Howardella ureilytica gen. nov., sp. nov., a Gram-positive, coccoid-shaped bacterium from a sheep rumen

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An unidentified obligately anaerobic, fastidious, Gram-positive, non-motile, non-spore-forming, non-fermentative coccoid-shaped bacterium (designated strain GPC 589^T) was isolated from the rumen fluid of a sheep. The major fatty acid constituents (>5 %) were C_{16:0} (29.2 %), C_{18:0} (40.7 %) and an unidentified compound (19.7 %) with an equivalent chain-length of 13.523. The G + C content of the DNA was 34 mol%. The organism was strongly ureolytic and generated ATP through the hydrolysis of urea. Comparative 16S rRNA gene sequence analysis demonstrated that strain GPC 589^T was far removed, phylogenetically, from the ruminococci and related Gram-positive anaerobic cocci but exhibited a phylogenetic association with *Clostridium* rRNA cluster XIVa [as defined by 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). *Int J Syst Bacteriol* **44**, 812–826]. Sequence divergence values of 12.5 % or more were observed between strain GPC 589^T and all other recognized species within this and related rRNA clostridial clusters. Phylogenetic analysis showed that strain GPC 589^T represents a new genus within cluster XIVa. On the basis of both phylogenetic and phenotypic evidence, it is proposed that strain GPC 589^T should be classified as representing a new genus and novel species, *Howardella ureilytica* gen. nov., sp. nov. The type strain is strain GPC 589^T (=DSM 15118^T=JCM 13267^T).

In the ruminant herbivore, primary digestion of the food is carried out by the micro-organisms of the rumen. For many of the ruminal bacteria, ammonia is the major nitrogen source. Urea is present in ruminant saliva and diffuses into the rumen through the rumen wall and, as urea is rapidly hydrolysed in the rumen (Pearson & Smith, 1943), it is an important source of ammonia especially in the recycling of nitrogen in the rumen. While urea is physiologically important to nitrogen metabolism in ruminants, it is also economically important to ruminant feeding. However, the microbial aspects of urea metabolism are not well understood, either regarding numbers relative to total rumen bacteria or whether the ureolytic

bacteria are widespread in rumen contents, or even whether bacteria are the major source of rumen urease (Farlin *et al.*, 1968).

Strain GPC 589^T was isolated from the rumen fluid of a penned cannulated sheep that had been fed on a diet of hay and grass with water ad libitum. The anaerobic culture technique of Hungate (1950) was used with oxygen-free CO₂ as the gas phase and incubation at 37 °C. Initially, a fresh rumen fluid sample (0.5 %, v/v) was enriched for 48 h (Cook, 1976) in medium GM comprising (w/v): Bacto casitone (0.5 %), Bacto yeast extract (0.5 %), KH₂PO₄ (0.25 %), Na₂HPO₄ (0.25 %), CH₃.COONa (0.1 %), mannitol (0.2 %), MgCl₂.6H₂O (0.05 %), sodium thio-glycollate (0.075 %) and resazurin (0.0001 %), pH 7.0; autoclaved at 103.5 kPa for 15 min. Filter-sterilized urea (0.2 % w/v, final concentration) was added to cooled medium. The enrichment was maintained by overnight subculturing (0.1 ml) in 8 ml medium G (mannitol-free GM) in Hungate tubes. An ureolytic Gram-positive coccus

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Abbreviation: ECL, equivalent chain-length.

