Eubacterium pyruvativorans sp. nov., a novel non-saccharolytic anaerobe from the rumen that ferments pyruvate and amino acids, forms caproate and utilizes acetate and propionate

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Two similar Gram-positive rods were isolated from 10^{-6} dilutions of ruminal fluid from a sheep receiving a mixed grass hay/concentrate diet, using a medium containing pancreatic casein hydrolysate as sole source of carbon and energy. The isolates did not ferment sugars, but grew on pyruvate or trypticase, forming caproate as the main fermentation product and valerate to a lesser extent. Acetate and propionate were utilized. One of these strains, I-6^T, was selected for further study. Strain I-6^T was a non-motile coccoid rod, $1.2 \times 0.4 \mu\text{m}$, with a Gram-positive cell wall ultrastructure and a G+C content of 56.8 mol%. No spores were visible, and strain I-6^T did not survive heating at 80 °C for 10 min. Its rate of NH_3 production was $375 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$, placing it in the 'ammonia-hyperproducing' (or HAP) group of ruminal bacteria. 16S rDNA sequence analysis (1296 bases) indicated that it represents a novel species within the 'low-G+C' Gram-positive group, for which the name *Eubacterium pyruvativorans* sp. nov. is proposed. Among cultivated bacteria, strain I-6^T was most closely related (89% identity) to other asaccharolytic *Eubacterium* isolates from the mouth and the rumen. It was 98% identical to uncultured bacterial sequences amplified by others from ruminal digesta.

Bacteria that form NH_3 rapidly from amino acids contribute to one of the most important inefficiencies in ruminant nutrition, namely that of poor nitrogen retention on high-protein diets (Leng & Nolan, 1984). So-called 'ammonia-hyperproducing' (also referred to as 'hyper-ammonia-producing' or HAP) bacteria, non-saccharolytic amino acid fermenters and rapid producers of ammonia from amino acids, are partly responsible for this inefficiency (Chen & Russell, 1988, 1989, 1990; Paster *et al.*, 1993; Russell *et al.*, 1988, 1991). The first HAP species isolated were *Clostridium aminophilum*, *Clostridium sticklandii* and *Peptostreptococcus anaerobius* (Chen & Russell, 1988, 1989; Paster *et al.*, 1993; Russell *et al.*, 1988, 1991). Subsequently, similar, though not the same, HAP bacteria were isolated

from cattle in New Zealand (Attwood *et al.*, 1998) and Australia (McSweeney *et al.*, 1999). This paper describes the isolation and properties of a HAP bacterial species isolated from sheep in the UK. It differs significantly from the other HAP species, not only phylogenetically but in its formation of higher levels of volatile fatty acids (VFAs) from acetate and propionate.

Isolation of bacteria and growth conditions

Strains I-6^T and I-8 were isolated from a 10^{-6} dilution of ruminal fluid from a sheep by virtue of their ability to grow anaerobically on the defined medium of Chen & Russell (1988) containing trypticase peptone (15 g l^{-1} ; Becton Dickinson Microbiology Systems) but no other energy source (CRT medium). The sheep received a maintenance diet of hay, barley, molasses, fish-meal and vitamins/minerals [$500, 299.5, 100, 91$ and $9.5 \text{ g (kg dry matter)}^{-1}$, respectively] twice daily. A sample of ruminal fluid was removed 3 h after feeding, kept warm and under CO_2 , and strained through four layers of muslin. Strained rumen fluid was diluted serially under CO_2 in basal CRT medium without added trypticase. The medium was adjusted to pH 7.0 before autoclaving. These dilutions were used to inoculate (1%, v/v) Hungate tubes containing CRT

Abbreviations: HAP, ammonia-hyperproducing; VFA, volatile fatty acid.

The GenBank accession number for the 16S rDNA sequence of strain I-6^T is AJ310135.

