An obligately anaerobic, coiled bacterium from Ace Lake, Antarctica

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A coiled or 'C-shaped', Gram-negative, non-motile bacterium was isolated from the anaerobic waters of Ace Lake, an Antarctic lake of salinity similar to seawater. The strain was obligately anaerobic and lacked oxidase, catalase and respiratory lipoquinones. It fermented glucose and peptones, and formed H₂, CO₂, and butyric, acetic and formic acids as major end-products. Pyruvate was metabolized to H₂, CO₂, and acetic and formic acids. The organism had an optimum temperature for growth of 15–16 °C, and no growth occurred at 22 °C. In synthetic medium, the generation time at 1.7 °C (*in situ* lake temperature) was 53 h. The optimum NaCl concentration for growth was 0.3 M, with poor growth at 0.1 M (5.8%, w/v). The mol% G + C of the DNA was 25.9 ± 0.4% (*T_m*). The bacterium could not be accommodated within current bacterial species based on phenotypic criteria. Although coiled bacteria have been previously observed in Antarctic anaerobic environments, they had not been cultivated, and their metabolic capability and possible ecological role were unknown.

Introduction

Ace Lake (68°24' S, 78°11' E) is one of two known methanogenic environments on continental Antarctica (Vincent, 1988). It is meromictic and increases in salinity with depth from about 6‰ at the surface (in summer) to 43‰ in its bottom waters at 24 m depth (Burton, 1980). The lake water is of marine origin (Masuda *et al.*, 1988), although the bottom waters are depleted in SO₄²⁻ (Mancuso *et al.*, 1990) due to sulphate reduction (Burton & Barker, 1979). Despite the low temperature of the bottom water, which is always less than 2 °C (Hand, 1980), it is saturated with methane, and lipid analysis of water column particulates suggests that large populations of methanogenic bacteria are present (Mancuso *et al.*, 1990).

Attempts to isolate the methanogens using trimethylamine and antibiotics in a synthetic marine salts medium with a low yeast extract concentration produced enrichments containing a non-motile, spiral or 'C-shaped' organism in addition to a methanogenic coccus. The majority of anaerobic curved bacteria have been isolated from the rumen (Drasar, 1986) or the intestinal tract of man or animals (Kaneuchi *et al.*, 1979; Himmelbloom & Canale-Parola, 1989). Curved bacteria have been ob-

served in the anaerobic hypolimnia of lakes (Caldwell & Tiedje, 1975), including Antarctic lakes of marine origin (McGuire *et al.*, 1987), by light, fluorescence and electron microscopy, but none have previously been isolated or characterized.

Methods

Media and conditions of cultivation. Synthetic basal salts comprised the following (dissolved in 1 litre distilled water): KCl, 0.335 g; MgCl₂·6H₂O, 6.0 g; MgSO₄·7H₂O, 1.0 g; NH₄Cl, 0.25 g; CaCl₂·2H₂O, 0.14 g; K₂HPO₄, 0.14 g; NaCl, 18.0 g; Fe(NH₄)₂(SO₄)₂·7H₂O, 2.0 mg. This medium is similar in composition to the salts used by Jones *et al.* (1983).

For enrichment of methanogens, 1.0 mg resazurin, 0.1 g yeast extract, 10.0 ml vitamin solution (Jones *et al.*, 1983), 10.0 ml mineral salts solution (Jones *et al.*, 1983), 0.1 ml sodium acetate and 2.0 g trimethylammonium chloride were added to the synthetic basal salts, prepared as a more concentrated solution in 900 ml distilled water. The solution was cooled under an atmosphere of O₂-free N₂, 2.0 g NaHCO₃ was added, and the medium was dispensed under N₂ and sterilized. A precipitate of salts formed in the medium but this redissolved slowly. The medium was completed by the addition of 1:20 (v/v) sterile stock solutions of 1.0% (w/v) cysteine hydrochloride and Na₂S·9H₂O, and 1:10 (v/v) filter-sterilized stock solution of 0.1% (w/v) vancomycin prepared in deoxygenated distilled water.

