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Methanoplanus limicola, a Plate-Shaped Methanogen Representing a Novel Family, the Methanoplanaceae

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Abstract. An angular plate-shaped weakly motile mesophilic methanogen was isolated from a swamp of drilling waste in Italy. Growth occurs on H_2/CO_2 or on formate. Acetate is required in addition. The optimal doubling time is 7 h at 40 °C. The cell envelope is composed most likely of glycoprotein subunits in hexagonal arrangement. The GC-content of its DNA is 47.5 mol%. On the basis of DNA-RNA hybridization it was found to represent a new family, the Methanoplanaceae within the order Methanomicrobiales.

Key words: Methanogens – Archaebacteria – Cell division – Glycoprotein – Acetate – Taxonomy

Recently, a square-shaped flat bacterium was discovered in a saturated salt brine (Walsby 1980; Stoeckenius 1981), which, however, cannot yet be cultivated in the laboratory. It was assumed (Walsby 1980) that the unusual shape may be explained by the absence of cell turgor in bacteria in a high ionic strength environment. From the composition of its envelope, Walsby (1980) speculates that this organism belongs to the archaebacteria.

Here, we report on the isolation and properties of another flat archaebacterium, which, however, grows at much lower ionic strength and which belongs to the methanogens.

Materials and Methods

Strains

Methanogenium marisnigri, DSM 1498, was obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen.

Culture Conditions

The isolate M3 was cultivated by using the technique described by Balch and Wolfe (1976). If not mentioned otherwise, the isolate was grown in "MG" medium, that is medium 3 of Balch et al. (1979), modified by the use of "Pepton aus Casein, tryptisch verdaut" (Merck) instead of Trypticase (BBL) and by adjusting the pH to 6.9 (H_2SO_4).

Twenty milliliter cultures were grown in stoppered pressurized 100 ml serum bottles (Bormioli, Italy) made of "type III"-glass by incubation in water bath shakers (New Brunswick) at 140 rpm and 37 °C.

Abbreviations: G + C: Guanine + Cytosine; SDS: Sodium dodecylsulfate (Sodium lauryl sulfate)

Methanogenium marisnigri, as a reference, was grown in the same medium.

Plating

Polysilicate plates were prepared as described (Stetter et al. 1981) except that they were equilibrated with MG-medium containing penicillin, vancomycin, kanamycin (each 150 µg/ml) and tetracycline (100 µg/ml) to prevent eubacterial contaminations.

Light Microscopy

The cells were viewed and photographed with a Leitz Ortholux II microscope, equipped with a vario-orthomat camera system (Leitz). Fluorescence was observed in a Zeiss Standard fluorescence microscope with an excitation filter H 436 and a selection filter LP 470.

Electron Microscopy

For thin sectioning, cell sediments were fixed in MG-medium not containing organic components with 20 g glutaraldehyde/l for 2 h and postfixed with 10 g OsO_4/l for 1 h. Durcupan (Fluka) epoxy resin was used for embedding and thin sections were contrasted with lead citrate (5 min), uranylacetate (5 min) and again with lead citrate (3 min).

For shadowing, the cells were fixed on parlodion coated grids and shadowcasted (Edwards vacuum coater 306) with a platinum-iridium alloy (angle 7°) for 15 s followed by carbon coating of the parlodion film for stabilization.

Electron micrographs were taken with a JEOL JEM 100 C electron microscope at 80 kV and with a 40 µm objective aperture.

Isolation of DNA

Two grams cells (wet weight) were suspended in 8 ml buffer (50 mM Tris-HCl, pH 8; 50 mM NaCl; 10 mM EDTA). Then, SDS and Na-deoxycholate were added up to final concentrations of 0.5% and 7 mM, respectively. This mixture was incubated for 15 min at 65 °C. Then, KCl was added to a final concentration of 0.5 M. After 1 h at 2 °C the precipitated potassium lauryl sulfate was removed by centrifugation (20 min, 21,000 rpm, rotor JA 21, Beckman J2-21). In the next step solid CsCl (0.92 g/ml) and ethidium bromide (20 µg/ml) were added to the supernatant and centrifuged 48 h at 45,000 rpm (rotor 50 Ti, 20 °C, Beckman L5-50). The DNA-band was isolated by suction with a syringe, ethidium

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bromide was removed by extraction 3 × with *n*-butanol and the solution was dialysed against 0.1 × SSC (0.015 M NaCl; 0.0015 M Na_3-citrate).

