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Characteristics of a novel, anaerobic, mycoplasma-like bacterium from Ace Lake, Antarctica

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Abstract: An obligately anaerobic bacterium that lacked a cell wall was isolated from the hypolimnion of Ace Lake, Antarctica. Cells were very pleomorphic, forming cocci, filaments up to 25 μm in length, and annular shapes. The organism was morphologically very similar to some members of the class Mollicutes which contains two genera of obligately anaerobic bacteria, *Anaeroplasma* and *Asteroleplasma*. Like members of the class, the isolate was resistant to high concentrations of penicillin (1000 Units ml^{-1}). Similar to *Anaeroplasma*, the organism had a low DNA G+C content (29.3±0.4) and produced hydrogen, carbon dioxide, acetic acid, lactic acid and succinic acid from the fermentation of glucose. However, the taxonomic status of the strain remained unclear as, unlike members of the class Mollicutes, the isolate had a relatively large genome size (2.26±0.11 x 10^9 daltons), did not pass through 0.45 μm pore size filters, and did not form typical mycoplasma-like colonies. The organism was psychrophilic with an optimum temperature for growth between 12°C and 13°C. A phenotypic description of the organism is given and the ecological role of the organism is inferred from its phenotype and the characteristics of its Antarctic habitat.

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Key words: anaerobic bacterium, psychrophile, Mollicutes, *Anaeroplasma*, methanogenic environment

Introduction

Many novel microbial communities and new taxa of microorganisms have been described through the detailed analysis of Antarctic ecosystems (Friedmann 1982, Miller & Leschine 1984, Franzmann *et al.* 1987). Ace Lake (68°24'S, 78°11'E) is of particular microbiological interest as it is one of two known methanogenic environments on continental Antarctica (Burton 1980, Vincent 1988). The lake water is of marine origin (Masuda *et al.* 1988) and although the salinity of the bottom water is about 44 $^{o}/_{oo}$ it is depleted in sulphate ions (Burton 1980) and contains 7 mmol l^{-1} sulphide (Mancuso *et al.* 1990). Despite the low temperature of the bottom water (1.7°C), it is saturated with methane, and lipid analysis of water column particulates suggests a large population of methanogenic bacteria are present (Mancuso *et al.* 1990).

As part of an investigation of the anaerobic microbiota of this ecosystem, a non-methanogenic, budding, pleomorphic, cell wall-less bacterium was isolated. Cell wall-less eubacteria are restricted to the class Mollicutes and are obligate parasites, commensals or saprophytes of man, animals, plants, or insects (Razin & Freundt 1984), although cell wall-less members of the domain Archaea occur in thermo-acidophilic environments (Darland *et al.* 1970). Wall-defective or wall-less L-phase variants of bacteria can occur which possess some mycoplasma-like morphological features. These are usually laboratory artifacts, which can revert to the bacterial form when the artifact-inducing substance (e.g. penicillin) is removed from the growth medium (Razin & Freundt 1984).

A cell wall-less bacterium isolated from the dark, cold, anaerobic hypolimnion of Ace Lake, which is devoid of animal or plant life, would not be expected to fit current taxonomic schemes for the cell wall-less bacteria. Phenotypic characterization of a single isolate of this organism was undertaken to increase our understanding of the microbiota of Ace Lake, and to assess the organism's place within current taxonomic schemes.

Materials and Methods

Media and conditions of cultivation

Artificial Marine Basal Salts Solution (AMBSS) was composed of KCl, 0.335 g; $MgCl_2.6H_20$, 6.0 g; $MgSO_4.7H_2O$, 1.0 g; NH_4Cl, 0.25 g; $CaCl_2.2H_20$, 0.14 g; K_2HP0_4, 0.14 g; NaCl, 18.0 g; $Fe(NH_4)2(S0_4)_2.7H_20$, 2.0 mg; dissolved in 1 l distilled water, and is similar in composition to the salts used by Jones *et al.* (1983).

