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Isolation and characterization of *Sporomusa acidovorans* sp. nov., a methylotrophic homoacetogenic bacterium

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Abstract. *Sporomusa acidovorans* sp.nov. was isolated from a pilot fermenter inoculated with effluent sample from the alcohol distillation industry. The isolate was a Gram-negative, motile, curved, spore-forming rod. The DNA base composition was 42% G + C. The temperature range for growth was 20 to 40°C, with an optimum at 35°C; growth occurred within a pH range of 5.4 to 7.5, with an optimum at pH 6.5. Growth substrates included methanol, H_2-CO_2, formate, fructose, ribose, fumarate, succinate and glycerol. Yeast extract was required for growth. The organism performed the homoacetogenic reaction.

Key words: *Sporomusa acidovorans* – Homoacetogenesis – Methanol – Hydrogen – Fructose

In absence of sulfate, the anaerobic oxidation of H_2 leads to methane or acetate production. Bacteria capable of reducing CO_2 to acetate were first observed in non-defined mixed cultures by Fischer et al. (1931). In 1936 Wieringa described the enrichment and the isolation of the homoacetogenic bacterium: *Clostridium aceticum*. Within the same genus, other homoacetogenic H_2 oxidizing bacteria have been discovered. *C. thermoautotrophicum* was isolated from mud or soil samples (Wiegel et al. 1981). A non-identified methanol degrading *Clostridium* strain has been recently described by Adamse and Velzeboer (1982).

Other spore-forming H_2-oxidizing bacteria stained Gram negative and belonged to the newly described genus *Sporomusa* (Möller et al. 1984). Three non spore-forming acetate producing bacteria have been isolated on H_2 and CO_2: *Acetobacterium woodii* (Balch et al. 1977), *A. wieringae* (Braun and Gottschalk 1982) and *Acetogenium kivui* (Leigh et al. 1981), a thermophilic homoacetogen.

We report on the isolation of a new Gram negative spore-forming homoacetogenic bacterium. This rod-shaped bacterium used methanol or H_2 as energy source and is proposed as a new species of the genus *Sporomusa*: *S. acidovorans*.

Materials and methods

Chemicals. All chemicals were of reagent quality unless otherwise noted. Gases were purchased from Airgaz (Marseille, France).

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Inoculum. Anoxic samples of a pilot fermenter feeded with waste water of the alcohol distillation industry (INRA, Narbonne, France) served as inoculum. The major substrates in the effluent were glycerol and lactic acid.

Enrichment and isolation. Enrichment cultures were incubated at 37°C with 10% inoculum from the pilot fermenter in 60 ml serum bottles containing 20 ml medium with methanol as substrate. Cultures were transferred (2 ml) every 2 weeks into fresh media. For isolation of axenic cultures of the homoacetogenic bacterium, the enrichment was serially diluted and inoculated into roll tubes (Hungate 1969).

Media. Sporomusa acidovorans was grown on complex medium containing the following: NH_4Cl, 1.0 g; $K_2HPO_4 \cdot 3H_2O$, 0.4 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; cystein-HCl, 0.5 g; fructose, 6 g; yeast extract (Difco), 1 g; resazurin, 0.001 g; mineral solution no 2 (Balch et al. 1979), 50 ml; trace element solution (Balch et al. 1979), 10 ml; distilled water, 1,000 ml. For roll tubes, agar (2%) was added in liquid medium. The medium was adjusted to pH 7.0 with KOH and was boiled under N_2. After cooling to room temperature, 20 ml of medium were transferred into 60 ml serum bottles inside an anaerobic glove box (La Calhène, Bezons, France). The bottles were stoppered with black butyl rubber closures (Bellco glass Inc., Vineland, NJ, USA) and outgassed with N_2-CO_2. After sterilization (110°C, 35 min), 0.25 ml Na_2CO_3 (10% w/v) and 0.2 ml $Na_2S \cdot 9H_2O$ (2% w/v) were dispensed into each bottle.