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was isolated from a Hungate roll tube of medium GM (4.5 ml containing 2% agar) that had been inoculated (0.5 ml) from the 10^{-7} dilution of a decimal dilution series (medium G) of an overnight subculture; this was identified presumptively as an enterococcus. After overnight incubation (about 18 h) in medium G, a decimal dilution series showed a hazy growth in the 10^{-9} dilution in contrast to the heavy growth present at lower dilutions: there was no visible growth in the higher dilutions. Over a 3-day incubation period during which time the ureolytic activity of this 10^{-9} dilution culture increased, there was no discernible increase in turbidity. The Gram-positive isolate from the 10^{-9} dilution tube was designated strain GPC 589^T. Urease activity was determined by measuring ammonia production in the presence and absence of urea. A 1 ml centrifuged sample of a broth culture was resuspended in an equal volume of 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. A 25 µl sample was incubated with 0.17 mmol urea in a final volume of 1 ml for 5 min at 37 °C. The reaction was terminated by the addition of phenate-hypochlorite reagents (Weatherburn, 1967), modified such that 1 ml of each reagent was used in an assay. The colour was developed for 25 min at 37 °C and read at 570 nm. Culture growth was measured by determining the viable count as turbidities were too low. Viable counts of cultures were carried out within the oxygen-free atmosphere of a flexible-film anaerobic chamber, using procedures described by Hartley *et al.* (1992). BHF plates of supplemented brain heart infusion blood agar (Barr *et al.*, 1987), stored anaerobically (10% CO₂, 10% H₂ in N₂, >90% RH) at 4 °C, were lightly air-dried at 37 °C before being transferred to the anaerobic chamber for overnight storage. The source of brain heart infusion used in this medium (Oxoid) was found to be important: other sources were inferior (data not shown). The agar medium was inoculated with triplicate 25 µl samples of a decimal dilution series of a culture prepared in brain heart infusion broth using a modification of the technique of Miles *et al.* (1938), as described previously by Hudson *et al.* (1984). The plates were removed from the chamber and incubated anaerobically at 37 °C for 72 h (Hartley *et al.*, 1992). Gram staining was based on the method of Preston & Morrell (1962) but with modifications to the iodine mordant (Magee *et al.*, 1975), the safranin counterstain (Holdeman *et al.*, 1977) and the use of acetone to fix the air-dried slides (Mangels *et al.*, 1984). Following publication of the procedure of Johnson *et al.* (1995), staining was done anaerobically to ensure uniformity of stain. Cell morphology was observed using a Leitz Dialux light microscope at a magnification of $\times 1000$ with cells grown in medium G overnight at 37 °C.

Strain GPC 589^T was characterized biochemically by using a combination of conventional tests as described in the VPI *Anaerobe Laboratory Manual* (Holdeman *et al.*, 1977). All biochemical tests were performed in triplicate. The following compounds did not augment the growth of

strain GPC 589^T when added to medium G and grown anaerobically at 37 °C: carbohydrates: glucose, sucrose, maltose, starch, mannitol and cellobiose; carboxylic compounds: pyruvate, lactate, malate, formate, fumarate, succinate and citrate; Tween 80; mixture of volatile fatty acids (propionic, n-butyric, n-valeric, iso-butyric, 3-methylbutyric and 2-methylbutyric; Holdeman *et al.*, 1977); nitrogen compounds (urea omitted): ammonia and nitrate; haemin; serum; vitamin K and bicarbonate. The following were required for optimum growth: casitone, yeast extract, acetate, magnesium, sodium/potassium phosphate, thioglycollate and urea. Thioglycollate was required for a reduced environment; cysteine was inhibitory. Acetate stimulated growth. Urea was demonstrated to be essential for growth of strain GPC 589^T; the minimal growth in medium without urea (Bacto casitone is a milk product and contains trace amounts of urea) contrasts with the 140-fold increase in the presence of urea at 0.2% (w/v). Whereas urea was essential for growth, ammonia, a product of the strong urease activity, did not support growth. The role for the hydrolysis of urea was to generate ATP. Thus, preincubation of strain GPC 589^T with the urease inhibitor fluorofamide (Millner *et al.*, 1982; Kenny, 1983) resulted in an 87% inhibition of ammonia production and a concomitant 92% reduction in ATP synthesis. Strain GPC 589^T did not grow at 20 or 50 °C. The G+C content of the DNA was determined by HPLC as described by Ezaki *et al.* (1990). DNA from peptostreptococci (*Peptostreptococcus asaccharolyticus*, *Peptostreptococcus prevotii* and *Peptostreptococcus magnus*), ruminococci (*Ruminococcus torques* and *Ruminococcus obeum*) and *Peptococcus niger* was used for reference. The G+C content of the DNA of strain GPC 589^T was found to be 34 mol%, which was consistent with the assignment of this isolate to the low G+C branch of the Gram-positive bacteria.