Trace element solution consisted of nitrilotriacetic acid, 1.5 g; $MgS0_4.7H_2O$, 3.0 g; $MnSO_4.2H_2O$, 0.5 g; NaCl, 1.0 g; $FeSO_4.7H_2O$, 0.1 g; $CoSO_4.7H_2O$, 0.18 g; $CaCl_2.2H_2O$, 0.1 g; $ZnSO_4.7H_2O$, 0.18 g; $CuSO_4.5H_2O$, 0.01 g; $KAl(SO_4)_2.12H_2O$, 0.02 g; $Na_2MoO_4.2H_2O$, 0.01 g; $NiCl_2.6H_2O$, 0.025 g;

$Na_2SeO_3.5H_2O$, 0.3 mg; dissolved in 1 l distilled water; pH adjusted to 7.0 with KOH. Vitamin solution consisted of biotin, 2.0 mg; folic acid, 2.0 mg; pyridoxine-HCl, 10.0 mg; thiamine-HCl, 5.0 mg; riboflavin, 5.0 mg; nicotinic acid, 5.0 mg; DL-calcium pantothenate, 5.0 mg; vitamin B_{12}, 0.1 mg; *p*-aminobenzoic acid, 5.0 mg; lipoic acid, 5.0 mg; dissolved in 1 l distilled water.

For methanogen enrichment medium, 1.0 mg resazurin, 0.1 g yeast extract, 10.0 ml vitamin solution, 10.0 ml mineral salts solution, 0.1 g sodium acetate, and 2.0 g trimethylammonium chloride were added to AMBSS, prepared as a more concentrated salts solution in only 900 ml distilled water. The solution was cooled under an atmosphere of nitrogen, 2.0 g $NaHCO_3$ were added, and the medium was dispensed under nitrogen and sterilized. A precipitate of salts formed in the medium but this redissolved slowly. The medium was completed with the addition of 1:20 (vol:vol) sterile stock solutions of 1.0% (wt/vol) cysteine hydrochloride and $Na_2S.9H_2O$, and 1:10 (vol:vol) filter-sterilized stock solution of 0.1 % (wt/vol) penicillin G prepared in anaerobic distilled water.

Maintenance Medium (MM) was prepared as the methanogen enrichment medium, but the trace salts solution, trimethylammonium chloride, sodium acetate, $Na_2S.9H_2O$, and penicillin G solution were omitted, and yeast extract was increased in concentration to 1.0 g l^{-1}. Sterile, anaerobic, glucose solution (10% wt:vol) was added (1:10, vol:vol) to complete the medium.

Peptone Yeast Extract Medium (PY) and Peptone Yeast Extract Glucose Medium (PYG) were prepared by the method of Holdeman *et al.* (1977) but AMBSS replaced the distilled water component in the recipe.

Cooked Meat AMBSS Medium was prepared as Cooked Meat Medium (Oxoid) by replacing the distilled water component with AMBSS.

Isolation

Anaerobic, water samples from depth 24 m in Ace Lake were maintained at 4°C in serum bottles with a gas phase of H_2:CO_2 (80:20) for one year. Microscopic examination of the sample showed the presence of a number of bacterial morphotypes, including a coccus which fluoresced under UV, and a pleomorphic, budding bacterium. Methanogen enrichment media (10 ml quantities) containing penicillin were inoculated with subsamples (0.2 ml) of the Ace Lake water sample. After incubation at 10°C for 14 days the enrichments were faintly turbid and contained (under UV light microscopy) non-fluorescing, pleomorphic, budding bacteria and blue fluorescing, methanogenic cocci. Use of the same medium solidified with 1.5 % (wt/vol) agar in "agar bottles" (Braun *et al.* 1979) with an N_2 gas phase enabled isolation of the budding pleomorphic organism from a single isolated colony. Subsequently, the organism was found to grow considerably better in MM which was used for maintenance of the strain.