For roll tubes preparation, agar was supplemented. Agar medium was dispensed anaerobically in portions of 4.5 ml into Hungate tubes (Bellco Glass Inc.). 0.08 ml Na_2CO_3 (10%) and 0.1 ml Na_2S (1%) were added to the medium. Stock solutions of Na_2CO_3 and Na_2S were prepared anaerobically under N_2 in 120 ml serum bottles and were autoclaved (110°C, 35 min). Sugar solutions were sterilized by filtration.

Analytical techniques. Volatile fatty acids, organic acids and H_2 were analysed as previously described (Garcia et al. 1982). Formic acid was measured using the technique of Lang and Lang (1972) as modified by Sleat and Mah (1984). Bacterial growth was quantified by measuring the optical density at 580 nm with a Spectronic 21 spectrophotometer (Baush and Lomb Inc., Rochester, NY, USA).

Microscopy. A Zeiss microscope equipped with epifluorescence was used to detect methanogenic bacteria which exhibited a blue-green fluorescence under UV illumination.

Phase contrast microscopy was performed using a Nikon microscope with an automatic camera for photography with Illford HPS film (ASA 400). Cells examined by electron microscopy were fixed with glutaraldehyde (1% v/v) and osmium tetroxide (1% w/v) and included in Epon-Araldite resin. Ultrathin sections were examined with electronic Jeol JEM 100 V microscope.

DNA preparation. DNA was isolated and purified by the method of Price et al. (1978). The mol % G + C was determined according to the method of De Ley (1970) from thermal denaturation in 0.015 M NaCl and 0.015 M trisodium citrate.

Results

Morphology. White and round colonies appeared after 3 weeks incubation in agar roll tubes at 37° C. Older colonies were dark brownish and as large as 3–4 mm. Three pure cultures were isolated from the last positive dilution (strains Mol, Mol_1 and Mol_2). All strains appeared morphologically similar in the microscope. They produced only acetate from complex medium containing sugars. They used fructose, ribose, methanol and H_2-CO_2. Strain Mol was subjected to further characterization.

The isolate was a slightly curved, spore-forming rod (5 µm length × 0.7 µm width) (Fig. 1) and stained Gram negative. It was motile by laterally inserted flagella. Its motility showed some resemblance with that of moving vibrios (Fig. 2). It occurred singly or as arrangements of two or more cells. Electron microscopy showed a multilayered cell wall (Fig. 3). Spore was terminal to subterminal. Spore suspensions survived pasteurization for 20 min at 80° C.

Fig. 1. Phase contrast photomicrograph of *Sporomusa acidovorans*. Bar is 5.0 µm. Note the curved rod and the subterminal spore

Substrates and optimal growth conditions. Nutritional studies were performed at 37° C. Growth occurred only in presence of yeast extract. Besides methanol, the bacterium fermented a variety of substrates as shown in Table 1. The pH and temperature profiles of strain Mol were examined during growth in basal medium with 0.1% yeast extract and 0.6% fructose as energy source. The pH optimum for growth was between 6.5 and 7.0 (Fig. 4). No growth occurred at pH 8.0 or pH 5.4. The optimum growth temperature was 35° C (Fig. 5). Nitrate, sulfate and sulfite were not used as electron acceptors when the bacteria were cultivated on methanol. In carbonate free medium, fructose was used, but not methanol. Acetate was the only organic end product formed. Alcohols or H_2 were never produced.

DNA base composition. The mol % G + C content was 42.

Discussion

Anaerobic enrichment cultures on methanol from a fermenter feeded with alcohol distillation wastes led to the development of homoacetogenic bacteria. Methylotrophic methanogenic bacteria (*Methanosarcina* sp.) were not observed. The ecological importance of the isolated homoacetogen in the pilot fermenter was presumably related to the high amount of glycerol used as growth substrate in the effluent.