Maintenance medium was prepared as for the methanogen enrichment medium, except that the trace-salts solution, trimethylammonium chloride, sodium acetate, Na₂S·9H₂O and vancomycin solution were omitted, and the yeast extract concentration was increased to 1.0 g l⁻¹. Sterile glucose (10%, w/v) prepared in deoxygenated distilled water was added (1:10, v/v) to complete the medium.

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Isolation. The methanogen enrichment medium (10 ml) in Hungate tubes with an atmosphere of N_2 was inoculated with 0.2 ml amounts of an Ace Lake water sample that had been collected from a depth of 24 m and maintained under H_2/CO_2 (80:20, v/v). After incubation at 10 °C for 7 d the enrichments were faintly turbid and on examination by UV light microscopy were seen to contain a relatively large number of non-fluorescing, 'C-shaped', and spiral organisms in addition to lesser numbers of blue-fluorescing cocci. Continued incubation led to an increase in the numbers of the coccus with a decrease in the numbers of the C-shaped organism. Subculture of the enrichments at weekly intervals in the same medium (omitting vancomycin) produced cultures in which the C-shaped bacterium was predominant. Employing the same medium gelled by the addition of 1.5% (w/v) agar in 'agar bottles' (Braun *et al.*, 1979) and incubated under N_2 enabled isolation of the C-shaped or spiral bacterium from a single isolated colony. Subsequently, the bacterium was found to grow considerably better in maintenance medium.

Morphological, physiological and biochemical tests. Unless otherwise stated, all tests were performed by the methods of Holdeman *et al.* (1977) using synthetic basal salts instead of distilled water in the preparation of test media. Colony morphology was determined for well separated colonies after anaerobic growth in agar bottles on maintenance medium agar for 1 month at 10 °C. Cell morphology and motility in wet mounts, and Kopeloff's modification of the Gram reaction were determined after growth for 48 h in maintenance medium. Cultures grown for 1 month on maintenance medium were tested for heat- or ethanol-resistant spores (Cato *et al.*, 1984). Susceptibility to antibiotics was tested by the broth-disc method (Holdeman *et al.*, 1977) in maintenance medium.

The growth response of the strain to temperature in maintenance medium was determined in a Toyo temperature gradient incubator, with growth measured by increase in OD_{550} .

For the determination of the optimum initial pH for growth, maintenance medium with the pH adjusted to 3.2 was prepared without $NaHCO_3$. Variable volumes of a deoxygenated 7.0% (w/v) $NaHCO_3$ solution were added to adjust the pH to a range of values between 3.2 and 8.0. Growth was determined by measuring OD_{550} .

Maintenance medium without NaCl and with all other sodium salts replaced by an equivalent weight of the corresponding potassium salts was used as the basal medium for the determination of growth response to variable Na^+ concentrations. Samples of basal medium (5 ml) with NaCl added to 0.1, 0.3, 0.5, 1.0, 1.5, 2.0 and 2.5 M were inoculated with 0.05 ml of a suspension of cells grown to exponential phase in maintenance medium, incubated at 4 °C and the increase in OD_{550} over a period of 14 d was determined. Stimulation of growth by the compounds listed below was determined in maintenance medium (without glucose or resazurin) by comparison of the final OD_{550} obtained in media with and without the test substrate after incubation for 14 d. The compounds used were: D(+)-glucose, 50 mM; D(-)-ribose, 50 mM; hydrogen/carbon dioxide, 80:20 (v/v) [2 bar overpressure in the headspace (1 bar = 10^5 Pa)]; sodium formate, 20 mM; sodium acetate, 20 mM; sodium pyruvate, 20 mM; glycerol, 20 mM; sodium lactate, 20 mM; betaine. H_2O , 20 mM; trimethylammonium chloride, 20 mM; and glycine, 20 mM.

