Clostridium schirmacherense sp. nov., an obligately anaerobic, proteolytic, psychrophilic bacterium isolated from lake sediment of Schirmacher Oasis, Antarctica

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A novel obligately anaerobic, proteolytic bacterium, designated AP15^T, was isolated from lake sediments of Schirmacher Oasis, Antarctica. The bacterium produced maximum cell mass between 5 and 10 °C in an anaerobic basal medium containing 0.5 % tryptone and peptone. The strain grew optimally at a pH around 8.0 and tolerated NaCl up to a concentration of 7.5 %. It contained diphosphatidylglycerol as the major phospholipid and C_{15:0}, C_{16:0} and C_{17:0} as the major cellular fatty acids. Several amino acids, including arginine, leucine, isoleucine, cysteine, glutamate and serine, supported growth. Glutamate was degraded to acetate, propionate, CO₂ and H₂. In addition, the strain degraded carbohydrates including glucose, raffinose, adonitol, ribose and rhamnose. The main fermentation products during growth on glucose were H₂, CO₂, formate, acetate, propionate and isovalerate. The DNA G + C content of the bacterium was 24 mol%. On the basis of a phylogenetic analysis, strain AP15^T is identified as a close relative of *Clostridium subterminale* ATCC 25774^T, with which it shares 99.5 % similarity at the 16S rRNA gene sequence level; however, it exhibits a low DNA–DNA binding value (55 %) to this strain at the whole-genome level. In addition to showing other major differences with respect to *C. subterminale* and other members of the genus *Clostridium*, AP15^T also exhibits phenotypic differences. On the basis of these differences, strain AP15^T is identified as representing a novel species of the genus *Clostridium*, for which the name *Clostridium schirmacherense* sp. nov. is proposed. The type strain is AP15^T (= DSM 17394^T = JCM 13289^T).

Prokaryotes dominate Antarctic ecosystems and play major roles in food chains, biogeochemical cycles and the mineralization of pollutants. Recent investigations using modern molecular biological techniques as well as classical procedures have demonstrated that many of the Antarctic micro-organisms reported to date represent novel species and exhibit broad phylogenetic diversity, since they include representatives from both the *Archaea* and the *Bacteria* (Alam *et al.*, 2003; Johnson & Bellinoff, 1981; Johnson *et al.*, 1981; Madden *et al.*, 1979; Miller & Leschine, 1984; Shivaji *et al.*, 1989, 1992).

Despite the extreme climatic conditions that persist in Antarctica, micro-organisms have been detected in all of the continent's distinctive habitats, e.g. lakes, ponds, rivers, streams, rocks and soil. These habitats differ from one another with respect to nutrients, temperature range, water activity and other physico-chemical parameters. Since these factors influence the survival and growth of micro-organisms, the microbial flora are bound to vary from one habitat to the other. Free water is available only during the summer, in oases that experience seasonal variation during late autumn and spring. The biology of these freshwater oases is therefore likely to be different from that of the rest of the continent. Microbiological studies in specific regions of continental Antarctica have been largely directed towards the isolation and characterization of psychrophilic micro-organisms. The number of newly isolated anaerobes has increased over the past few years, the main emphasis being on their morphological/physiological characterization and

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Details of glucose fermentation and amino acid fermentation by strain AP15^T are available as supplementary tables in IJSEM Online.

phylogenetic analysis (Dixit *et al.*, 2005; Attwood *et al.*, 1996; Broda *et al.*, 2000a, b; Engle *et al.*, 1996; Toda *et al.*, 1988). Anaerobic microbial communities and micro-organisms have been described by various workers during investigations of the Antarctic ecosystem (Franzmann *et al.*, 1991; Franzmann & Rohde, 1991; Franzmann & Dobson, 1992; Mountfort *et al.*, 1997). Proteolytic bacteria play a crucial role in food chains and in the aquatic ecosystems of lakes and other bodies of water in Antarctica, including dry valleys, coastal lakes and the McMurdo Ice Shelf. These investigations have provided clues as to the photosynthesis and carbon flux associated with benthic cyanobacterial mats.

