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Two new species of anaerobic oxalate-fermenting bacteria, *Oxalobacter vibrioformis* sp. nov. and *Clostridium oxalicum* sp. nov., from sediment samples

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Abstract. Two types of new anaerobic bacteria were isolated from anoxic freshwater sediments. They grew in mineral medium with oxalate as sole energy source and with acetate as main carbon source. Oxalate as well as oxamate (after deamination) were decarboxylated to formate with growth yields of 1.2–1.4 g dry cell matter per mol oxalate degraded. No other organic or inorganic substrates were used, and no electron acceptors were reduced. Strain WoOx3 was a Gram-negative, non-sporeforming, motile vibrioid rod with a guanine-plus-cytosine content of the DNA of 51.6 mol%. It resembled the previously described genus *Oxalobacter*, and is described as a new species, *O. vibrioformis*. Strain AltOx1 was a Gram-positive, spore-forming, motile rod with a DNA base ratio of 36.3 mol% guanine-plus-cytosine. This isolate is described as a new species of the genus *Clostridium*, *C. oxalicum*.

Key words: Anaerobic oxalate degradation – Membrane energetization – *Oxalobacter vibrioformis* sp. nov. – *Clostridium oxalicum* sp. nov.

Oxalic acid and its sodium, potassium, ammonium, or calcium salts occur in tissues of many species of plants and algae (Thimann and Bonner 1950) and is ingested with the plant material by man and animals. In humans, oxalate is present in the blood, in urine, and in kidney stones (Hodgkinson 1977). Also in aquatic sediments, oxalate was detected in concentrations of 0.1–0.7 mmol per liter of sediment (Smith and Oremland 1983).

Several species of aerobic bacteria are known to be able to grow with oxalate (e.g. Jakoby and Bhat 1958; Chandra and Shethna 1975) and the oxidation of oxalic acid was studied intensively with *Pseudomonas oxalaticus* (e.g. Quayle 1961; Dijkhuizen et al. 1977).

Little is known about the anaerobic decomposition of oxalate, although it is degraded readily by ruminal microorganisms (Morris and Garcia-Rivera 1955; Allison et al. 1977; Dawson et al. 1980) and in sediments (Smith and Oremland 1983). So far, only two different strains of oxalate-degrading anaerobes have been isolated: *Oxalobacter formigenes* (Allison et al. 1985) from the rumen of a sheep, the intestine of a pig, and from human feces, as well as from sediments. Strain Ox-8 (Smith et al. 1985) was isolated from freshwater lake sediments but only partially characterized. Bhat (1966) mentioned the isolation of an oxalate-degrading *Clostridium* strain but did not give any description.

All these bacteria decarboxylate oxalate to formate and have to synthesize ATP only from this energy-yielding reaction with the small free energy change of −25.8 kJ/mol (Thauer et al. 1977). Cell yields, as far as determined, were very low, about 1 g dry matter/mol oxalate. Acetate was needed for cell carbon synthesis (Allison et al. 1985; Smith et al. 1985).

In this paper, the isolation and characterization of two new strains of anaerobic oxalate-fermenting bacteria from freshwater sediments is described. One isolate resembles physiologically the known strain of *Oxalobacter*, the other one is a Gram-positive, spore-forming rod, representing a new *Clostridium* species.

Materials and methods

Sources of isolates

Enrichment cultures were inoculated with 5 ml anoxic mud from various habitats. Freshwater sediment samples were taken from polluted creeks near Konstanz, Marburg, Tübingen (all FRG), and Woods Hole (Massachusetts, USA). Anaerobic sludge was obtained from the municipal sewage treatment plant in Tübingen. Subsamples were pasteurized (15 min, 80 °C) before incubation. Anoxic marine sediment samples were taken from the North Sea coast (Schobüll, FRG), Sippewissett salt marsh (near Falmouth, Mass., USA), and from several channels in Venice, Italy.

Media and cultivation

Carbonate-buffered, sulfide-reduced mineral medium was prepared and dispensed as described earlier (Widdel and Pfennig 1981; Schink and Pfennig 1982). It contained a 7-vitamin solution (Widdel and Pfennig 1981), selenite-tungstate solution, and the trace element solution SL10 (Widdel et al. 1983). The pH was 6.8–7.0, and growth temperature was 30 °C. Substrates were added from sterile, neutralized stock solutions. To enrichment cultures under N_2/CO_2

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(90%/10%) gas headspace containing 50 ml freshwater or saltwater medium, 20 mM di-ammonium oxalate, 3 mM acetate, and, in some cases, 10 mM sulfate were added.

Growth of pure cultures was determined by measuring turbidity in 20 ml-screw cap tubes in a Spectronic 20 spectrophotometer (Milton Roy Company, Rochester, NY, USA). For these experiments, a medium containing only 0.05 mM $CaCl_2$ was used to prevent precipitation of calcium oxalate. Also a commercial media system (API 20A, Bio-Mérieux, Nürtingen, FRG) was applied under anaerobic conditions for further characterization.