Morphological, physiological and biochemical tests

Unless otherwise stated all tests were performed by the methods of Holdeman *et al.* (1977) using MM without glucose or MM instead of Peptone Yeast Extract Medium (PY) or Peptone Yeast Extract Glucose Medium (PYG) as the basal media for tests, although the production of acid from carbon sources was tested in PY medium (Holdeman *et al.* 1977) prepared with AMBSS instead of distilled water. After inoculation, test media were incubated at 10°C for one month unless otherwise stated. Colony morphology was determined for well separated colonies after anaerobic growth (agar bottles) on MM agar for one month at 10°C. Cell morphology and motility in wet mounts, and Kopeloff's modification of the Gram reaction were determined after growth for seven days in MM. Cultures grown for one month in MM were tested for heat or ethanol resistant spores (Cato *et al.* 1984).

Discs containing 100 μg digitonin were prepared by drying an appropriate volume of sterile 1.5 % ethanolic digitonin on sterile filter paper discs. Sterile discs containing 2 mg thallous acetate were also prepared. Susceptibility to antibiotics (Becton Dickinson discs), digitonin and thallous acetate was tested by the broth-disc method (Holdeman *et al.* 1977) in MM. In addition, susceptibility to higher concentrations of penicillin G (1000 Units ml^{-1}) and vancomycin (100 μg ml^{-1}) were tested in MM with addition of appropriate amounts of anaerobic, filter sterilized antibiotic solutions.

Cell filterability was tested by passage of 1.0 ml of turbid culture through 0.2 and 0.45 μm pore-size filters (Sartorius filters in syringe filter holders) into MM using N_2 gassed filters and syringes. The receiving MM was examined for cell growth after two months incubation at 10°C.

Bacteriolysis of cells of *Escherichia coli* was tested by the method of Robinson (1984) except that the cells of *E. coli* DSM 30083^T were suspended in solidified MM, and the test was incubated for two months at 10°C before examination of the inoculated agar for zones of clearing.

The growth response to temperature of the strain in MM was determined in "Hungate" tubes placed in a Toyo temperature gradient incubator. Growth in the tubes was measured by increase in absorbency at 550nm.

For the determination of the optimum initial pH for growth, MM with pH adjusted to pH 3.2 was prepared without $NaHCO_3$. Variable volumes of an anaerobic 7.0% $NaHCO_3$ solution were added to give a pH range between 3.2 and 8.0. Growth was measured by the increase in absorbency determined at 550 nm.

For the determination of growth response to variable sodium concentrations, MM without sodium chloride and with all other sodium salts replaced by an equivalent weight of the potassium salt was used as basal medium. Basal medium with NaCl concentrations adjusted to 0.0, 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, and 2.5 M were inoculated, incubated at 10°C and the increase of absorbency at 550 nm was determined.

Stimulation of growth by D(+)glucose, 50 mM; D(-)ribose,

50 mM; H_2:CO_2 (80:20) 2 bar overpressure in headspace; or 20 mM of either sodium formate, sodium acetate, sodium pyruvate, glycerol, sodium lactate, betaine.H_2O, trimethylammonium chloride, or glycine, was tested in MM (without 1.0% glucose or resazurin) by comparison of the final absorbency at 550 nm reached in media with the test substrate and without it after incubation for 2 months.

Fermentation end-products from D(+)glucose were determined in the spent media from the growth stimulation tests, and endproducts in PY (in Artificial Marine Basal Salts) and PYG (in Artificial Marine Basal Salts) were determined using gas chromatography (Shimadzu). Headspace gases were determined on a packed column of 100/120 Carbosieve (Supelco), isothermal at 170°C, using N_2 carrier gas at 30 ml min^{-1}, with thermal conductivity (150°C, 75mA). Alcohols and volatile fatty acids were separated by a Nukol™ capillary column (Supelco), with hydrogen as carrier (linear gas rate 20 cm sec^{-1}; split 100:1), and flame ionization detection. Alcohols and volatile fatty acids in ether extracts of culture media (Holdeman *et al.* 1977) were separated with a temperature program (3 min at 75°C, 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 weight 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 seven days in MM at 10°C were fixed in 3.0% glutaraldehyde (wt/vol) in AMBSS. Scanning electron microscopy (SEM) was performed on fixed cells deposited on polylysine-coated Nuclepore filters by filtration. For SEM of cells on paper filters (Schleicher & Schuell, No. 595), the filter was placed in an actively growing culture for 1 hr to allow the cells to attach to the filter. The filters were then fixed in 3.0% glutaraldehyde in AMBSS. Preparations were dehydrated with acetone, dried at the critical point of liquid CO_2, sputter-coated with gold (10 nm), and examined in a Zeiss DSM 940 SEM.