Cold-active anaerobic bacteria, which produce extracellular proteases, potentially can be utilized for the biodegradation of organic wastes that are rich in protein, e.g. night-soil (human faeces), in low-temperature areas, as the hydrolysis of biopolymers is the first step in the anaerobic digestion process. These extracellular proteases from psychrophilic anaerobes might possess unique adaptive and structural features not present in those from other aerobic, mesophilic or thermophilic microbes. In this communication, we describe the characterization of an anaerobic proteolytic bacterium from Antarctic lake sediment. On the basis of the phenotypic and genotypic data, we conclude that AP15^T represents a novel species.

Strain AP15^T was isolated from a lake-sediment sample collected from Schirmacher Oasis (Antarctica) between 20 January and 20 February 1994 during the 14th Indian Summer Scientific Expedition. The geographical coordinates of the site are 70° 45' 12" S and 11° 46' E. Sealed vials were transported to the laboratory at 4 °C and stored at -20 °C. The sediment sample was enriched for proteolytic bacteria in M5 medium containing 0.5 % tryptone and peptone (Dube *et al.*, 2001). The medium was supplemented with trace element solution (5 ml) and 10 ml reducing solution (11.25 g Na₂S and 0.125 g cysteic acid per 10 ml) per litre. The pH was adjusted to 7.0 by using 2 M NaOH. Serum vials (60 ml), each containing 30 ml medium, were prepared anaerobically under a continuous flow of N₂ and H₂ (80:20). The medium was inoculated with 2 % sample and incubated at 10 °C for 1 week. Subculturing was done by transferring 5 % inoculum to fresh bottles under similar conditions.

Tenfold serial dilutions of enriched cultures were prepared anaerobically in normal saline; 0.1 ml from each dilution step was spread on the surface of agar containing M5 medium with casein (0.5 %) in an anaerobic workstation (Don Whitley). Plates were incubated at 10 °C for 7 days; colonies showing zones of proteolysis were transferred to M5 broth. Several pure cultures were obtained, one of which was designated strain AP15^T and studied further. The cells were grown anaerobically in M5 broth that was devoid of casein but contained 0.5 % each of tryptone and peptone. The effect of pH on growth and protease production was studied by growing cells in 30 ml M5 broth in 60 ml serum

vials. The pH of the media was adjusted between 5 and 13, at increments of 1 pH unit, using 10 mM buffers. The following buffers were used for different pH ranges: sodium acetate (pH 3.0–5.0), sodium citrate (pH 3.0–5.0), potassium phosphate (pH 6–8), Tris/HCl (pH 7.0–9.0), sodium borate (pH 9.0–10.0) and glycine/NaOH (pH 11.0–13.0). Vials were inoculated with 2 % (v/v) freshly grown culture (OD₆₀₀ = 0.4) and incubation was carried out at 10 °C. Subsequently, the effect of pH was observed in greater detail (using increments of 0.5 pH units) at pH values from 6 to 9. Growth was observed by determining the OD₆₀₀. Similarly, the effect of NaCl was evaluated by using M5 medium at various NaCl concentrations (1–15 %). Growth was monitored after 6 and 12 days incubation at 10 °C. For animal toxicity determinations, supernatants of early and late stationary phase cultures were titrated to ascertain the minimum lethal dose, using serial twofold dilutions made in gelatin diluent (Hall *et al.*, 1985). An aliquot (0.5 ml) of each dilution was inoculated intraperitoneally into each of six 20 g mice. The mice were observed for 4 days: flaccid paralysis and deaths were recorded. A *Clostridium botulinum* type E culture supernatant was used as the control in the mouse bioassay. Toxicity was not observed with the culture supernatant of strain AP15^T.

The shape of the bacterium was determined by using light microscopy and scanning electron microscopy. Motility was examined by using the hanging drop technique (Pelczar *et al.*, 1993). For scanning electron microscopy, cells were grown at 20 °C for 6 days and collected by using low-speed centrifugation (6000 g for 5 min at 4 °C). The pellet was washed twice with 50 mM phosphate buffer (pH 7.5). Glutaraldehyde (3 % final concentration) and osmium tetroxide (1 % final concentration) were used for pre-fixation before serial dehydration in acetone. The cells were examined under a JEOL 840 scanning electron microscope at 5 kV after gold coating of the mounted cells (JFC 1100 sputter-coating unit; JEOL).