Analysis of the Cell Wall

The existence of rigid cell wall sacculi was checked for as described by Stetter et al. (1981). Hydrolysates of whole cells were analysed for muramic acid with an amino acid analyzer (König and Stetter 1982, in preparation).

For the identification of proteins and glycoproteins, cell envelopes were obtained after sonification (Sonifier B 12, Branson Sonic Power Company) of the cells at 50 W for 20 s. The lysate was suspended in basal salt medium (MG-medium without organic components), containing 1 mg Deoxribonuclease I (Boehringer, Mannheim)/l, and was afterwards centrifuged at 40,000 rpm (rotor 50 Ti, 20°C, Beckman L5-50). The pellet was washed twice with basal salt solution, solubilized in Laemmli's sample buffer (Laemmli 1970) and applied onto exponential polyacrylamide gels, which were prepared according to Laemmli (1970) and Mirault and Scherrer (1971). The gradient ranged from 5 to 25% polyacrylamide. The volume of the closed mixing vessel was 18 ml for a 20 ml gel. The gel was stained for protein and carbohydrates with coomassie blue and periodate-Schiff-reagent (Segrest and Jackson 1972).

Temperature Measurement

Temperature in the field was determined with an electronic thermometer (Metratherm 1200 d, BBC Metrawatt, Germany) equipped with electrode T 126.

Methane Detection

Methane was quantitated by gas chromatography using a Hewlett Packard gas chromatograph, model 5880 A. It was determined on a 6 feet glass column filled with Carbosieve S (Supelco) at 70°C isothermal.

Results

Collection of the Sample

The sample MII/3 was taken from a small swamp composed of drilling waste, which was left from drilling the neighbouring steam well "Mofete II" near Baia in the Naples area (Italy). The swamp is provided with water by a streamlet flowing out from the condensor of the well.

Within the swamp, there were some places, were gas bubbles ascended continuously to the surface. From one of them, about 3 m away from the embankment, a sample (MII/3) of the loose greyish mud sediment was drawn in a depth of about 0.2 m with a 1 l beaker mounted on a long stick. The original temperature of the sample was 19°C, the pH was 7.0 and the conductivity was 5 mS, that is about 70% of sea water. The sample was immediately filled into a sterile 100 ml storage bottle, which was sealed with a rubber stopper after the addition of 0.1 ml of resazurin (0.1% w/v in water). Then, in order to lower the redox potential, 1 ml of an aqueous solution of each 1.2% (w/v) of L-cystein · HCl · H_2O and $Na_2S \cdot 9\,H_2O$ (pH 7.0, adjusted with NaOH) and 1 ml of a freshly prepared aqueous solution of sodium dithionite (0.2% w/v) were injected into the sample through the stopper with 1 ml syringes. The sample was then carried to the laboratory at room temperature (around 20°C).

Enrichment

In a Freter type anaerobic chamber (Aranki and Freter 1972), 100 ml serum bottles containing 20 ml MG-medium and 10 µg vancomycin/ml were inoculated with 1 ml of sample MII/3. After sealing with stoppers the serum bottles were pressurized (200 kPa $H_2 : CO_2 = 80 : 20$; Balch and Wolfe 1976) and then incubated in a water bath shaker (New Brunswick) at 30°C. After 3 days, low but significant amounts of methane could be detected in the gas atmosphere of the culture vessels. In the UV-fluorescence microscope, some emerald green fluorescing almost crystal-plate shaped particles were observed among large amounts of rods, spirilli, and, due to vancomycin, atypical spheres. A strong enrichment of this novel methanogen, designated M3, was obtained by the simultaneous addition of vancomycin, penicillin, kanamycin (each 150 µg/ml) and tetracycline (100 µg/ml) into the MG-medium.

Isolation Procedure

The enriched methanogen M3 could be isolated by serial dilutions in MG-medium in serum bottles. At the 10^{-8} dilution, no infection could be detected in the microscope even without antibiotics. To obtain single colonies, the culture was streaked parallelly onto polysilicate and onto agar plates, both prepared with MG-medium. After 3 months incubation at 30°C, round, smooth, bright ochre-colored colonies about 2 mm in diameter became visible on the polysilicate plates. No growth occurred on agar. From single colonies liquid cultures could be obtained over night.