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medium. The cultures were incubated at 39 °C for 7 days, and 20 µl of the 10⁻⁶ dilution was then streaked cross-wise onto plates of CRT medium and incubated again for 7 days. Two colonies were picked and grown in the liquid form of ruminal fluid-containing medium M2 (Hobson, 1969); bacteria were then reisolated by repeating the streaking and colony selection on CRT plates. Isolates were stored in medium M2.

Bacterial identification

The bacteria were characterized using conventional methods based on those of Holdeman *et al.* (1977). The presence of spores was investigated by incubating overnight M2 cultures at 80 °C for 10 min and then reinoculating fresh tubes of M2 liquid medium. Fermentation products were determined by derivatization and capillary GLC of supernatants from cultures grown in liquid M2 (Richardson *et al.*, 1989). Some fermentation tests were carried out using API 20 A strips (bioMérieux) under anaerobic conditions to determine sugar utilization and indole production. Gelatin liquefaction was assessed as described by Holdeman *et al.* (1977) except that the liquid form of medium M2 (Hobson, 1969) was the basal medium. Sugar utilization was also tested by adding the substrate to CRT medium to a final concentration of 5 g l⁻¹ and determining the growth response turbidimetrically at 650 nm using a Novaspec II spectrophotometer (Amersham Pharmacia Biotech). Staining for cytoplasmic inclusions and chemical analysis for storage polysaccharide and poly-β-hydroxybutyrate were carried out as described by Hanson & Phillips (1981). The G+C content was determined by means of the differential dye-binding method of Apajalahti *et al.* (2001), using regression analysis ($r^2 > 0.99$) of data obtained from gradients containing standard DNA samples of known G+C content (*Clostridium perfringens*, *Escherichia coli* and '*Micrococcus lysodeikticus*'). Sample DNA was analysed in triplicate. SDS-PAGE was carried out using pre-cast acrylamide gradient gels (ExcelGel SDS, gradient 8–18%T; Amersham Pharmacia Biotech). Whole cells were sedimented from liquid M2 cultures by centrifugation (at 15 000 g for 15 min) and the pellet was resuspended in SDS sample buffer, boiled and then centrifuged once more (at 12 000 g for 10 min) before being applied to the gel.

Ammonia production rate

The method used here was based on that described by Russell *et al.* (1988). Bacteria were grown overnight in 10 ml CRT medium; 1 ml was removed anaerobically into a microcentrifuge tube on ice and centrifuged (12 000 g for 10 min), 1 ml 150 g trypticase l⁻¹ was added to the remaining 9 ml in the culture tubes and the tubes were then incubated at 39 °C for 6 h. A further 1 ml sample was taken and centrifuged. Ammonia concentrations were determined for the supernatant fluids by using an automated phenol/hypochlorite method (Whitehead *et al.*, 1967) and protein in the pellet was determined using the Folin reagent following alkaline digestion (Herbert *et al.*,

1971). Ammonia production rates were calculated as the ammonia produced divided by the mean protein concentration in the 0 h and 6 h samples. Results are means obtained from three separate cultures.

Electron microscopy

Bacteria were fixed for 2 h in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, then post-fixed for 1 h in 1 % osmium tetroxide in the same buffer. The fixed bacteria were washed in the cacodylate buffer and embedded in 1.5 % agarose. Small blocks of the agarose-embedded sample (1 mm²) were dehydrated in a graded ethanol series, cleared in propylene oxide and infiltrated overnight in a 50:50 mixture of propylene oxide and Araldite resin (Agar Scientific). The infiltrated specimens were embedded in fresh Araldite resin and the blocks were polymerized for 3 days at 70 °C. Ultrathin sections of embedded samples were contrasted with uranyl acetate and lead citrate and examined in a JEOL 1200 EXB transmission electron microscope operating at an accelerating voltage of 80 or 100 kV. Whole bacteria were also examined in the electron microscope after negative staining in 2.5 % ammonium molybdate, pH 6.5.