Isolation and cytological characterization

Pure cultures of oxalate-degrading bacteria were obtained by repeated application of the agar shake culture technique (Pfennig and Trüper 1981). Purity was checked microscopically and by growth tests in complex medium (AC medium, Difco, Detroit, MI, USA) with and without added oxalate.

Gram-straining was carried out as described by Magee et al. (1975) with *Acetobacterium woodii* and *Klebsiella* sp. as controls. Flagella were stained according to Blenden and Goldberg (1965).

Cytochromes were assayed in French press cell extracts by redox difference spectroscopy in an Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland).

Chemical analyses

Sulfide formation from sulfate was analyzed by the methylene blue method (Cline 1969). Oxalate was determined in its methylated form by capillary gas chromatography using malonate as internal standard as described earlier (Dehning and Schink 1989). Formate was quantified colorimetrically according to Lang and Lang (1972).

The DNA base composition was determined by thermal denaturation as described by De Ley (1970) after extraction according to Marmur (1961). *Escherichia coli* strain K12 was used as reference strain.

Enzyme assays

Cell suspensions were prepared in anoxic 50 mM potassium phosphate buffer, pH 7.2, and cells were permeabilized by addition of 0.1 mg of cetyltrimethylammonium bromide per mg protein (0.05–0.2 mg protein per assay). The activities of carbon monoxide dehydrogenase and formate dehydrogenase were assayed with benzyl viologen as electron acceptor according to Dickert and Thauer (1978) using for the latter 20 mM formate instead of CO.

Chemicals

All chemicals (analytical grade) were obtained from Fluka, Neu-Ulm; Merck, Darmstadt; Sigma, München; all FRG.

Results

Enrichment and isolation

Enrichment cultures with oxalate as substrate and various sediment samples as inocula showed after 3–5 weeks gas production and an increase of pH from 7.0 to 7.7 indicating substrate utilization. In subcultures, turbidity was observed after 2–3 weeks, and the pH rose again whereas gas production decreased. After five to six transfers, isolation was tried by two subsequent dilution series in agar medium with oxalate and 5 mM acetate. White, lens-shaped (strain WoOx3) or globular colonies developed within 2–3 weeks of incubation.

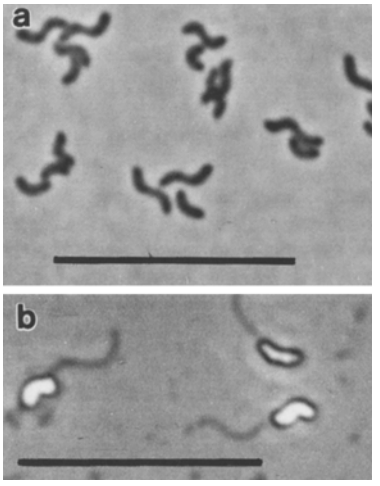

Fig. 1a, b. Phase contrast photomicrographs of the isolate WoOx3 showing **a** vegetative cells and **b** flagella staining. Bar equals 10 μm

Pure cultures were isolated from sulfate-free enrichments with non-pasteurized freshwater sediment samples (strains WoOx1, WoOx2, WoOx3), from sulfate-free enrichments with marine sediment of Rio Marin, Venice (strains MaOx1, MaOx2), and from pasteurized, freshwater sediment samples and sewage sludge (strains LaOx1, AltOx1, OyOx1, KWTOx1 which all looked similar in the microscope and showed the same growth characteristics). From parallel enrichment cultures supplemented with sulfate, no oxalate-degraders different from those mentioned could be isolated.

All strains decarboxylated oxalate to formate and assimilated acetate for cell carbon synthesis. Strains WoOx3 and AltOx1 were chosen for further characterization.

Morphological and cytological characterization

Cells of strain WoOx3 appeared in phase contrast microscopy as motile vibrioid rods, 1.8–2.4 × 0.4 μm in size, occurring singly, in pairs, or sometimes in spiral chains (Fig. 1a). Similar strains (strains MaOx1, MaOx2) were also obtained from marine sediments. Motility was observed only in growing cultures, and flagella staining revealed 1–2 polar flagella (Fig. 1b). Spore formation was never detected, even after growth in sporulation medium supplemented with 20% soil extract, 1% tryptone, 0.1% xylose, and 30 μM manganese sulfate. The Gram-staining reaction was negative. The guanine-plus-cytosine content of the DNA was 51.6 ± 0.6 mol%. No cytochromes were detected.