Culture and Storage

Liquid cultures are routinely transferred after 2 – 3 days into fresh medium (5% inoculation). In order to preserve the strain for longer periods, it was grown for 2 days and then, after renewing the gas atmosphere, it was simply frozen and stored at −20°C.

Optimal Growth Temperature

The isolate M3 grows between 17°C and 41°C (Fig. 1) with an optimum around 40°C. At 43°C and at 15°C no growth occurs.

Growth Requirements

H_2 and formate serve as substrates for growth. In addition, acetate is essentially required (Table 1). 0.1% acetate is sufficient (data not shown). Optimal growth is obtained by combination of acetate with yeast extract or with peptone and vitamins (Table 1). No growth occurs on acetate alone, methanol and methylamines (data not shown) as substrates.

M 3 grows in the presence of 0.4 – 5.4% NaCl (Fig. 2). The optimal salt concentration is around 1% NaCl. The optimal pH for growth is between pH 6.5 – 7.5.

Morphology

In the light microscope, slowly wobbling plates with sharp crystal-like edges 1 – 3 µm long and 1 – 2 µm wide can be seen

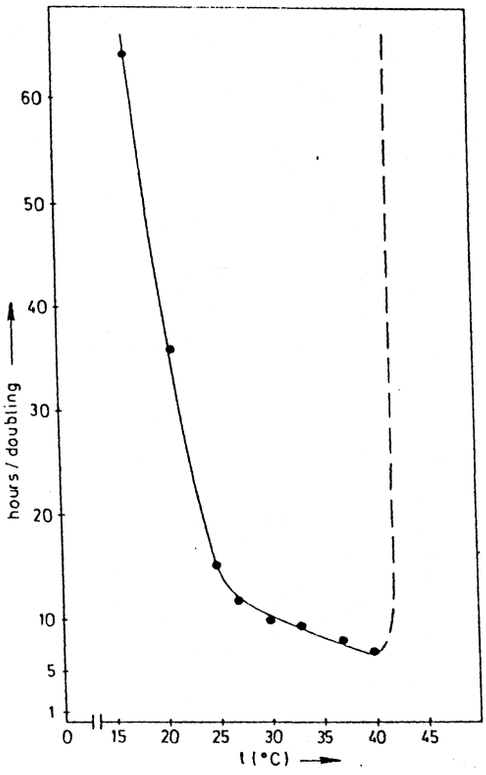

Fig. 1. Optimal growth temperature. Growth was determined several times during the exponential phase by O.D.$_{578}$-measurement. The hours/doubling were calculated from the slopes of the growth curves (not shown)

Table 1. Influence of organic components on growth. The basal salt medium (medium 3 of Balch et al. 1979, but without organic ingredients) was supplemented with organic components in the following concentrations: Acetate 0.1%; peptone 0.2%; yeast extract 0.2%; vitamins (trace vitamins according to Balch et al. 1979) 0.2 ml/20 ml medium. Gas phase: H_2/CO_2. The samples (20 ml) were inoculated with 1 ml of a culture grown in a modified MG-medium, in which all organic components were reduced to 1/10 of the normal concentration. The initial O.D.$_{578}$ was around 0.03

Organic components	Maximal absorbance (O.D.$_{578}$)
– (Control)	–
Peptone	–
Yeast extract	–
Vitamins	–
Acetate	0.21
Peptone + yeast extract	–
Peptone + vitamins	–
Yeast extract + vitamins	–
Acetate + yeast extract	0.62
Acetate + peptone	0.45
Acetate + vitamins	0.18
Peptone + yeast extract + vitamins	--
Acetate + yeast extract + peptone	0.37
Acetate + peptone + vitamins	0.60
Acetate + vitamins + yeast extract	0.49
Acetate + yeast extract + peptone + vitamins	0.47

(Fig. 3a). In the profile (see arrows), they appear as rods about 0.2 – 0.3 µm in diameter. The cells showed a negative gram reaction. In the electron microscope angular plates 1.6 – 2.8 µm long and 1.5 µm wide are visible (Figs. 3b, 4b),