rDNA analysis

DNA from strain I-6^T was extracted using methods described by McEwan *et al.* (1994). A partial sequence of 16S rDNA was amplified using the universal primers 5'-AGAGTTTGATCATGGCTCAG-3' and 5'-ACGGCTACCTTGTTACGACTT-3' (Weisburg *et al.*, 1991) in a mixture that contained 1 µM each primer, 200 µM each dNTP, 50 mM KCl and 1.5 M MgCl₂ in 10 mM Tris/HCl (pH 8.3) buffer. Next, 0.1 µg DNA and 2.5 U *Taq* DNA polymerase were added to 100 µl of this mixture and amplification was carried out using 45 cycles of 94 °C for 1 min followed by 2 min at 55 °C and 3 min at 72 °C. Amplification was confirmed on an agarose gel.

Amplified DNA was cloned into the pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions. Plasmids were isolated from recombinant colonies (Maniatis *et al.*, 1982) and the nucleotide sequence of the insert was determined using an ABI Prism 377XL DNA sequencer (Perkin Elmer). A DNA similarity search was performed using the DDBJ BLAST server (<http://www.ddbj.nig.ac.jp/E-mail/homology.html>). The phylogenetic tree was constructed using CLUSTAL W analysis (<http://www2.ebi.ac.uk/clustalw/>) followed by programs within PHYLIP version 3.57 (Felsenstein, 1989). Analysis within PHYLIP was performed by first using the DNADIST and NEIGHBOR programs. The validity of the resulting tree was determined using 1000 bootstrap iterations (SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs). The initial tree was viewed using TreeView (Page, 1996) and bootstrap values inserted using FREEHAND (version 9).

Identification and phylogenetic analysis

Whole-cell extracts from isolates I-6^T and I-8 produced identical banding patterns in SDS-PAGE. The patterns were distinct from banding patterns of other ruminal bacteria, including other non-saccharolytic *Clostridium*/*Eubacterium* species and the HAP species identified previously, *P. anaerobius*, *C. sticklandii* and *C. aminophilum* (results not shown). The morphology of isolate I-6^T was typical of a Gram-positive organism, having dimensions of approx. $1.2 \times 0.4 \mu\text{m}$ and a thin, layered cell wall (Fig. 1). More distinctive were inclusions of low electron density, which appeared under transmission electron microscopy (Fig. 1a, b), and membrane-like structures associated with the septum of dividing cells and in a polar location in non-dividing cells (Fig. 1c, d). The former gave the appearance of storage materials, but staining and chemical analysis for glycogen and poly- β -hydroxybutyrate did not give positive results.

Neither isolate appeared, by fermentation-strip methods, to utilize any of the most common sugars, including

glucose, mannitol, lactose, sucrose, maltose, salicin, xylose, arabinose, glycerol, cellobiose, mannose, melezitose, raffinose, sorbitol, rhamnose and trehalose. Liquid-culture studies confirmed that, of the substrates tested, growth increased above that provided by trypticase (final $\text{OD}_{650} = 0.4$) only with sodium pyruvate (final $\text{OD}_{650} = 1.2$) and, to a lesser extent, with sodium DL-lactate (final $\text{OD}_{650} = 0.5$). No increase in OD_{650} was observed with glucose, maltose, cellobiose, fructose, glycerol, xylose, ethanol, sodium fumarate, sodium succinate, sodium acrylate or sodium citrate. Isolate I-6^T did not grow in medium M2 in the presence of $5 \mu\text{M}$ monensin. It grew at 45°C but not at 25°C . No haemolysis was observed in blood-agar plates. Its G+C content was 56.8 mol% (SE 0.10%), at the upper end of G+C values for *Clostridium*/*Eubacterium* species (Cato & Stackebrandt, 1989) and similar to the G+C values found for *C. aminophilum*, another ruminal HAP species (Paster *et al.*, 1993).