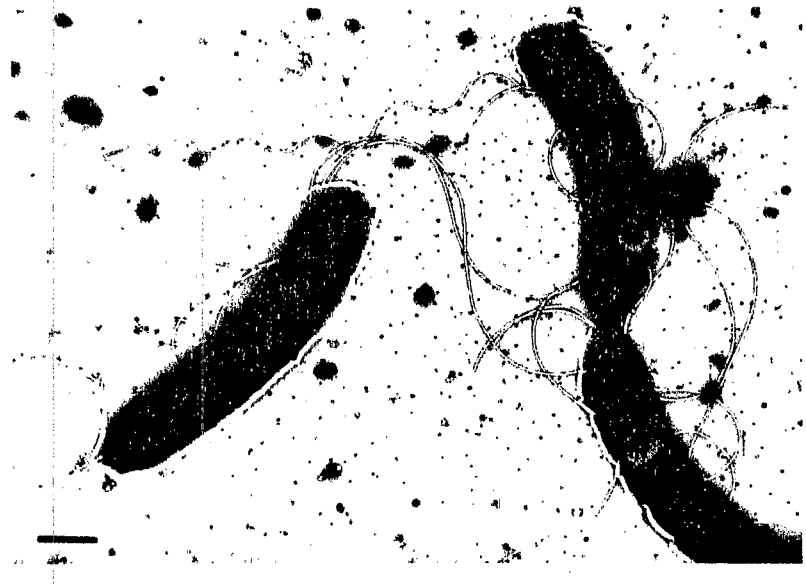

Fig. 2
Transmission electron micrograph of *Sporomusa acidovorans*. Note the flagella distributed at the concave side. Bar is 0.5 µm

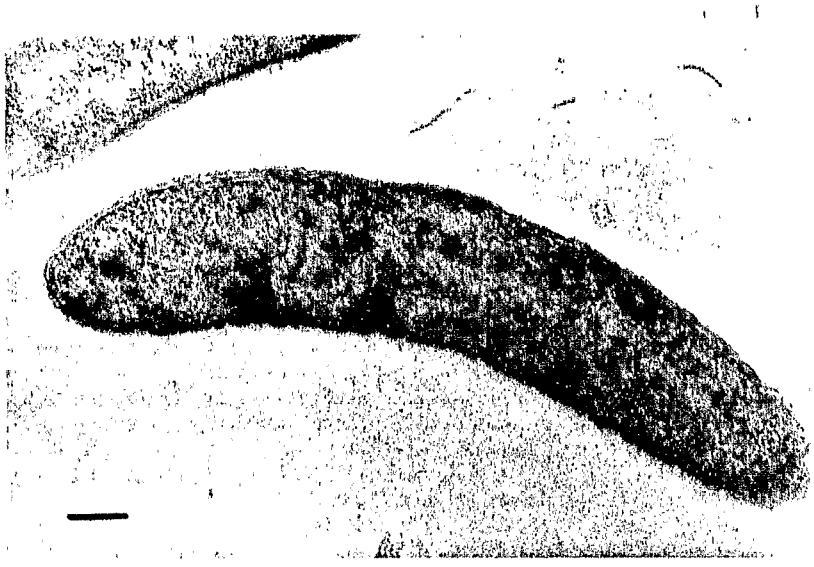

Fig. 3
Electronmicrograph of thin sections showing a multilayered cell wall. Bar is 0.5 µm

Table 1. Utilization of various substrates by *Sporomusa acidovorans*, *S. sphaeroides* and *S. ovata*

Substrate	*S. acidovorans*	*S. sphaeroides*[a]	*S. ovata*[a]
H_2-CO_2	+	+	+
Methanol	+	+	+
Ethanol	−	+	+
Glycerol	+	+	−
Formate	+	+	+
Lactate	−	+	+
Pyruvate	+	+	+
Fumarate	+	−	−
Succinate	+	−	−
Malate	+	nd	nd
Oxaloacetate	+	nd	nd
Glutamate	+	nd	nd
Trimethylamine	−	+	−
L-Serine	+	+	−
Fructose	+	−	+
Ribose	+	−	−

[a] Data from Möller et al. (1984); −, no growth; +, growth; nd, not determined
The medium contained 0.5% of the organic substrate and 0.05% yeast extract. Additional compounds tested which did not support growth: malonate, citrate, lysine, threonine, glucose, maltose, lactose, xylose, melibiose, arabinose, dulcitol, galactose, saccharose, cellobiose, starch, cellulose, gelatin