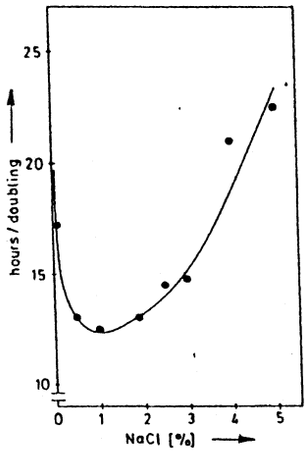

Fig. 2. Effect of NaCl on growth. The NaCl was added to a MG-medium prepared without NaCl supplement. This basal medium already contains 0.33% NaCl, which are not considered in the diagram

showing large indentations in the center (Fig. 3b). In cross-sections, often "bone-shaped" (Fig. 3c) profiles with a diameter of only 0.07 µm in the center, and 0.1 – 0.25 µm on the ends are seen besides normal rod-shaped profiles. Sometimes also *y*-shaped cross-sections (Fig. 3e) and profiles with convexities, possibly buds, can be detected. Septa or diaphragm-like indentations were never seen. The cells often contain electron dense round inclusions, possibly granules of reserve material (Figs. 3b, 3d, 4b). In thin sections the granules frequently seem to be shrunken (Fig. 3d) or often to be broken out, leaving behind a less electron dense hole (Fig. 3c, 3d).

A polar tuft of flagella can be observed (Fig. 4a). Each flagellum is about 13.3 nm in diameter and up to 32 µm long (not shown).

Cell Envelope

The cell envelope shows a hexagonal surface pattern (Fig. 4a). The distance of the subunits – center to center – was determined from EM-photographs to be 14.0 nm. Preparations of the cell envelope show one dominating protein band (Fig. 5, lane 3) with an apparent molecular weight of 143,000 as determined by co-electrophoresis with molecular weight standards in the SDS-gel (Fig. 5, lane 1). This band is also stained with the periodate-Schiff-reagent (Fig. 5, lane 5), and therefore seems to be a glycoprotein. We believe this band to consist of the main envelope protein. No rigid cell wall sacculi could be isolated. In accord, the cells were completely lysed with 2% SDS at room temperature. No muramic acid could be detected.

DNA Base Composition

The DNA contains 47.5 mol% GC as determined by the melting point in 0.1 × SSC (Marmur and Doty 1962), using calf thymus DNA (42 mol% GC) as a reference.

Discussion

The new isolate M3 occurs as plates with sharp edges, strongly reminding of flat crystals, which in the electron microscope show an almost "pneumatic boat"-like appearence: between a surrounding puffed up roll, 0.1 – 0.25 µm in diameter, there is an inner plate, only 0.05 –