Isolates I-6^T and I-8 were unusual in their VFA metabolism. During growth on M2 medium, which contains clarified

Fig. 1. Transmission electron micrographs (a, b) and negatively stained electron micrographs (c, d) of cells of strain I-6^T. Note the presence of apparent storage materials, indicated by arrows in (a), and membranous structures associated with polar regions of non-dividing cells (c) and the septum of dividing cells (d).

Table 1. VFA production by strain I-6^T in liquid medium M2

Results are means from three cultures. The mean final cell density in M2 medium was 0.60 mg protein ml⁻¹.

Medium	VFA initial concentration or net production (mM)								
	Formate	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Caproate	Lactate
Concentration in uninoculated medium	0.50	11.06	2.99	0.13	1.94	0.11	0.19	0.09	75.66
Final concentration	0.81	9.53	0.39	0.35	1.79	0.22	1.25	7.70	74.00
VFA produced (+) or utilized (-)	+0.31	-1.53	-2.60	+0.22	-0.15	+0.11	+1.06	+7.61	-1.66

ruminal fluid and therefore VFAs, there was a consistent decrease in the concentrations of acetate and propionate during growth (Table 1). Caproate was the major product and, to a lesser extent, valerate was also formed. The concentration of caproate formed was higher than any we have encountered previously with any ruminal species and the utilization, rather than production, of propionate is, as far as we are aware, unique among ruminal species of bacteria.

The other properties of isolate I-6^T, based on non-molecular methods of classification, were less distinctive, and all of the reactions in the API 20 A strips were negative, including those for indole production, urease, gelatin liquefaction, aesculin production and catalase activity, so identification by conventional means was made difficult by insufficient discriminatory information. Molecular phylogenetic analysis proved more useful. Analysis of the 16S rDNA sequence placed strain I-6^T within the 'low-G+C' *Clostridium*/*Eubacterium* group (Collins *et al.*, 1994; Fig. 2). It represents a novel species, located on a different branch with respect to the original HAP bacteria isolated by Russell and his colleagues (Fig. 2). Isolate I-6^T is most closely related, among published isolates, to HAP isolate C2 of Attwood *et al.* (1998) (89 % identity), two asaccharolytic species, *Eubacterium minutum* and *Eubacterium infirmum*, from the mouth (Cheeseman *et al.*, 1996; Wade *et al.*, 1999a, b) (also 89 % identity), and another oral species, *Eubacterium brachy* (Hamid *et al.*, 1994) (88 % identity). Its 16S rDNA sequence is very similar to three sequences obtained from the rumen by Tajima *et al.* (2000) (accession nos AB034093, AB034092 and AB034148). These bacteria do not fall into any of the clusters proposed by Collins *et al.* (1994), being taxonomically quite distant from the closest group, cluster XI (Attwood *et al.*, 1998; Cheeseman *et al.*, 1996). The bacteria share an asaccharolytic physiology as well as a common location in the digestive tract, suggesting a common progenitor.

The rate of NH₃ production from pancreatic casein hydrolysate (trypticase) was 375 ± 56 nmol (mg protein)⁻¹ min⁻¹ when grown in CRT medium in a 6 h incubation. This activity was similar to the 346, 318 and 367 nmol (mg protein)⁻¹ min⁻¹ observed with *P. anaerobius*, *C. aminophilum* and *C. sticklandii* (Russell *et al.*, 1991), though not as

high as some of the rates, up to 946 nmol (mg protein)⁻¹ min⁻¹, observed with isolates from grazing ruminants in New Zealand (Attwood *et al.*, 1998).