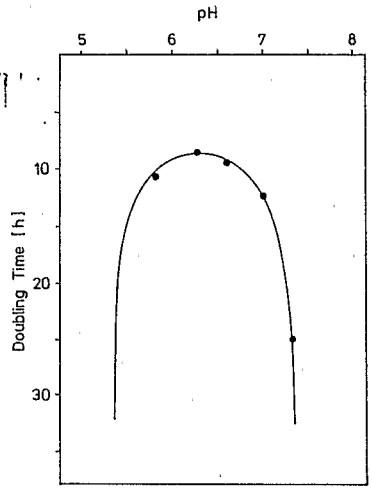

Fig. 4. Effect of pH on generation time of *Sporomusa acidovorans*. Cultures were incubated at 37°C. Medium contained 0.6% fructose and 0.1% yeast extract

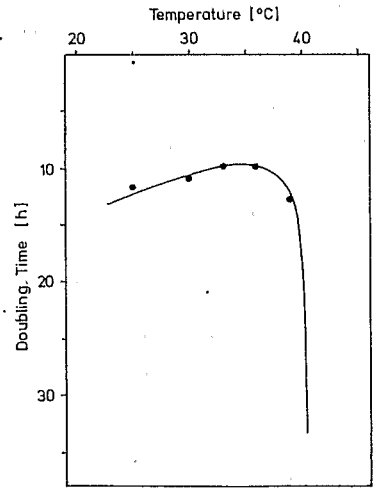

Fig. 5. Effect of temperature on generation time of *Sporomusa acidovorans*. Medium contained 0.6% fructose and 0.1% yeast extract

The isolated homoacetogenic spore-forming bacterium showed a typical Gram-negative cell wall with lateral flagella. These features exclude it from the genus *Clostridium*. Other anaerobic H_2-oxidizing genera were ruled out for the following reasons: *Acetobacterium* sp. (Balch et al. 1977; Braun and Gottschalk 1982) stained Gram-positive, did not form spores and was motile by peritrichous flagella. The thermophilic *Acetogenium kivui* (Leigh et al. 1981) was non-motile and did not produce spores. Unlike *Desulfotomaculum* species (Campbell and Postgate 1965; Pfennig et al. 1981), strain Mol could not use sulfate as electron acceptor.

The banana-shaped cell form, the truly Gram-negative cell wall with laterally inserted flagella as well as the reduction of CO_2 to acetate indicated that strain Mol is a member

of the newly described genus *Sporomusa* (Möller et al. 1984). Within this genus, two species have been described. Strain Mol differed from *S. sphaeroides* by using sugars. Furthermore, strain Mol did not grow on trimethylamine. Unlike *S. ovata*, the current isolate used ribose, glycerol, L-serine and did not form oval spores. It could be distinguished from both species by the unability to use lactate, ethanol, and by requiring yeast extract for growth. *S. sphaeroides* and *S. ovata* grew on betaine in chemically defined medium containing minerals and vitamins. In contrast to *S. sphaeroides* and *S. ovata*, strain Mol grew on fumarate or succinate.

Thus we propose to place strain Mol in the genus *Sporomusa* and name it *S. acidovorans* in recognition to the utilization of acidic compounds (succinate, fumarate...).

Sporomusa acidovorans sp.nov. (a.ci.do'vo.rans; L. neut. n. *acidum* acid; L.v. *voro* to devour; M.L.part. adj. *acidovorans* acid-devouring).

Morphology. Sporulating, curved rods 2–8 μm × 0.7–1 μm with Gram-negative cell wall. Motile by laterally inserted flagella. Occurs singly or in short chains of cells. Colonies are white to darkly brown, entire and convex in shape.

Metabolism. Obligate anaerobe. Degrades $H_2 - CO_2$, methanol, formate, pyruvate, succinate, fumarate, malate, oxaloacetate, glutamate, glycerol, serin, fructose, ribose. Yeast extract is required for growth. The only fermentation product is acetate; CO_2 is the only electron acceptor.

DNA % G + C. The mol % G + C of DNA is 42.

Source. Pilot fermenter feeded with effluent from the alcohol distillation industry.

Type strain. The type strain is Mol (DSM no. 3132). Its description is the same as the species given above.

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