For embedding and thin sectioning, cells were fixed for 1 h in 3% glutaraldehyde (wt/vol) on ice and embedded in 1.5 % agar prepared with AMBSS. The agar was cut into small cubes, washed with AMBSS, fixed with 1.0 % osmium tetroxide (wt/vol) for 1 h on ice, washed several times with AMBSS, dehydrated with acetone and embedded in Spurr resin (Spurr 1969). Ultra-thin sections were post-stained with uranyl acetate and lead citrate before examining in a Zeiss EM 10B transmission electron microscope at an acceleration voltage of 80 kV at calibrated magnifications.

Cell components

Cell components were determined on cells grown in MM for 30 days at 10°C. Cells were examined for the presence of lipoquinones (Tindall 1990). For the determination of fatty acid methyl esters of whole cells, freeze dried cells (10 mg) were hydrolyzed in methanol: toluene : conc. H_2SO_4 (5:5:0.2) at 50°C overnight (Minnikin *et al.* 1977). Fatty acid methyl esters were analysed on a Hewlett Packard fatty acid methyl-ester bacterial identification system.

DNA base composition

DNA was isolated by the method of Marmur (1961). Cells lysed on suspension in saline-EDTA (sodium chloride 8.75 g l^{-1}; ethylenediaminetetraacetic acid disodium salt, 37.2 g l^{-1}. pH 8.0). The T_m of each DNA was determined by thermal denaturation in 0.1 X SSC (SSC = 8.76 g NaCl, 4.41 g trisodium citrate.$2H_2O$ per litre of 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 1968), was used as standard DNA in each determination. G+C content was determined from the formula $(G+C)x = (G+C)_{std} + 2.44\ (T_{mx} - T_{mstd})$ (Sly *et al.* 1986)

Genome size

Genome size was determined from DNA renaturation rates using a modification (Huss 1983) of the method of Gillis *et al.* (1970). *Clostridium butyricum* DSM 552^T DNA, with known genome size (5.62 x 10^9 daltons; Huss 1983) and G+C content (27–28 mol %; Cummins & Johnson 1971), served as reference DNA.

Results & discussion

Isolation

A single strain of a pleomorphic, budding bacterium, strain ACE-P, was isolated at 10°C from anaerobic, bottom water of Ace Lake. The strain developed in anaerobic, artificial seawater media which had been intended for the enrichment of methanogenic bacteria, and which contained a low concentration of yeast extract, trimethylamine, and penicillin G. Enrichments were streaked on solidified medium in bottles with a N_2 gas phase and the single strain was isolated from a well separated colony. Methanogen Enrichment Medium did not support good growth and a much better cell yield was achieved in MM. The organism did not show fluorescence under UV light microscopy, did not produce methane in culture headspaces and grew optimally when supplied with a fermentable carbon source such as glucose. It was clearly not a methanogen.

Cell morphology

Cells were very pleomorphic with unequal/budding type division. Cells did not change morphology when continually