For biochemical tests, the cultures were grown in M5 broth and tests were performed as described by Lanyi (1987) and Smibert & Krieg (1994). Substrate-utilization studies were performed in basal medium containing complex substrates at 0.5 % (trypticase, yeast extract, gelatin, peptone, carboxymethyl cellulose and Casamino acids), carbohydrates at 20 mM (sucrose, fructose, arabinose, mannose, adonitol, mannitol, raffinose, glucose, lactose, melibiose, inositol, trehalose, maltose, dulcitol, xylan, arabitol, xylose, inulin, cellobiose, amygdalin, glycogen, galactose, rhamnose, ribose, sorbitol) and amino acids at 10 mM (alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, 4-hydroxy-L-proline, isoleucine, lysine, methionine, threonine, tryptophan, tyrosine, valine, 2-aminobutyric acid, ornithine, phenylalanine, serine, proline) as described by Mechichi *et al.* (1999). Tests were scored positive when a difference of 0.1 in the OD_{600nm} was observed relative to cells grown in basal medium without carbon sources. Growth was also correlated with a fall in

pH in the supernatant at full growth (as ascertained by the absence of any further increase in the OD₆₀₀).

Chemicals were obtained from Sigma or Hi-Media and were of reagent grade. Tests for possible electron acceptors were carried out using basal media containing glucose, supplemented with 20 mM sulfate, sulfite, thiosulfate, elemental sulfur or fumarate.

Quantitative analysis of glucose and its products in the culture media was carried out. Glucose was assayed with glucose oxidase (Merckotest; Merck). Volatile fatty acids and alcohols were determined by GC with a flame-ionization detector (Shimadzu) by using the method described by Mountfort & Rhodes (1991). H₂, CO₂ and H₂S were measured using GC with a thermal conductivity detector.

The phenotypic properties of strain AP15^T are given in the species description. Characteristics useful for distinguishing strain AP15^T from its closest relatives are listed in Table 1. *Clostridium subterminale* ATCC 25774^T and *Clostridium perfringens* ATCC 13124^T were used as controls in biochemical tests and in studies relating to morphology, motility, identification of fatty acids and DNA–DNA hybridization.

DNA was isolated according to the procedure of Marmur (1961), and the small-subunit rRNA gene was amplified using the two primers 16S1 (5'-GAGTTTGATCCTGGC-TCA-3') and 16S2 (5'-CGGCTACCTTGTTACGACTT-3'). The purified DNA product, which was approximately 1.5 kb

in length, was sequenced using five forward primers and one reverse primer, as described previously (Reddy *et al.*, 2000). The closest match to the deduced sequence was determined by a BLAST search (at <http://www.ncbi.nlm.nih.gov>). Pair-wise evolutionary distances were computed using the DNADIST program with the Kimura two-parameter model (Kimura, 1980). Phylogenetic trees were constructed by using four tree-making algorithms: UPGMA, KITSCH, FITCH and DNAPARS from the PHYLIP package (Felsenstein, 1993). The stability among the clades of a phylogenetic tree was assessed by evaluating 1000 replicates of the dataset, and was analysed using the programs SEQBOOT, DNADIST, UPGMA and CONSENSE of the PHYLIP package.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that AP15^T was a member of the low-G + C-content Gram-positive bacteria (Fig. 1) and was grouped within rRNA cluster I (Collins *et al.*, 1994). The closest relatives of strain AP15^T were *C. subterminale* and *Clostridium argentinense*, which showed 99.58 and 99.4 % sequence similarity, respectively. DNA–DNA hybridization was performed by using the membrane filter method (Tourova & Antonov, 1987), as described previously (Reddy *et al.*, 2000; Shivaji *et al.*, 1992). DNA of strain AP15^T exhibited only 55 % binding with *C. subterminale* ATCC 25774^T at the whole-genome level.

Cells were grown in M5 medium, and cellular fatty acid methyl esters were prepared according to the method of

Table 1. Differential characteristics of strain AP15^T, *C. subterminale* and *C. argentinense*

All three taxa are negative for indole production and positive for H₂S production. Cells of all three taxa are rods. NR, Not reported.