Fatty acid methyl esters were obtained from 40 mg cells scraped from Petri dishes by saponification, methylation and extraction, using minor modifications of the methods of Miller (1982) and Kuykendall *et al.* (1988). The fatty acid methyl ester mixtures were separated using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID), which consisted of an Agilent model 6890N gas chromatograph fitted with a 5% phenyl-methyl-silicone capillary column (0.2 mm $\times$ 25 m), a flame-ionization detector, Agilent model 7683A automatic sampler and an HP-computer with MIDI database (Hewlett Packard Co.). Peaks were integrated automatically and fatty acid names and percentages were calculated using the MIS standard software (Microbial ID). The gas chromatographic parameters were as follows: carrier gas, ultra-high-purity hydrogen; column head pressure, 60 kPa; injection volume, 2 µl; column split ratio, 100:1; septum purge, 5 ml min⁻¹; column temperature, 170–270 °C at 5 °C min⁻¹; injection port temperature, 240 °C; and detector temperature, 300 °C (Kämpfer & Kroppenstedt, 1996). The cellular fatty acid composition of strain GPC

589^T consisted of straight-chain saturated and unsaturated fatty acids. The principal fatty acids were the saturated acids C_{16:0} (29.2 %) and C_{18:0} (40.7 %). There were also smaller amounts of the saturated fatty acids C_{14:0} (0.7 %) and C_{17:0} (1.7 %). The unsaturated acids C_{16:1 ω 9c} (2.4 %), C_{18:1 ω 9c} (4.5 %) and C_{18:1 ω 11c} (1.1 %) were also present. An unidentified compound with an equivalent chain-length (ECL) of 13.523 was detected (19.7 %), but could not be assigned to a known fatty acid or a dimethylacetal.

The 16S rRNA gene sequence was determined from the isolated DNA (Luton *et al.*, 2002) using the primers of Hutson *et al.* (1993) and PCR was performed using the protocol of Innis & Gelfand (1990). The 16S rRNA gene was amplified by PCR using the universal eubacterial primers (Lane, 1991). Sequenced 16S rRNA gene fragments were assembled into one contiguous sequence. Phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein, 1993) and TreeView (Page, 1996), after alignment of data using ARB (Ludwig *et al.*, 2004). Distances (Kimura two-parameter model) and clustering by neighbour-joining methods were determined by using bootstrap values based on 1000 replicates (Fig. 1). The 16S rRNA gene sequence of strain GPC 589^T was a continuous stretch of 1531 bp. Sequence database searches showed that strain GPC 589^T displayed the highest sequence relatedness to members of the *Clostridium* subphylum (data not shown), consistent with the low G+C content. On the basis of sequence similarities, the closest named relatives of strain GPC 589^T corresponded to species within *Clostridium* rRNA cluster XIVa (see Collins *et al.*, 1994, for rRNA group designations). Sequence similarity calculations indicated that the closest relatives of strain GPC 589^T were *Catonella morbi* ATCC 51271^T (86.5 %), *Johnsonella ignava* ATCC 51276^T (85.3 %) (these were placed in cluster XIVa by Willems & Collins, 1995), *Eubacterium saburreum* C27KA (87.4 %), *Clostridium polysaccharolyticum* DSM 1801^T (88.3 %), *Acetitomaculum ruminis* 139B^T (87.2 %) and *Eubacterium hallii* ATCC