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subcultured in media free of penicillin G, and cells of the same morphology could be recognized in the original anaerobic water sample which had not been subjected to treatment with antibiotics. The majority of cells in young cultures (7 days) occurred as chains of cells attached by thin threads (Figs. 1b, 1f, 1h). Cells sometimes showed rudimentary branching (Fig. 1i). A minority of cells were spherical with phase clear centres (Fig. 1a). In old cultures (one month), cells were mostly of the spherical form. Cells deposited with suction onto polylysine coated Nuclepore filters were flattened in appearance (Fig. 1c). Cell size was very variable, ranging from coccoid shapes 1.0 μm in diameter to spheres 3.0 μm across, to filaments 25 μm long (Fig. 1c). Cells allowed to attach to paper filters without suction were not flattened (Fig. 1d). Scanning electron micrographs showed that the clear centres seen by phase microscopy (Fig. 1a) were cavities in annular cell (Figs 1d, 1e). No cell wall was observed in thin sections (Figs 1g–1j) and cells were bounded by a single membrane with an exterior coating of unknown material (Fig. 1j). Attempts to isolate purified cell walls and analyse for peptidoglycan by the methods of Schleifer & Kandler (1972) were not successful (personal communication, N. Weiss). Thin sections of annular cells showed a continuous cell membrane (Fig. 1g). The cells were very similar in morphology to cells of strains of the class Mollicutes (Boatman 1979, figs 4 & 7, Rodwell & Mitchell 1979, figs 3 & 4). Cells disintegrated when Gram stained and the resultant debris stained Gram negative. Cells were fragile and cells lysed when cell pellets (collected by centrifugation of cell grown in MM) were resuspended in distilled water. Cells were not motile. Colonies were punctate (<0.5 mm in diameter), white, circular and did not appear to penetrate the agar surface after growth on MM (solidified with 1.0% Noble agar) for one month at 10°C.

Physiological and biochemical tests

Strain ACE-P was an obligate anaerobe and did not grow in an anaerobic jar unless the agar was poured and inoculated in an anaerobic chamber; the jar being sealed within the chamber, and an overpressure of anaerobic gas being maintained in the jar. The organism lacked quinones, catalase and oxidase, it did not reduce nitrate or produce hydrogen sulphide. Obligately anaerobic bacteria which lack cell walls are currently restricted to the genera *Anaeroplasma* and *Asteroleplasma* (Robinson & Freundt 1987). Biochemical test results for strain ACE-P are given in Table I, where strain attributes are compared with those from descriptions of species of *Anaeroplasma* and *Asteroleolasma*.

In addition to the attributes listed in Table I, strain ACE-P had other characteristics. The organism did not hydrolyse gelatin, lecithin, starch, Tween 80, or esculin. It did not produce any change in milk, and did not digest meat particles. Indole was not produced. Arginine and urea were not hydrolyzed. Yeast extract was required for growth.

Fermentation end products in PY-Artificial Marine Basal Broth were hydrogen (1.2%; vol/vol in gas phase), carbon dioxide (0.7 %), acetic acid (6.8 mM), lactic acid (2.3 mM) and succinic acid (7.6 mM). In PYG-Artificial Marine Basal

Table I. Attributes of strain ACE-P compared with those of members of the genera *Anaeroplasma* and *Asteroplasma*[1].

Attribute	ACE-P	*Anaeroplasma* spp.	*Asteroleplasma anaerobium*
Cells pass through a 0.45 μm pore	-	+	+
Bacteriolytic	-	+[2]	-
Digest casein	-	+[2]	-
Susceptibility to:			
digitonin 20 μg ml^{-1}	-	+	-
penicillin 1000 U ml^{-1}	-	-	-
0.2 % thallous acetate	-	+	+
Cholesterol required	-	+	-
Acid from:			
glucose	+	+	NA
starch	-	+	NA
cellobiose	+	-[2]	NA
arabinose	±	+[2]	NA
lactose	-	+[2]	NA
Fermentation products from starch (glucose for ACE-P)			
acetic acid	+	+	NA
formic acid	-	+	NA
succinic acid	+	+	NA
lactic acid	+	+	NA
ethanol	+	+	NA
pyruvic acid	+	-	NA
CO_2	+	+	NA
H_2	+	v	NA
Optimum temperature	12°C	37°C	37°C
Mol % G+C	29.3±0.4	29–34	40–41
Genome size (daltons.10^9)	2.26±0.11	*c.* 1.0	*c.* 1.0