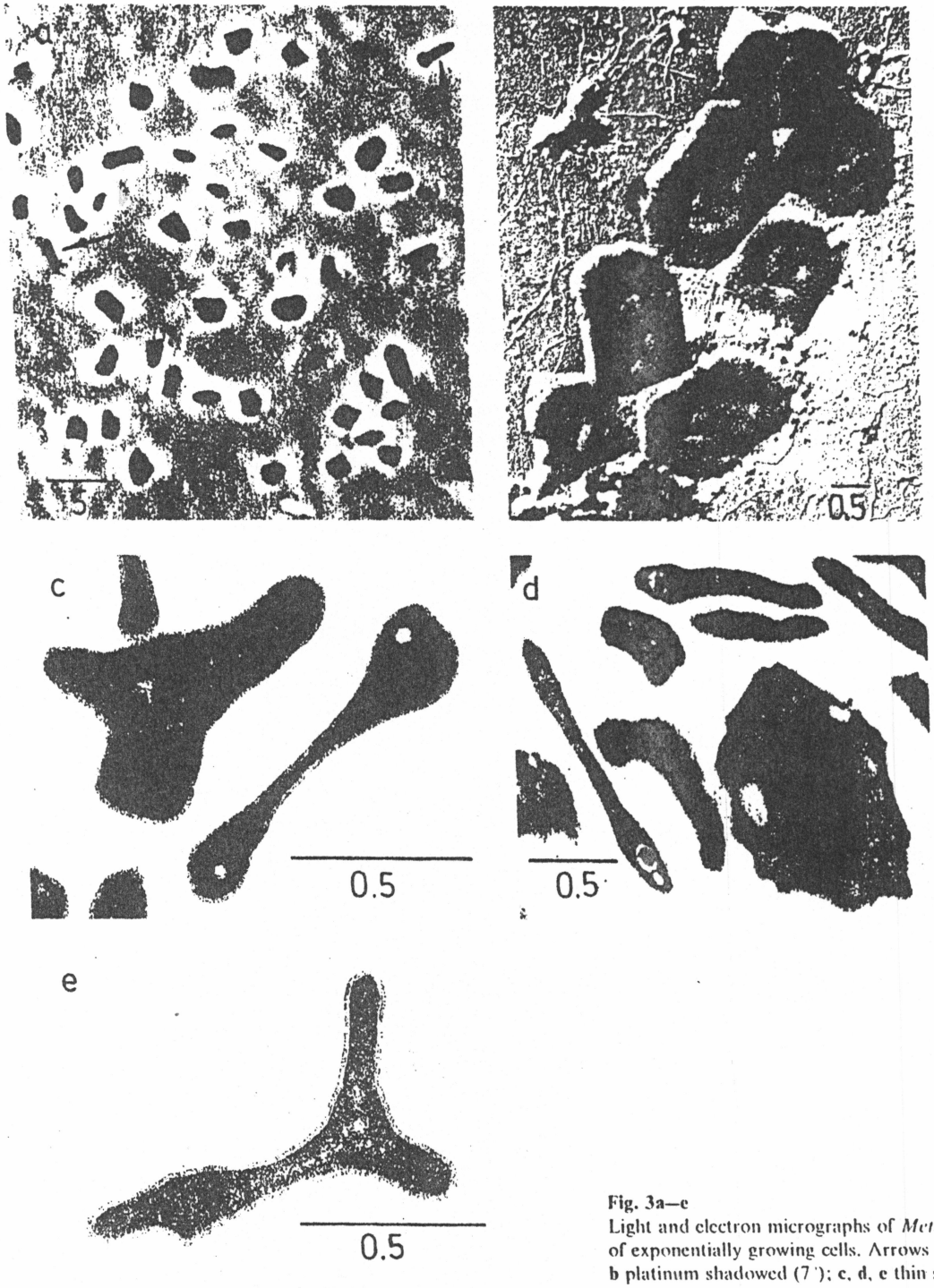

Fig. 3a—e
Light and electron micrographs of *Methanoplanus limicola*. **a** Phase contrast of exponentially growing cells. Arrows indicate bacteria appearing in profile; **b** platinum shadowed (7°); **c**, **d**, **e** thin sections. Statements of size in μm

0.07 μm thick, usually showing some humps. Although not quadratic, the flat shape with the sharp edges strongly reminds of the square halophilic bacterium discovered by Walsby (1980). The novel methanogen, however, grows at much lower ionic strength. Even after a transfer in destilled water, its flat shape is perfectly stable at least for hours (data not shown). The shape-maintaining principle remains unclear at the moment. One could speculate, that the organism may either possess an efficient osmoregulation or, more likely, contain internal structures supporting the envelope in order to obtain the flat shape.

No septa formation could be detected, indicating that cell division does not occur by the usual binary fission. The *y*-shaped profiles and the profiles with swellings in thin sections point to an unusual budding mechanism.

From its ability to form methane from H_2 and CO_2 and from its green fluorescence the new organism is clearly defined to be a methanogen. The obligate requirement for acetate in the medium, which, however, by itself cannot serve as a substrate, was already reported for *Methanogenium cariaci* (Romesser et al. 1979). Other organic compounds, such as a mixture of peptone and vitamins or yeast extract are not essential, but stimulate growth. No methanol or methylamines can be used, indicating that this bacterium does not belong to the Methanosarcinaceae. This is proven in addition by the lack of a rigid cell wall, the possession of flagella and