27751^T (87.5 %). These bacteria were isolated from such diverse habitats as the human buccal cavity, the rumen and human faeces. The monospecific genera *Catonella* and *Johnsonella*, described by Moore & Moore (1994), were isolated from the human gingival crevice and periodontal pockets (Moore *et al.*, 1985) and were originally referred to as 'Bacteroides D42' and 'Bacteroides D19', respectively. Holdeman & Moore (1974) described *E. hallii*, which was isolated from human faecal flora. *A. ruminis* was isolated from the bovine rumen and described by Greening & Leedle (1989). In 1980, van Gylswyk isolated *Fusarium polysaccharolyticum* from the sheep rumen, which was later renamed *Clostridium polysaccharolyticum* (van Gylswyk *et al.*, 1980). The description of the anaerobic filamentous micro-organisms isolated from human dental plaque and gingival crevices but unnamed by Theilade & Gilmour (1961) was used by Holdeman *et al.* (1977) in their description of *E. saburreum* (Skerman *et al.*, 1980). A BLAST search using the 16S rRNA gene of strain GPC 589^T revealed that uncultured bacterial clones have been identified from humans, which are closer to strain GPC 589^T than the species in *Clostridium* rRNA cluster XIVa. Ley *et al.* (2006) identified two clones, RL249 (GenBank accession no. DQ805137) and RL181 (DQ798309) from human faeces and Eckburg *et al.* (2005) identified clone MH95 (AY982262) from a biopsy of the human colon mucosa and a further three clones from biopsies of the human colon, MG76 (AY982192), LY31 (AY976352) and MG71 (AY916289).

From the data presented, it is evident that the Gram-positive, asporogenous, coccoid-shaped strain GPC 589^T, recovered from a sheep rumen, represents an unknown line within the *Clostridium* subphylum of the Gram-positive bacteria (Collins *et al.*, 1994). Phylogenetically, strain GPC 589^T has an affinity with rRNA cluster XIVa, but not with the Gram-positive anaerobic cocci that are interspersed throughout a variety of phylogenetic groups. The association of strain GPC 589^T with members of *Clostridium*

Fig. 1. Phylogenetic tree based on analysis of the 16S rRNA gene sequences of strain GPC 589^T, representative species within cluster XIVa of the *Clostridium* subphylum, and representatives of other clusters of the *Clostridium* subphylum showing the position of the novel genus *Howardella*. The dendrogram was produced with DNADIST (neighbour-joining) analysis using 1317 bp of aligned sequence. The phylogenetic tree was rooted to *Clostridium butyricum* ATCC 19398^T (GenBank accession no. M59085; not shown). Bootstrap values above 50 % are shown from 1000 replicates. Bar, 10 % sequence divergence.

rRNA group XIVa was only loose, with strain GPC 589^T branching deeply at the periphery of the cluster. Treeing analysis showed that strain GPC 589^T did not have a close relationship with any individual member of *Clostridium* rRNA group XIVa, although the branching at the base of the grouping was supported by a high bootstrap value. The distinctiveness of strain GPC 589^T was also evident from divergence values of greater than 12 % with members of rRNA cluster XIVa and of greater than 20 % with other recognized Gram-positive anaerobic cocci.

Clostridium rRNA cluster XIVa embraces a diverse range of organisms including *Catonella morbi*, *J. ignava*, *E. hallii*, *A. ruminis*, *E. saburreum* and a variety of clostridial species, *Clostridium aerotolerans*, *Clostridium oroticum*, *Clostridium polysaccharolyticum*, *Clostridium proteolyticum*, *Clostridium ramosum* and *Clostridium formicaceticum*. In addition to high sequence divergence values and tree topology considerations, support for the separateness of strain GPC 589^T from other organisms within *Clostridium* rRNA cluster XIVa also comes from phenotypic considerations (Table 1). In particular, strain GPC 589^T can be readily distinguished from all other taxa within this cluster by its small, coccoid cells and the synthesis of ATP from the hydrolysis of urea. Furthermore, strain GPC 589^T can be distinguished from *Clostridium polysaccharolyticum* by not producing endospores. The non-fermentative characteristic distinguishes strain GPC 589^T from *Catonella morbi*, *E. hallii*, *E. saburreum* and *A. ruminis*. The phenotypic distinctiveness of strain GPC 589^T, together with its long rRNA line branching at the base of *Clostridium* rRNA cluster XIVa, merits its inclusion in a new genus and species, *Howardella ureilytica* gen. nov., sp. nov.