+ = positive; - = negative; NA = not available; v = variable result.
[1]Descriptions given in references (Robinson *et al.*, 1975, Robinson & Allison, 1975, Robinson, 1979, Robinson, 1984, Robinson & Freudt, 1987).
[2]Reaction differs for *Anaeroplasma abactoclasticum.*

Fig. 1. Photomicrographs (**a.** & **b.**), scanning electron micrographs (**c.–f.**) and transmission electron micrographs of thin sections (**g–j**) of a cell wall-less anaerobic Antarctic bacterium, strain ACE-P, grown in MM for 7 days at 10°C. The position of "arrow heads" indicate the threadlike connection between dividing cells in **b.**, **f.**, and **h.**, and the unknown material exterior to the cell membrane in **j.** Bars = **a.** & **b.** 5.0 μm, **c.** 2.0 μm, **d.** 1.0 μm, **e.** to **i.** 0.25 μm, **j.** 0.1 μm.

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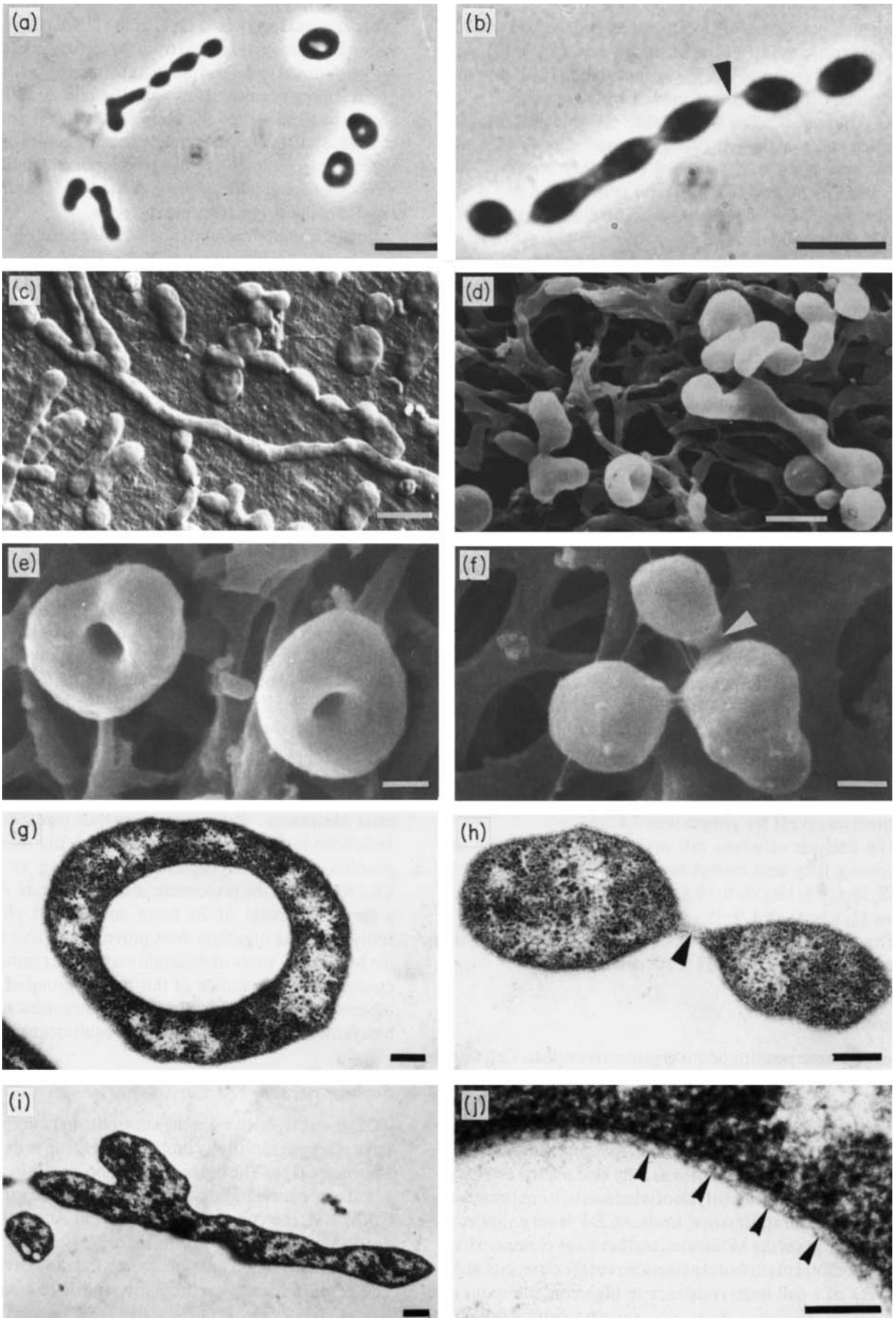