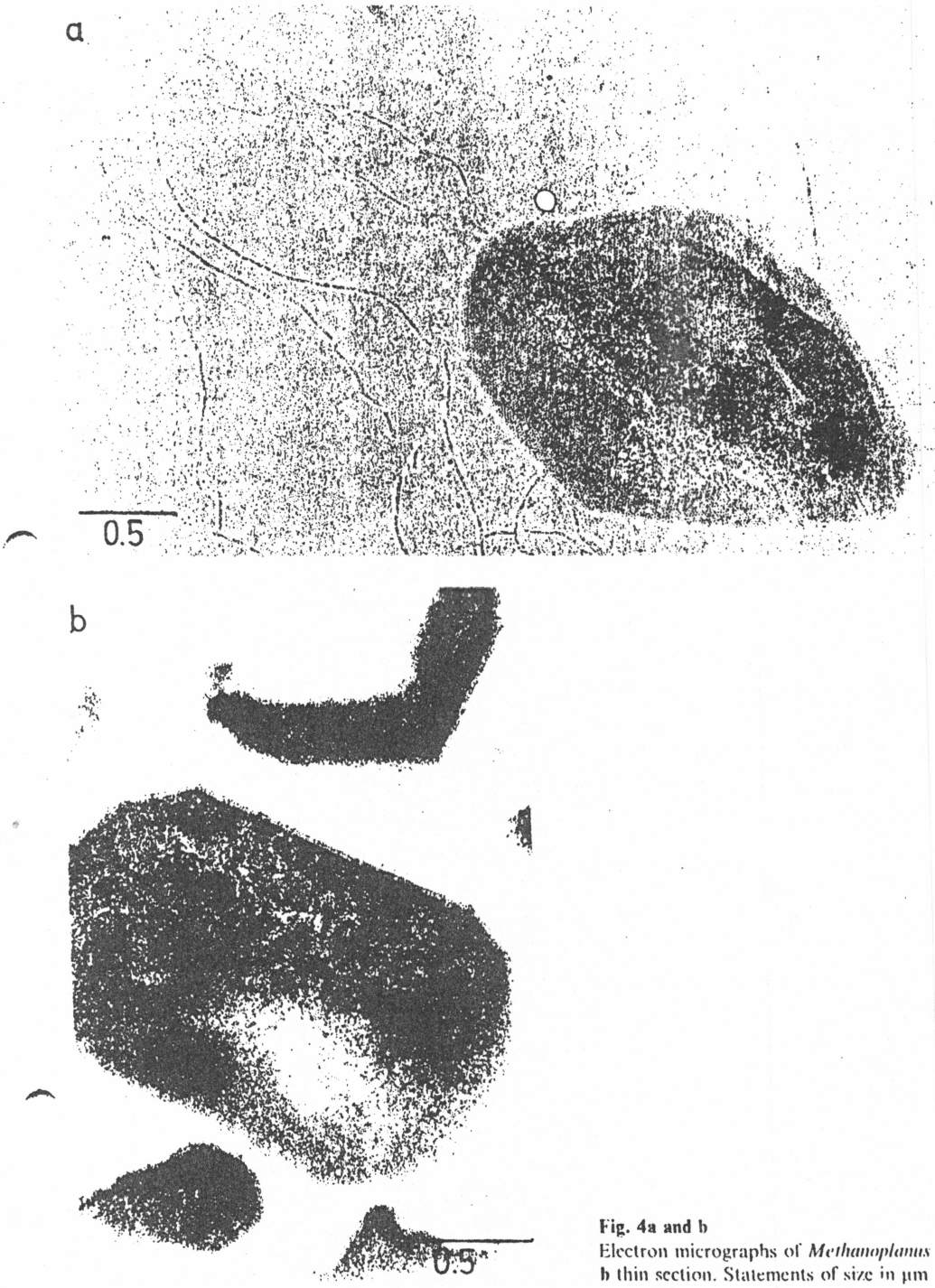

Fig. 4a and b
Electron micrographs of *Methanoplanus limicola*. **a** Rotary shadowed (7 ..
b thin section. Statements of size in µm

the negative gram reaction of the isolate. A close relationship to the Methanococcales, which similar to the isolate can grow on formate, was very unlikely because of a much higher GC-content of the isolate. Due to the latter feature and based on the polyamine composition (S. Schoberth, personal communication), however, the isolate seems to belong to the Methanomicrobiales. DNA-RNA hybridization experiments (Tu et al. 1982) with DNA and ^{32}P-labelled 16s r-RNA substantiated this assumption: the hybrid of the M3 DNA with the Methanogenium RNA showed a higher thermostability (fs = fractional stability = 0.59) than that with the Methanococcus RNA (fs = 0.43), further indicating that M3 belongs to the Methanomicrobiales. Within this ord.. Methanogenium RNA yielded an even more stable hybrid with the Methanosarcina DNA (fs = 0.62) than with .. DNA, demonstrating that the two families are closer .. to each other than to M3. Therefore, we believe the new isolate to be a member of at least a new family, which we name the Methanoplanaceae.

