

(*P. ruminis* and *P. xylanivorans*) and *B. fibrisolvens* contained $C_{16:0}$ fatty acid in the highest proportion (Van Gylswyk *et al.*, 1996). Other major straight-chain saturated and unsaturated fatty acids detected were $C_{14:0}$, $C_{15:0}$, $C_{16:1}$ and $C_{18:1}$, which is in total accordance with the results of Miyagawa (1982). The different relative proportions of these fatty acids and the detection of some fatty acids unique to some groups of isolates enabled us to cluster our isolates into five separate species.

There have been many attempts to reclassify *Butyrivibrio*-like isolates (Hungate, 1966; Hespell *et al.*, 1993; Forster *et al.*, 1996). Our data fully support the proposal of two novel bacterial species: the non-proteolytic and non-fibrolytic species *B. hungatei* and the fibrolytic species *P. xylanivorans*. Both species are common in the rumen content of sheep and cow.

Description of *Butyrivibrio hungatei* sp. nov.

Butyrivibrio hungatei (hun.ga'te.i.N.L. gen. n. *hungatei* named after Robert E. Hungate, an American microbiologist, who isolated similar strains in the 1960s).

Cells are Gram-negative, straight to slightly curved rods ($0.3\text{--}0.5 \times 1.5\text{--}2.5\ \mu\text{m}$) that do not form endospores. Motile by means of a single polar or subpolar flagellum (Fig. 2). Growth occurs anaerobically at 39°C , but not at 25°C . Limited growth is observed at 45°C . No growth is observed in the presence of oxygen. Arabinose, cellobiose, aesculin, glucose, lactose, maltose, mannose, raffinose, salicin, sucrose and xylose are utilized by the type strain. Tryptophan, urea and gelatin are not utilized. The following substrates do not support growth of the type strain: glycerol; mannitol; melezitose; sorbitol; rhamnose; trehalose. Medium M10 with glucose (22 mM) is fermented to

Fig. 2. Electron micrograph of *Butyrivibrio hungatei* JK 615^T cells showing the size and flagellar arrangement. Bar, 1 μm .

formate (12 mM), acetate (2.2 mM), butyrate (10.8 mM) and ethanol (3.4 mM). No lactate or succinate production is observed. No growth occurs in the absence of fermentable carbohydrates. Does not show any significant fibrolytic or proteolytic activity, utilizing mainly oligo- and monosaccharides as growth substrates. The type strain does not liquefy gelatin, produce hydrogen sulfide, reduce nitrate or hydrolyse urea and is catalase-negative. α -Galactosidase, α -arabinosidase, phenylalanine arylamidase and leucine arylamidase are produced. No amylase, xylanase, β -endoglucanase, laminarinase, pectin hydrolase, proteinase or DNase activities are detected. The major cellular fatty acid components of strain JK 615^T grown in DSM medium no. 330 without rumen fluid are saturated, branched-chain anteiso- $C_{17:0}$ (17.4%) and iso- $C_{16:0}$ (14.5%), and unsaturated straight-chain $C_{16:1}$ (13.2%) fatty acids. Also present are $C_{16:0}$ 2-OH (6.9%), $C_{14:0}$ 2-OH (4.3%) and iso- $C_{17:1}$ (3.4%). Branched-chain fatty acids represent the prevailing proportion of cellular fatty acids in this species. Strains are common isolates in our set of anaerobic rumen bacteria. In the rumen, they participate in the utilization of intermediates of fibre degradation. Strains belonging to *Butyrivibrio hungatei* are listed in the footnote of Table 3. The type strain is JK 615^T (=DSM 14810^T = ATCC BAA-456^T). The DNA G+C content of the type strain is 44.8 mol% (as determined by the HPLC method).

Description of *Pseudobutyrvibrio xylanivorans* sp. nov.

Pseudobutyrvibrio xylanivorans (xy.la.ni.vo'rans. N.L. n. *xylanum* xylan; L. part. adj. *vorans* devouring, digesting; N.L. part. adj. *xylanivorans* xylan-digesting).

Cells are Gram-negative, straight to slightly curved rods ($0.4\text{--}0.6 \times 1.5\text{--}3.0\ \mu\text{m}$) that do not form endospores. Cells are motile by means of a single polar or subpolar flagellum (Fig. 3). Growth occurs anaerobically at 39°C , but not at 25°C or 45°C . No growth is observed in the presence of oxygen. However, exposure of grown liquid culture to air for several hours does not destroy the ability of the species to recover under anaerobic conditions. Substrates that support growth of the type strain are cellobiose, aesculin, glucose, lactose, maltose, mannose, melezitose, salicin, sucrose and xylose. Tryptophan, urea and gelatin are not utilized. The following substrates do not support growth of the type strain: glycerol; mannitol; raffinose; sorbitol; rhamnose; trehalose. Medium M10 with glucose (22 mM) is fermented to formate (15.1 mM), butyrate (17.6 mM), lactate (10.9 mM), succinate (0.3 mM) and ethanol (1.1 mM). Acetate is utilized. No growth occurs in the absence of fermentable carbohydrates. Strain Mz 5^T does not liquefy gelatin or produce hydrogen sulfide; neither does it reduce nitrate, hydrolyse urea nor produce catalase. It produces α -galactosidase, α -arabinosidase, xylanase, amylase, β -endoglucanase, pectin hydrolase, proteinase and DNase. The major cellular fatty acid components of strain Mz 5^T grown in DSM medium no. 330 without rumen