Ecological niche

Strain I-6^T thus appeared to be typical of the non-saccharolytic HAP bacteria isolated from the rumen first by Russell and his colleagues (Chen & Russell, 1988, 1989; Paster *et al.*, 1993; Russell *et al.*, 1988) and subsequently by Attwood *et al.* (1998) and McSweeney *et al.* (1999). It was similar to these other isolates in its ability to grow on trypticase in the absence of sugars, its non-saccharolytic metabolic properties and its sensitivity to monensin. Like most of the isolates obtained in this way, with the exception of the *Clostridium botulinum*-like isolate Lp1284 (McSweeney *et al.*, 1999), it was not proteolytic. Most importantly from the point of view of ruminal ecology,

Fig. 2. Phylogenetic position of *Eubacterium pyruvivorans* sp. nov. relative to the most closely related members of the *Clostridium*/*Eubacterium* cluster and to other HAP bacteria (*C. aminophilum*, *C. sticklandii* and *P. anaerobius*) based on 16S rDNA sequences. *Frankia* sp. ORS020606 was used as the outgroup. Bar, 10 % difference. Bootstrap values are shown as percentages. Accession numbers are shown in parentheses.

isolate I-6^T produced NH₃ rapidly from amino acids. Thus, despite the relatively small numbers of these bacteria, representing around 1 % of the population, they may still play a significant role in ruminal ammonia formation (Russell *et al.*, 1991).

Phylogenetic analysis based on rDNA similarity indicated that strain I-6^T was a novel species. Strain I-6^T was metabolically and morphologically similar to two other groups of *Clostridium*/*Eubacterium* species isolated from sheep on the same diet, but phylogenetically separate and having different whole-cell SDS-PAGE banding patterns (S. C. P. Eschenlauer, N. McKain, N. D. Walker, N. R. McEwan, C. J. Newbold and R. J. Wallace, unpublished data). Isolate I-6^T grew rapidly on pyruvate, in contrast to related species and other HAP bacteria, which were reported to ferment pyruvate only weakly (Attwood *et al.*, 1998) or not at all (Chen & Russell, 1989; Russell *et al.*, 1991). Pyruvate is not present at high concentrations in ruminal fluid (Wallace, 1978). The ability of strain I-6^T to grow rapidly on pyruvate presumably reflects only the central role of pyruvate in metabolism. The niche that strain I-6^T occupies may be one of a scavenger, converting amino acids and other compounds formed as the result of fermentation of feedstuffs and autolysis of other rumen microbial species to pyruvate to generate energy via the oxidation of pyruvate to acetate. The interconversion of VFAs to form caproate may be a mechanism for regenerating oxidized cofactors to enable the fermentation to proceed, a scenario similar to that observed in *Clostridium kluyveri* (Smith *et al.*, 1985).

Description of *Eubacterium pyruvativorans* sp. nov.

Eubacterium pyruvativorans (pyr.uv.at'i.vor.ans. N.L. n. *pyruvatum* pyruvate; L. v. *vorans* devouring, eating greedily; N.L. neut. adj. *pyruvativorans* devouring pyruvate).

Cells are Gram-positive, straight to slightly curved rods, 0.3–0.5 µm wide and 1.0–1.5 µm long. Cells occur in short chains. No flagella are present and spores are not evident. The G + C content of the DNA is 56.8 mol%. Heating in the spores test eliminates viable cells. Overnight growth in M2 broth produces a uniformly opalescent suspension. Colonies on M2 solid medium are, after 72 h, light tan in colour and 2 mm in diameter and have slightly irregular edges. Growth is supported by pyruvate and, to a lesser extent, lactate. Amino acids can be used as the sole source of carbon and energy, with yields much lower than those obtained with pyruvate. Sugars are not fermented. No growth occurs aerobically. The main fermentation product is caproate in rich, ruminal fluid-containing medium.

The type strain, I-6^T (= ATCC BAA-574^T = NCIMB 13911^T), was one of two isolates obtained from the sheep rumen using a medium selective for bacteria that can grow on peptides as the sole source of carbon and energy. The other strain, I-8, had an identical whole-cell protein SDS-PAGE pattern,

similar fermentation activities and products and similar NH₃-producing activity with respect to strain I-6^T.

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