The distribution and the biotope of the isolate is not clear at the moment. From its original habitat, its growth .. the mesophilic temperature range and its salt requirement and tolerance, the organism can be assumed to be comm.. swamps of freshwater and seawater. There it may ha..

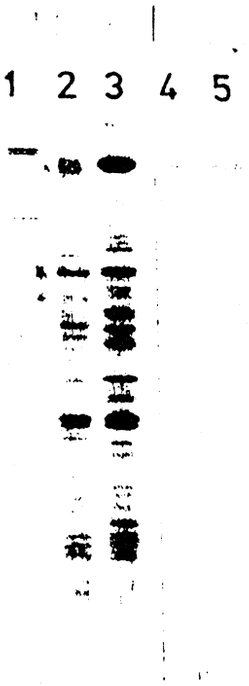

Fig. 5. SDS-polyacrylamide gel. Electrophoresis was performed at 110 V for 14 h. (*1*) Molecular weight standard: RNA polymerase subunits of *Escherichia coli* (from top to the bottom): β' (160,000), β (155,000), σ (92,000), α (37,000); (*2*) and (*4*) envelope preparation of *Methanogenium marisnigri*; (*3*) and (*5*) envelope preparation of *Methanoplanus limicola*; (*1*)–(*3*): coomassie staining; (*4*) and (*5*): periodate-Schiff staining

previously overlooked because of its transparence and its unusual shape. Due to morphology and habitat, we name the new isolate M3 *Methanoplanus limicola*.

Description and Classification of the Methanoplanaceae

Order Methanomicrobiales, Balch and Wolfe (1976).

Family I, Methanomicrobiaceae, Balch and Wolfe (1976).

Family II, Methanosarcinaceae, Balch and Wolfe (1976).

Family III, Methanoplanaceae, Wildgruber, Thomm and Stetter (fam. nov.) Methanoplanaceae, Me. tha. no. pla. na. ce' ae. M. L. neut. n. *Methanoplanus* type genus of the family; -aceae ending to denote a family; M. L. fem. pl. n. Methanoplanaceae the *Methanoplanus* family. The Methanoplanaceae belong to the order Methanomicrobiales, Balch and Wolfe (1976). The family Methanoplanaceae contains one genus.

Gram-negative cells, occurring as thin plates with sharp edges. The cell envelope shows a hexagonal surface pattern. Cells oxidize H_2 or formate as the sole energy source for growth and methane production.

Genus I *Methanoplanus*, Wildgruber, Thomm and Stetter (gen. nov.) Me. tha. no. pla'nus. M. L. n. *methanum* methane; M. L. adj. *planus* flat; M. L. masc. n. *Methanoplanus* the methane (-producing) plate. The description of the genus is the same as that of the family. *Methanoplanus limicola*, Wildgruber, Thomm and Stetter (sp. nov.) li. mi'co. la. L. *limicola*, L. masc. n. inhabitant of a swamp on account of its habitat.

Angular, crystal-like plates 0.07–0.30 µm thick and 1.6–2.8 µm long and 1.5 µm wide, occurring singly. The cells are sometimes branched, without septa. The cell envelope shows a hexagonal surface pattern and contains a dominating glycoprotein. No sacculus is present. A polar tuft of flagella can be seen, each flagellum about 13.3 nm in diameter and up to 32 µm long. The cells contain electron dense round inclusions, about 0.1–0.2 µm in diameter. On polysilicate plates round, smooth, bright, ochrecolored, flat colonies, about 2 mm in diameter are formed. Weakly motile. Anaerobic. Gram-negative. Growth between 17°C and 41°C, optimum 40°C. Optimal salt concentration is 1% NaCl, optimal pH is 7. H_2 and formate serve as substrates for growth and methane production. In addition, acetate is strictly required. No growth occurs on acetate alone, on methanol, and on methylamines. Cells are resistent against vancomycin, penicillin, kanamycin, and tetracycline.

The DNA forms complexes with 16s rRNA from *Methanogenium marisnigri* with a fractional stability of 0.59.

The G + C content of the DNA is 47.5 mol%.

Lives possibly in seawater and freshwater swamps.

Type strain: DSM 2279.

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