Fermentation end-products from D(+)-glucose and sodium pyruvate were analysed in the spent media from the growth stimulation tests, and end-products in peptone/yeast extract synthetic basal salts (PY) and peptone/yeast extract/glucose synthetic basal salts (PYG) were determined using gas chromatography (Shimadzu). Headspace gases were determined on a 2.5 m long, 3 mm column of 100/120 mesh Carbosieve (Supelco), operated isothermally at 170 °C, using N_2 as the carrier gas at a flow rate of 30 ml min^{-1} , with a thermal conductivity detector (150 °C, 75 mA). Alcohols and volatile fatty acids were separated by a 30 m Nukol capillary column (Supelco), with H_2 as the carrier gas (linear gas flow rate, 20 $cm s^{-1}$; split 100:1), and measured

with a flame ionization detector. Alcohols and volatile fatty acids in ether extracts of the culture broths (Holdeman *et al.*, 1977) were separated with a temperature programme (3 min at 75 °C, followed by heating at 32 °C min^{-1} to 170 °C, with the final temperature maintained for 6 min). Chloroform extracts of methyl esters of low molecular mass, non-volatile fatty acids (Holdeman *et al.*, 1977) were determined isothermally at 140 °C. Formate in the supernatants of centrifuged, spent media was determined by the colorimetric method of Lang & Lang (1972).

Electron microscopy. Cells grown for 3 d in maintenance medium at 10 °C were fixed in 3.0% (w/v) glutaraldehyde in synthetic basal salts. Scanning electron microscopy was performed using fixed cells deposited on Nuclepore filters (Costar UK). Preparations were dehydrated with acetone, dried at the critical point of liquid CO_2 , sputter coated with gold (10 nm), and examined with a Zeiss DSM 940 scanning electron microscope.

For embedding and thin sectioning, cells were fixed with a solution of 3.0% (w/v) glutaraldehyde and 0.15% (w/v) ruthenium red in PBS (50 mM potassium phosphate buffer containing 0.9% w/v, NaCl, pH 6.9) on ice. After washing with 0.15% (w/v) ruthenium red in PBS, cells were fixed for a further 3 h in 3.0% (w/v) glutaraldehyde and 1.0% (w/v) osmium tetroxide in PBS at room temperature. Cells were washed in PBS, embedded in 1.5% (w/v) agar, dehydrated with acetone and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were post-stained with uranyl acetate and lead citrate before examining in a Zeiss EM 10B electron microscope at an acceleration voltage of 80 kV at calibrated magnifications.

Cell components. Cell components were determined on cells grown in maintenance medium for 10 d at 10 °C. Cells were examined for the presence of lipoquinones (Tindall, 1990) and fatty acids (Kroppenstedt *et al.*, 1990).

DNA base composition. DNA was isolated by the method of Marmur (1961) with the recovery of ethanol- or propan-2-ol-precipitated DNA by centrifugation, not by spooling. A treatment with cetyltrimethylammonium bromide (CTAB) (Murray & Thompson, 1980) was required to remove contaminating polysaccharide. The T_m of each DNA was determined by thermal denaturation in 0.1 × SSC (1 × SSC is 8.76 g NaCl and 4.41 g hydrated trisodium citrate per litre of double-distilled, deionized water) with a Gilford 2400 spectrophotometer and thermo-programmer. DNA of known G + C content, purified from *Escherichia coli* DSM 30083^T (ATCC 11775^T) (G + C = 51.7; Starr & Mandel, 1969), was used as the standard DNA in each determination. G + C content (the mean and standard error of four determinations) was determined from the formula $(G + C)_x = (G + C)_{std} + 2.44 (T_{mx} - T_{mstd})$ (Sly *et al.*, 1986).

Results

The bacterium was normally C-shaped (Fig. 1a, c), with a cell width of 0.6–0.8 μm . Coils (Figs 1b and 2) of diameter 1.0–1.4 μm and length up to 40 μm (Fig. 2a) were more prevalent in young cultures and were wound anticlockwise (Fig. 2b). Cross-walls or septa were not observed in thin sections of cells in long coils (Fig. 2c, d). Straight cells were rarely observed. The cells stained Gram-negatively, and possessed a laminated cell wall with two membranes (OM and CM, Fig. 1d). The cells were non-motile and lacked flagella. Endospores were absent and the bacterium showed no heat- or alcohol-resistance.

Fig. 1. Light and electron micrographs of the coiled or C-shaped isolate from Ace Lake after growth in maintenance medium for 4 d at 10 °C. (a) Light micrograph showing C-shaped morphology. (b) Scanning electron micrograph showing coil and C-shaped morphology. (c, d) Transmission electron micrographs of ultrathin sections showing (c) the C-shape and (d) cytoplasmic membrane (CM) and outer membrane (OM). Bars; (a) 5 μ m, (b) 1 μ m and (c, d) 0.1 μ m.