Fig. 3. Electron micrograph of *Pseudobutyrvibrio xylanivorans* Mz 5^T cells showing (a) the size and arrangement (bar, 100 nm) and (b) flagellar basis (bar, 0.5 µm).

fluid are C_{16:0} (39.2 %) and anteiso-C_{17:1} (6.4 %), followed by iso-C_{17:0} 3-OH (3.9 %) and C_{14:0} (4.3 %). This species is characterized by a higher content of straight-chain cellular fatty acids and a lower content of branched-chain fatty acids. Produces high xylanase activity. Most strains also produce amylase, proteinase and DNase. Laminarinase is detected in some species. Strains belonging to *Pseudobutyrvibrio xylanivorans* are listed in the footnote of Table 3. The most common butyrate-producing anaerobic bacterium in our set of bacteria isolated from the rumen of sheep and cow. The type strain is Mz 5^T (=DSM 14809^T =ATCC BAA-455^T). The DNA G+C content of the type strain is 42.1 mol% (as determined by the HPLC method).

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REFERENCES

Attwood, G. T. & Reilly, K. (1995). Identification of proteolytic rumen bacteria isolated from New Zealand cattle. *J Appl Bacteriol* **79**, 22–29.

Attwood, G. T., Reilly, K. & Patel, B. K. C. (1996). *Clostridium proteoclasticum* sp. nov., a novel proteolytic bacterium from the bovine rumen. *Int J Syst Bacteriol* **46**, 753–758.

Avguštin, G., Wallace, R. J. & Flint, H. J. (1997). Phylogenetic diversity among ruminal isolates of *Prevotella ruminicola*: proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. and redefinition of *Prevotella ruminicola*. *Int J Syst Bacteriol* **47**, 284–288.

Barcenilla, A., Pryde, S. E., Martin, J. C., Duncan, S. H., Stewart, C. S., Henderson, C. & Flint, H. J. (2000). Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl Environ Microbiol* **66**, 1654–1661.

Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.

Diez-Gonzalez, F., Bond, D. R., Jennings, E. & Russell, J. B. (1999). Alternative schemes of butyrate production in *Butyrivibrio fibrisolvens* and their relationship to acetate utilization, lactate production and phylogeny. *Arch Microbiol* **171**, 324–330.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Felsenstein, J. (1989). PHYLIP – Phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.

Forster, R. J., Teather, R. M., Gong, J. & Deng, S. J. (1996). 16S rDNA analysis of *Butyrivibrio fibrisolvens*: phylogenetic position and relation to butyrate-producing anaerobic bacteria from the rumen of white-tailed deer. *Lett Appl Microbiol* **23**, 218–222.

Forster, R. J., Gong, J. & Teather, R. M. (1997). Group-specific 16S rRNA hybridization probes for determinative and community structure studies of *Butyrivibrio fibrisolvens* in the rumen. *Appl Environ Microbiol* **63**, 1256–1260.

Gregg, K., Cooper, C. L., Schafer, D. J., Sharpe, H., Beard, C. E., Allen, G. & Xu, J. (1994). Detoxification of the plant toxin fluoracetate by a genetically modified rumen bacterium. *Biotechnology* **12**, 1361–1365.

Hall, T. A. (1999). BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.

Hazlewood, G. P., Theodorou, M. K., Hutchings, A., Jordan, D. J. & Galfre, G. (1986). Preparation and characterization of monoclonal antibodies to a *Butyrivibrio* sp. and their potential use in the identification of rumen butyrivibrios, using enzyme-linked immunosorbent assay. *J Gen Microbiol* **132**, 43–52.

Hespell, R. B., Kato, K. & Costerton, J. W. (1993). Characterization of the cell wall of *Butyrivibrio* species. *Can J Microbiol* **39**, 912–921.

Holdeman, L. V., Cato, E. P. & Moore, W. E. C. (1977). *Anaerobe Laboratory Manual*, 4th edn. Blacksburg, VA: Virginia Polytechnic Institute and State University.

Hudman, J. F. & Gregg, K. (1989). Genetic diversity among strains of bacteria from the rumen. *Curr Microbiol* **19**, 313–318.