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Broth, end products were hydrogen (2.9%), carbon dioxide (1.0%), acetic acid (9.0 mM), lactic acid (5.1 mM), and succinic acid (6.2 mM). A heavy inoculation (10% vol/vol) was usually required before growth would occur in PY- or PYG-Artificial Marine Basal Salts. In glucose-MM, end products were hydrogen (9.6%), carbon dioxide (7.1 %), acetic acid (11.4 mM), ethanol (1.5 mM), succinic acid (8.5 mM), lactic acid (0.8 mM) and pyruvic acid (0.6 mM). End products in Cooked Meat AMBSS Medium were hydrogen (38%), carbon dioxide (1.1 %), acetic acid (3.0 mM), lactic acid (2.2 mM) succinic acid (3.7 mM). Propionate was not produced from threonine. Glycine, trimethylamine, betaine, methanol, lactate, formate, pyruvate or glycerol were not utilized as single energy sources for growth.

In addition to the carbon sources listed in Table I, acid was produced from mannose, ribose, sorbitol, glycogen, trehalose, galactose, maltose, xylose, and sucrose. Acid was not produced from esculin, hippurate, mannitol, amygdalin, salicin, raffinose, rhamnose, glycerol, sorbose, erythritol, adonitol, dulcitol, melibiose, inositol, inulin, melezitose.

The organism was resistant to bacitracin (2 μg ml^{-1}), vancomycin (100 μg ml^{-1}), kanamycin (10 μg ml^{-1}), ampicillin (20 μg ml^{-1}), polymyxin B (300 Units ml^{-1}), penicillin G (1000 Units ml^{-1}) and digitonin (200 μg ml^{-1}) but was susceptible to erythromycin (15 μg ml^{-1}), chloramphenicol (60 μg ml^{-1}) and tetracycline (30 μg ml^{-1}).

The optimum temperature for growth was 12°C to 13°C with a generation time of 22 h in MM. No growth occurred at 19°C. At 1.7°C, the temperature of Ace Lake *in situ* at depth 24 m, the generation time was 55 h. The organism did not grow without added NaCl or at concentrations of 1.0 M (*c.* 5.8 %) or above. The optimum NaCl concentration for growth was 0.3 M (*c.* 1.7 %), and poor growth occurred at 0.1 M. The organism required at least 0.01 mM Mg^{2+} for growth. The optimal initial pH for growth was 7.4.

The analysis of whole cell methanolysates revealed the following fatty acid methyl esters (% = wt/total wt): 14:0, 16.8; 16:1, 9.5; 16:1 cis 9, 49.8; 16:0, 17.2; 18:1 cis 9, 2.1; 18:1 trans 11, 2.8; 18:0, 1.9.

The mol % G+C of the DNA was 29.3±0.4 % and the genome size was 2.26±0.11 x 10^9 daltons.

Taxonomy

The taxonomic position of this organism is unclear. Cell wall-less bacteria are limited to the eubacterial class Mollicutes or the genus *Thermoplasma* of the domain Archaea. Within the class Mollicutes, obligately anaerobic strains are restricted to the genera *Anaeroplasma* or *Asteroleplasma*. Strain ACE-P is not a member of the Archaea as fatty acid methyl esters are produced on acid methanolysis of whole cells; it contains ester linked lipids. In appearance, strain ACE-P is very similar to some members of the Mollicutes, and has many characteristics which conform to attributes of members of the class, including the lack of a cell wall, resistance to high concentrations of penicillin and low mol % G+C of the DNA. In addition, the suite of end products formed from the fermentation of glucose are very similar to the end products formed by members of the genus *Anaeroplasma*. However, if strain ACE-P could be accommodated within the class Mollicutes it could not be classified within the two currently existing genera of anaerobic mycoplasmas as it shows considerable phenotypic differences with members of both genera (see Table I). The strain would represent a new genus within the class.