The bacterium was an obligate anaerobe and did not grow in an anaerobic jar (Oxoid) unless the agar was poured and inoculated in an anaerobic chamber. The bacterium lacked quinones, catalase and oxidase, and it did not reduce nitrate or produce H_2S . Colonies were cream to faintly yellow in colour, low convex in morphology, 2.0 mm in diameter and smooth after growth on maintenance medium for 1 month at 10 °C. Yeast extract stimulated but was not required for growth, but growth did not occur if the vitamin solution was omitted from the maintenance medium in the absence of yeast extract. The vitamin solution was not required if yeast extract was present.

Fermentation end-products in PY broth were H_2 (3.2%, v/v), CO_2 (3.5%, v/v), acetic acid (12.5 mM), butyric acid (11.4 mM), formic acid (1.0 mM), propionic acid (1.1 mM) and isovaleric acid (0.4 mM).

In PYG broth, the end-products were hydrogen (4.9% by vol.), carbon dioxide (5.4% by vol.), butyric acid (26.6 mM), acetic acid (3.2 mM), formic acid (1.1 mM) and propionic acid (0.4 mM). In maintenance medium plus glucose, the end-products were H_2 (23.7%, v/v), CO_2 (6.8%, v/v), acetic acid (18.9 mM), formic acid (10.4 mM) and butyric acid (7.7 mM). The end-products in cooked meat medium-synthetic basal salts broth were H_2 (5.8%,

v/v), CO_2 (6.8%, v/v), butyric acid (11.6 mM), acetic acid (9.1 mM), formic acid (1.0 mM), with traces of *isobutanol*, *isopentanol* and *isovaleric acid*. Pyruvate was metabolized to H_2 , CO_2 , acetic acid and formic acid; no butyric acid was formed. Propionate was not produced from threonine. Glycine, trimethylamine, betaine, methanol, lactate, formate and glycerol were not utilized as sole energy sources for growth.

Acid was produced from only a limited number of the carbohydrates tested in PY broth, including D-glucose and D-fructose, and a weak acid reaction was produced with D-mannose and D-mannitol. Acid was not produced from aesculin, hippurate, starch, D-amygdalin, salicin, D-ribose, D-raffinose, D-galactose, sucrose, L-rhamnose, glycerol, D-sorbose, D-sorbitol, erythritol, D-cellobiose, adonitol, D-lactose, dulcitol, D-melibiose, *meso*-inositol, glycogen, L-arabinose, maltose, D-trehalose, inulin, D-xylose and D-melezitose.

The organism was susceptible to penicillin G (2 Units ml^{-1}), ampicillin (4 $\mu\text{g ml}^{-1}$), tetracycline (6 $\mu\text{g ml}^{-1}$), polymyxin B (60 Units ml^{-1}), and chloramphenicol (12 $\mu\text{g ml}^{-1}$) but was resistant to bacitracin (2 $\mu\text{g ml}^{-1}$), erythromycin (3 $\mu\text{g ml}^{-1}$), vancomycin (6 $\mu\text{g ml}^{-1}$) and kanamycin (10 $\mu\text{g ml}^{-1}$).

The optimum growth temperature was 15–16 °C with

Fig. 2. Light and electron micrographs of the coiled or C-shaped isolate from Ace Lake after growth in maintenance medium for 4 d at 10 °C. (a) Light micrograph and (b) scanning electron micrograph of the coil wound anticlockwise. (c, d) Transmission electron micrographs of ultrathin sections through coiled cells. Bars; (a) 5 µm, and (b-d) 1 µm.

a generation time of 6.7 h in maintenance medium. No growth occurred at 22 °C. At 1.7 °C, the *in situ* temperature of Ace Lake at 24 m depth, the generation time was 53 h. The bacterium did not grow without added NaCl or at NaCl concentrations of 1.0 M (ca. 5.8%, w/v) or above. The optimum NaCl concentration for growth was 0.3 M (ca. 1.7%, w/v), and poor growth occurred at 0.1 M. The bacterium required at least 0.01 mM-Mg²⁺ for growth. The optimal initial pH for growth was about 8.0.

The bacterium did not hydrolyse urea, gelatin, lecithin, starch, Tween 80 or aesculin. It did not produce any visible change in milk, and did not digest meat particles. Indole was not produced.