Characteristic	Strain AP15 ^T	<i>C. subterminale</i>	<i>C. argentinense</i>
Habitat	Lake sediment, Schirmacher Oasis, Antarctica	Marine sediments, soil, bovine and human faeces	Soil, human autopsy samples
Cell size (µm)	2–4 × 0.5–0.7	1.6–11.0 × 0.5–1.9	1.3–1.9 × 1.6–9.4
Growth characteristics			
Temperature range (°C)	5–35	25–45	25–45
pH optimum	8.0	6.0–6.4	6.2–6.3
NaCl tolerance (%)	7.5	6.5	6.5
Biochemical characteristics			
Aesculin hydrolysis	+	–	–
H ₂ production*	1	4	4
β-Haemolysis	–	+	+
Amino acids utilized	Arg, Leu, Ile, Cys, Glu, Ser	Arg, Gly, Lys, Ser, Trp, Phe, Tyr	Val, Leu, Lys
Major end-products on PYG medium†	F, A, P, IV	A, B, IV	A, B, IB, IV, PhA
Cellular fatty acid methyl esters			
Major	C _{15:0} , C _{16:0} , C _{17:0}	C _{12:0} , C _{14:0} , C _{16:0}	NR
Minor	C _{14:0} , C _{16:1} , C _{18:0} , C _{18:1}	iso-C _{15:0} , C _{16:7} , C _{16:9}	NR
DNA G + C content (mol%)	24	28	29

*H₂ production is given on a scale from '0' (no production) to '4+' (abundant).

†PYG medium contains 0.5 % peptone, 2.0 % yeast extract and 0.4 % glucose. A, Acetate; B, butyrate; F, formate; IB, isobutyrate; IV, isovalerate; P, propionate; PhA, phenylacetate.

Fig. 1. UPGMA phenogram showing the phylogenetic relationships between strain AP15^T and related species of the genus *Clostridium*, based on 16S rRNA gene sequence analysis. Bootstrap values are given at the nodes. This is a consensus tree and the branch lengths in the phenogram are not to scale.

Sato & Murata (1988). Methyl esters were analysed by GC/MS (Thermo Finnigan Trace GC/MS). Cell walls were prepared and peptidoglycan was analysed by using the methods described by Schleifer & Kandler (1972). Amino acids and peptides were separated and characterized by TLC on cellulose sheets. Phospholipids were analysed using silica-gel TLC as described by Komagata & Suzuki (1987). Isolation of the DNA and determination of the DNA G + C content (mol%) were performed by using the method of Reddy *et al.* (2000). The mean value from two independent experiments is given; the variation between the experiments was less than 2%.

The predominant fatty acids in cells of strain AP15^T were C_{15:0}, C_{16:0} and C_{17:0}, and there were only minor amounts of C_{14:0}, C_{16:1}, C_{18:0} and C_{18:1}. The cells contained diphosphatidylglycerol as the major phospholipid, and *meso*-diaminopimelic acid was present in the cell wall. The DNA G + C content was low (24 mol%).

A few examples of anaerobic bacteria isolated from various habitats of Antarctica (e.g. lake-water columns, sediments and samples from low-salinity ponds; Mountfort *et al.*, 1997) have been described. Reports of true psychrophiles are scant, and, to the best of our knowledge, no proteolytic

anaerobes from this icy continent have been described. This communication provides the first description of a proteolytic, psychrophilic *Clostridium* species from Antarctica.

Strain AP15^T was assigned to the genus *Clostridium* on the basis of its main characteristics: it is fermentative, Gram-positive, spore-forming, strictly anaerobic and does not utilize sulfate or nitrate as an electron acceptor (Cato *et al.*, 1986). Phylogenetic analysis, based on 16S rRNA gene sequences, indicated that strain AP15^T is closely related to the genus *Clostridium* and is grouped within rRNA cluster I. Although strain AP15^T exhibited a maximum sequence similarity of 99.5 % with *C. subterminale* at 16S rRNA gene level, it showed only 55 % overall DNA–DNA binding at the whole-genome level. Interestingly, the DNA G + C content was low (24 mol%) relative to that of *C. subterminale* and *C. argentinense* (28 and 29 mol%, respectively; Cato *et al.*, 1986; Suen *et al.*, 1988).