Hungate, E. R. (1966). *The Rumen and its Microbes*. New York: Academic Press.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kopečný, J. & Bartoš, S. (1990). Activity of hydrolases in the gastrointestinal tract of goats. *Small Rumin Res* **3**, 25–35.

Kopečný, J., Marinšek-Logar, R. & Kobayashi, Y. (2001). Phenotypic and genetic data supporting reclassification of *Butyrivibrio fibrisolvens* isolates. *Folia Microbiol* **46**, 45–48.

- McAllister, T. A., Cheng, K. J., Rode, L. M. & Forsberg, C. W. (1990). Digestion of barley, maize and wheat by selected species of ruminal bacteria. *Appl Environ Microbiol* **56**, 46–53.
- Maidak, B. L., Larsen, N., McCaughey, M. J., Overbeek, R., Olsen, G. J., Foge, K., Blandy, J. & Woese, C. R. (1994). The ribosomal database project. *Nucleic Acids Res* **22**, 3485–3487.
- Mannarelli, B. M., Stack, R. J., Lee, D. & Ericsson, L. (1990). Taxonomic relatedness of *Butyrivibrio*, *Lachnospira*, *Roseburia*, and *Eubacterium* species as determined by DNA hybridization and extracellular-polysaccharide analysis. *Int J Syst Bacteriol* **40**, 370–378.
- Margherita, S. S., Hungate, R. E. & Storz, H. (1964). Variation in rumen *Butyrivibrio* strains. *J Bacteriol* **87**, 1304–1308.
- Mayberry, W. R., Lambe, D. W., Jr & Ferguson, K. P. (1982). Identification of *Bacteroides* species by cellular fatty acid profiles. *Int J Syst Bacteriol* **32**, 21–27.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Miyagawa, E. (1982). Cellular fatty acid and fatty aldehyde composition of rumen bacteria. *J Gen Appl Microbiol* **28**, 389–408.
- Moore, L. V. H., Bourne, D. M. & Moore, W. E. C. (1994). Comparative distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic Gram-negative bacilli. *Int J Syst Bacteriol* **44**, 338–347.
- Moore, W. E. C., Johnson, J. L. & Holdeman, L. V. (1976). Emendation of *Bacteriodaceae* and *Butyrivibrio* and description of *Desulfomonas* gen. nov. and ten new species in the genera *Desulfomonas*, *Butyrivibrio*, *Eubacterium*, *Clostridium*, and *Ruminococcus*. *Int J Syst Bacteriol* **26**, 238–252.
- Mrázek, J. & Kopečný, J. (2001). Development of competitive PCR for detection of *Butyrivibrio fibrisolvens* in the rumen. *Folia Microbiol* **46**, 63–65.
- Robards, A. W. & Wilson, A. J. (1993). *Procedures in Electron Microscopy*. New York: John Wiley.
- Roche, C., Albertyn, H., Van Gylswyk, N. O. & Kistner, A. (1973). The growth response of cellulolytic acetate-utilizing and acetate-producing butyrivibrios to volatile fatty acids and other nutrients. *J Gen Microbiol* **78**, 253–260.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Stack, R. J. (1988). Neutral sugar composition of extracellular polysaccharides produced by strains of *Butyrivibrio fibrisolvens*. *Appl Environ Microbiol* **54**, 878–883.
- Stewart, C. S., Flint, H. J. & Bryant, M. P. (1997). The rumen bacteria. In *The Rumen Microbial Ecosystem*, pp. 35–38. Edited by P. N. Hobson & C. S. Stewart. London: Blackie Academic & Professional.
- Stoakes, L., Kelly, T., Schieven, B., Harley, D., Ramos, M., Lannigan, R., Groves, D. & Hussain, Z. (1991). Gas-liquid chromatographic analysis of cellular fatty acids for identification of Gram-negative anaerobic bacilli. *J Clin Microbiol* **29**, 2636–2638.
- Tajima, K., Aminov, R. I., Nagamine, T., Ogata, K., Nakamura, M., Matsui, H. & Benno, Y. (1999). Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. *FEMS Microbiol Ecol* **29**, 159–169.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Van der Toorn, J. J. K. T. & Van Gylswyk, N. O. (1985). Xylan-digesting bacteria from the rumen of sheep fed maize straw diets. *J Gen Microbiol* **131**, 2601–2607.
- Van Gylswyk, N. O., Hippe, H. & Rainey, F. A. (1996). *Pseudobutyrovibrio ruminis* gen. nov., sp. nov., a butyrate-producing bacterium from the rumen that closely resembles *Butyrivibrio fibrisolvens* in phenotype. *Int J Syst Bacteriol* **46**, 559–563.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
- Willems, A., Amat-Marco, M. & Collins, M. D. (1996). Phylogenetic analysis of *Butyrivibrio* strains reveals three distinct groups of species within the *Clostridium* subphylum of the Gram-positive bacteria. *Int J Syst Bacteriol* **46**, 195–199.