The analysis of whole cells revealed the following fatty acids in amounts greater than 0.5% of the total material extracted: 12:0, 0.81%; 11:0 3OH, 0.67%; 12:0 3OH, 1.86%; 14:0, 1.97%; 14:1 *cis*-7 DMA (DMA = dimethyl acetal), 1.0%; 14:0 DMA, 1.26%; 15:1 *iso*, 1.25%; 15:2 (isomer position unresolved), 0.62%; 15:1 (isomer

position unresolved), 18:88%; 15:0, 2.91%; unknown, 2.20%; 16:1 *cis*-9, 33.44%; 16:1 (isomer position unresolved), 1.89%; 16:0, 13.99%; 17:1 (isomer position unresolved), 2.71%; 18:1 (isomer position unresolved) 0.94%; and 18:0, 0.83%.

Although the cells possessed a Gram-negative cell wall structure and stained negatively in the Gram reaction, lysis in SDS required lysozyme pretreatment. The mol% G + C was 25.9 ± 0.4 . DNA from lysed cells could not be spooled due to the large amounts of polysaccharide which precipitated with the DNA in ethanol.

Discussion

A distinctive feature of this Antarctic isolate is its morphology, reminiscent of the morphology of members of the family *Spirosomaceae* (Raj & Maloy, 1990) and the ring-forming clostridia *Clostridium methylpentosum* (Himmelbloom & Canale-Parola, 1989), *Clostridium coeleatum* and *Clostridium spiroforme* (Kaneuchi *et al.*, 1979). Members of the family *Spirosomaceae* are obligately aerobic, contain menaquinone-7 as a respiratory lipoquinone and have DNA with G + C contents in the range 38–53% (Raj & Maloy, 1990). Unlike members of the genus *Clostridium*, the Antarctic isolate is non-sporeforming, Gram-negative and contains hydroxy fatty acids that have not been reported for *Clostridium* spp. (O'Leary & Wilkinson, 1988).

Gram-negative, curved, anaerobic rods that produce butyric acid but not succinic acid as a major end-product from glucose fermentation are currently limited to the genus *Butyrivibrio*. The low mol% G + C content of the DNA of the Antarctic isolate ($25.9 \pm 0.4\%$) is well below the range of G + C contents of the DNAs of members of the genus *Butyrivibrio* (36–41%; Bryant, 1984). In addition, a range of phenotypic characteristics (non-motility, low temperature optimum, non-production of lactic acid, requirement for NaCl) suggests inclusion of this bacterium in the genus *Butyrivibrio* may be inappropriate.

Conclusive statements regarding the taxonomy of this Antarctic bacterium requires phylogenetic analysis; however, reporting of its phenotype, source, and method of isolation at this stage will assist with further study of the non-motile, coiled bacteria of anaerobic aquatic environments, which have been regarded as 'non-cultivable' bacteria. As the majority of curved, obligately anaerobic, fermentative bacteria have previously been isolated from the intestine or rumen, it is not surprising that an essentially marine, cold-adapted isolate is difficult to accommodate within existing taxa, at least by phenotypic comparisons.

The bacterium was isolated from anoxic water samples collected from Ace Lake after passage through enrichments prepared for the isolation of trimethylamine-utilizing methanogens. It did not grow with trimethylamine as a sole source of energy, nor did it hydrolyse any of the macromolecules tested. Presumably it has an intermediate role in the breakdown of biomass, fermenting peptides and a limited number of sugars and producing the precursors for methanogenesis and acetogenesis.

Although the bacterium grew at the *in situ* temperature (1.7 °C) and sodium ion concentration (0.5 M) (Masuda *et al.*, 1988) of Ace Lake bottom water, it is not perfectly adapted to this environment, as the optimal growth conditions for the bacterium in synthetic media were 15–16 °C and 0.3 M-NaCl. The numbers of C-shaped bacteria in the water column of Ace Lake have not been determined, but in the anoxic hypolimnion of nearby Burton Lake, which has an *in situ* temperature of –0.5 °C and a sodium ion concentration of 0.56 M, curved and coiled bacteria reach a population density of 10^5 cells ml⁻¹ (McGuire *et al.*, 1987).

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