But the question remains; does the strain truly belong in the Mollicutes? Unlike strain ACE-P, all members of the class thus far described produce very small cells which pass through 0.45 μm pore size filters, have small genome sizes (4.5 x 10^8 to 1.0 x 10^9 daltons), and most produce characteristic colonies with a "fried egg" type morphology (Razin & Freundt 1984). Given these differences, placement of strain ACE-P within the Mollicutes would considerably alter the concept of the class. In addition, all members of the class, with the exception of some species of the genus *Acholeplasma*, are obligate parasites, commensals or saprophytes of man, animals, plants or insects. Species of *Acholeplasma* were first isolated from sewage, compost and soil although little evidence was available that they could persist in these environments (Tully 1984). The widespread distribution of *Acholeplasma* in animal tissues and secretions, as well as in plant material, suggests their recovery from soil could be related to their presence in animal wastes, which raises questions about their free living existence (Tully 1984). As the cold, anaerobic hypolimnion of Ace Lake is devoid of animal and plant life, ACE-P must be free living. Unlike all previously described cell wall-less bacteria, ACE-P is psychrophilic.

The strain clearly represents a new taxon of cell wall-less bacteria. Phenotypic data alone do not yield sufficient information to allow classification of strain ACE-P within the class Mollicutes. Phylogenetic analysis (sequencing of the 16S rRNA in this case) has been used to define the taxonomic position of most mycoplasmas (Weisburg *et al.* 1989). Clarification of the taxonomic position of strain ACE-P and a formal proposal of its name must await phylogenetic analysis. If the organism does prove to be a true member of the Mollicutes, study of the strain may answer some questions concerning the evolution of this unique group of organism, especially concerning hypotheses of genome reduction, reduced biosynthetic capabilities, and host requirements.

Ecology

ACE-P was isolated from the anaerobic hypolimnion of Ace Lake. Oxygen sensitivity limits the organism to depths below 10 m in the lake. The organism grew optimally in media with a sodium concentration of 0.3 M Na^+ and grew well at 0.5 M Na^+, the concentration of Na^+ in Ace Lake at 24 m depth (Masuda *et al.* 1988). As the organism lysed in distilled water its distribution must be limited to saline environments. The organism was psychrophilic, and it had an optimum

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temperature for growth (12–13°C) that was well above the lake temperatures *in situ* at 24 m depth. A reasonable growth rate (g = 55 h) occurred at *in situ* temperatures at depth 24 m (1.7°C) if nutrients were not limiting. Although the concentration of nutrients has not been determined for Ace Lake at depth 24 m, total organic carbon at 22 m depth was about 60 mg l^{-1} (Burton 1980). In the anaerobic hypolimnion of Ace Lake, sodium ion concentrations vary between 0.5–0.5 mM (Masuda *et al.* 1988) and temperatures vary from 10°C to about 1°C (Burton 1980). Given the strain's requirement for salt, low temperature and an oxygen-free environment, it may inhabit Antarctic marine and deep sea sediments in addition to Antarctic saline lakes.

Ace Lake is a methanogenic environment (Mancuso *et al.* 1990). The organism probably plays an intermediate role in the mineralization of organic carbon, metabolizing simple sugars and peptides to hydrogen and simple organic acids (acetate, lactate and succinate), which in turn are utilized by methanogens and acetogens. Mycoplasma-like organisms have been implicated previously in anaerobic degradation and methanogenesis in sewage sludge digesters, but the taxonomic position of the organisms involved remained unresolved (Rose & Pirt 1981, Robinson 1984).

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