Furthermore, strain AP15^T differs from the type strains of both *C. subterminale* and *C. argentinense* with respect to its psychrophilic nature. The isolate tolerated up to 7.5 % NaCl as against 6.5 % NaCl for both *C. subterminale* and *C. argentinense* (Cato *et al.*, 1986). It grew optimally at pH 8.0, which is higher than the optimal pH values reported for *C.*

subterminale (pH 6.0–6.4) and *C. argentinense* (pH 6.2–6.3). The predominant fatty acids in cells of strain AP15^T are C_{15:0}, C_{16:0} and C_{17:0}, and there are only minor amounts of C_{14:0}, C_{16:1}, C_{18:0} and C_{18:1}. In the type strain of *C. subterminale*, the predominant fatty acids are C_{12:0}, C_{14:0} and C_{16:0}, and there are only minor amounts of iso-C_{15:0}, C_{16:7} and C_{16:9} (Elsden *et al.*, 1980). In addition to the above major differences, strain AP15^T differed from both *C. subterminale* and *C. argentinense* in that it could hydrolyse aesculin, did not produce β -haemolysis of sheep red blood cells and produced moderate amounts of H₂ (Cato *et al.*, 1986; Suen *et al.*, 1988). It also differed from *C. subterminale* in that it was able to utilize leucine, isoleucine, cysteine and glutamate but not glycine, lysine, tryptophan, tyrosine or phenylalanine (Cato *et al.*, 1986). Strain AP15^T did not utilize valine or isoleucine, unlike *C. argentinense*. The fermentation products of glucose are consistent with clostridial fermentation, but the stoichiometry differs from that of *C. subterminale* and *C. argentinense* in that it produces a large amount of propionate and a negligible amount of butyrate. Thus, these differentiating characteristics support novel species status for strain AP15^T within the genus *Clostridium*, and we propose the name *Clostridium schirmacherense* sp. nov.

Description of *Clostridium schirmacherense* sp. nov.

Clostridium schirmacherense (schir.ma.cher.en'se. N.L. neut. adj. *schirmacherense* pertaining to Schirmacher Oasis in Antarctica).

Cells are motile, Gram-positive rods (2–4 × 0.5–0.7 µm) that form spores. Spores are subterminal and distend the cell. Strictly anaerobic and chemo-organotrophic. Grows at temperatures in the range 5–35 °C and produces maximum cell mass at 5–10 °C. The pH range for growth is between 6 and 9, with an optimum at pH 8.0. NaCl is not required for growth but is tolerated up to a concentration of 7.5 % (Table 1). Hydrolyses aesculin, gelatin and casein, produces H₂S but does not reduce nitrate to nitrite. Negative for catalase, oxidase, lipase, indole production and β -haemolysis. Utilizes raffinose, glucose, adonitol, rhamnose and ribose but not sucrose, arabinose, mannose, mannitol, lactose, inositol, trehalose, maltose, dulcitol, xylan, arabinol, xylose, inulin, amygdalin, glycogen, galactose or sorbitol. Complex substrates such as yeast extract, Casamino acids, peptone and gelatin are also fermented. Some amino acids, including arginine, serine, leucine, isoleucine, cysteine and glutamate, are utilized whereas asparagine, proline, glutamine, aspartate, glycine, lysine, methionine, threonine, tryptophan, tyrosine, valine, 2-aminobutyric acid, ornithine and phenylalanine are not utilized. Sulfite and thiosulfate are reduced but sulfate, elemental sulfur and fumarate are not reduced. The major fermentation end-products are as follows: acetate, propionate, H₂ and CO₂ from glutamate; acetate and butyrate from isoleucine; acetate, propionate, H₂ and CO₂ from cysteine; formate, acetate and propionate from arginine; acetate, propionate, H₂ and CO₂ from leucine; and

acetate, isobutyrate and butyrate from serine. Details of the amino acid fermentation products are available as supplementary data (Supplementary Table S1) in IJSEM Online. Glucose is fermented to formate, acetate, propionate, butyrate, isovalerate, H₂ and CO₂ (Supplementary Table S2 in IJSEM Online). Contains diphosphatidylglycerol as the major phospholipids, and *meso*-diaminopimelic acid is present in the cell wall. The major cellular fatty acids are C_{15:0}, C_{16:0} and C_{17:0}. The G + C content of the DNA is 24 mol%.

The type strain, AP15^T (=DSM 17394^T=JCM 13289^T), was isolated from lake sediments of Schirmacher Oasis, Antarctica.

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