Description of *Howardella* gen. nov.

Howardella (Ho.war.del'la. N.L. fem. dim. *Howardella* to honour the New Zealand microbiologist Bernard Howard, in recognition of his many contributions to anaerobic microbiology).

Gram-positive-staining, non-spore-forming, non-motile, coccoid cells. Growth occurs under strictly anaerobic conditions. Carbohydrates are not fermented. Nitrate is not reduced. Acetate stimulates growth. Member of the *Clostridium* subphylum of the Gram-positive bacteria and is phylogenetically loosely associated with *Clostridium* rRNA group XIVa. The type species is *Howardella ureilytica*.

Description of *Howardella ureilytica* sp. nov.

Howardella ureilytica (u.re.i.ly'ti.ca. N.L. n. urea urea; Gr. adj. *lutikos* able to dissolve; N.L. adj. *lyticus* -a -um dissolving; N.L. fem. adj. *ureilytica* urea dissolving).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.77–0.83 µm long × 0.61–0.67 µm wide and occur singly, in pairs, although occasionally in short chains of 6–8 cells. After 72 h anaerobic incubation at 37 °C under a gas phase of N₂, CO₂ and H₂ (80:10:10, by vol.) on BHF/blood agar, colonies are minute to 1 mm in diameter, circular, entire, domed and white. Urea is obligatory. Carbohydrates are not fermented. Hydrolysis of urea by cytosolic urease generates ATP. Long-chain cellular fatty acids consist of a complex mixture including an unknown with an ECL of 13.523, C_{16:0} and C_{18:0}. The G + C content of the DNA of the type strain is 34 mol%. Habitat, gastrointestinal tract.

Table 1. Differential characteristics of strain GPC 589^T (*Howardella ureilytica* gen. nov., sp. nov.) and phylogenetically closely related species

Strains: 1, strain GPC 589^T; 2, *Catonella morbi* ATCC 51271^T (data from Moore & Moore, 1994; Moore *et al.*, 1985); 3, *J. ignava* ATCC 51276^T (Moore & Moore, 1994; Moore *et al.*, 1985); 4, *E. saburreum* C27KA (Theilade & Gilmour, 1961; Holdeman *et al.*, 1977; Skerman *et al.*, 1980); 5, *E. hallii* ATCC 27751^T (Holdeman & Moore, 1974); 6, *A. ruminis* 139B^T (Greening & Leedle, 1989); 7, *Clostridium polysaccharolyticum* DSM 1801^T (van Gylswyk, 1980; van Gylswyk *et al.*, 1980). –, Negative; +/–, weak; +, positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7
Gram stain	+	–	–	+/–	+	+	–
Cell size (µm)	0.61–0.67 × 0.77–0.83	0.7–1.2 × 1.6–4.0	0.8 × 3.7–6.4	0.7–1.1 × 6.0–18.0	0.8–2.4 × 4.7–25.0	0.8–1.0 × 2.0–4.0	0.6–1.1 × 2.0–15
Motility	–	–	–	–	–	+/–	+
Spore formation	–	–	–	–	–	–	+
Utilization of:							
Cellobiose	–	+	–	–	–	+	+
Glucose	–	+	–	+	+	+	ND
Maltose	–	+	–	+/–	+	–	–
Starch	–	–	–	–	–	–	+
Sucrose	–	+	–	+	–	ND	–
DNA G + C content (mol%)	34	34	32	ND	38	32–36	42

The type strain, GPC 589^T (=DSM 15118^T =JCM 13267^T), was isolated from a sheep rumen.

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