Cells of strain AltOx1 were motile, spore-forming, straight rods with rounded ends, measuring 2.5–4.8 × 0.7–0.9 μm, occurring singly or in pairs (Fig. 2a). This cell type was isolated from all freshwater sediments, however, only after pasteurization of the inoculum. Heat-resistant, oval spores (1.3 × 0.9 μm) were formed subterminally to centrally and were released into the medium (Fig. 2a, b). Flagella staining revealed peritrichous flagellation (Fig. 2c). The

Table 1. Fermentation stoichiometry and growth yields

Strain	Oxalate degraded (mmol)	Cell matter formed (mg)[a]	Acetate assimilated (mmol)[b]	Formate assimilated (mmol)[b]	Formate produced (mmol)	Electron recovery (%)	Growth yield (mg/mmol)
WoOx3	10.2	13.1	0.17	0.40	9.2	104	1.28
	19.5	23.6	0.31	0.73	17.9	101	1.21
AltOx1	10.4	13.9	0.18	0.43	9.1	98	1.42
	19.8	25.7	0.33	0.79	18.3	103	1.38

Experiments were carried out in 500 ml-bottles with 20 or 40 mM oxalate and 3 mM acetate

[a] Cell dry weights were determined directly with 500 ml cultures

[b] Synthesis of cell material was calculated as follows: $8\ C_2H_4O_2 + 19\ CH_2O_2 \rightarrow 6\ \langle C_4H_7O_3\rangle + 11\ CO_2 + 14\ H_2O$

(strain AltOx1) oxalate (Fig. 5). Higher substrate concentrations inhibited growth. During growth, the pH rose from 6.8 to 8.1 in the presence of 80 mM oxalate. No further increase could be observed (Fig. 5).

Enzymes were assayed in permeabilized cell suspensions of both strains. Benzyl viologen-dependent formate dehydrogenase activity of strain AltOx1 was 80–150 mU/mg protein, and 100–180 mU/mg protein with strain WoOx3. No carbon monoxide dehydrogenase activity (≤ 2 mU/mg) was found in either isolate.

Discussion

Physiology

The new strains of anaerobic bacteria described in this study were enriched and isolated from freshwater mud samples with oxalate as sole energy source and acetate as main carbon source. Oxalate was decarboxylated to formate:

$$C_2O_4^{2-} + H_2O \rightarrow HCO_2^- + HCO_3^- \quad \Delta G_0' = -25.8\ \text{kJ/mol}\,.$$

Although the Gibbs free energy change (calculated after Thauer et al. 1977) of this reaction is small, it supplies enough energy to support the whole energy metabolism of these bacteria. Neither substrate-linked nor electron transport-linked phosphorylation for ATP synthesis are possible. Growth by decarboxylation of dicarboxylic acids has been described so far for *Propionigenium modestum* (Schink and Pfennig 1982) fermenting succinate, for *Oxalobacter formigenes* (Allison et al. 1985) degrading oxalate, and for *Malonomonas rubra* (Dehning and Schink 1989) and *Sporomusa malonica* (Dehning et al. 1989) growing with malonate. All these bacteria obtained low growth yields in the range of 1.1–2.4 g dry matter per mol substrate utilized which reflect the small changes of free energy connected with the respective decarboxylation reactions.

Two different systems have been described so far for coupling decarboxylation reactions to the establishment of an ion gradient across the membrane. In *P. modestum*, a membrane-bound, sodium-pumping decarboxylase converts the decarboxylation energy directly into a sodium-ion gradient which drives ATP synthesis by a sodium ion-dependent ATPase (Hilpert et al. 1984). In *O. formigenes*, an electrogenic oxalate^{2-}/formate$^-$ antiporter generates a transmembrane proton gradient by linking the simultaneous substrate import and product export to a net proton extrusion (Anantharam et al. 1989).

With our new isolates we examined the hypothesis whether oxalate is cleaved by decarbonylation to CO_2 by a carbon monoxide dehydrogenase, similar to acetate decarbonylation by acetate-fermenting methanogens (Krzycki and Zeikus 1984). Conversion of carbon monoxide to formate could be coupled to membrane energetization in a similar manner as is CO fermentation to CO_2 and hydrogen in *Acetobacterium woodii* (Dickert et al. 1986) and a phototrophic bacterium (Uffen 1976). However, neither CO dehydrogenase activity nor CO as intermediate could be detected in our isolates. The found formate dehydrogenase activities provide reducing equivalents for the formation of sugars from CO_2 and acetate which serves as main carbon source for cell material synthesis (see legend to Table 1). It will be subject of further research in our laboratory to examine whether oxalate degradation by our new isolates utilizes an energy coupling system similar to that of *O. formigenes*.

No oxalate-fermenting sulfate reducers were isolated from our enrichment cultures. Postgate (1963) described a new subspecies of *Desulfovibrio, D. vulgaris* ssp. *oxamicus*, which grew with oxamate or oxalate in the presence of sulfate and yeast extract. But growth tests with this strain (DSM 1925) in our laboratory revealed that neither oxamate nor oxalate were degraded, and no sulfate was reduced with these substrates within 20 days of incubation. At the moment, we cannot explain this failure. Since this strain is maintained at the culture collection with lactate as electron donor in a complex medium, it may have lost its oxalate-degrading capacity.

Taxonomy

The Gram-negative strain WoOx3 is morphologically and physiologically similar to the strain Ox-8 isolated and described by Smith et al. (1985). The major differences are its motility, the higher growth rate, and the utilization of oxamate. There are also many similarities with *O. formigenes* (Allison et al. 1985) including the Gram-reaction, the DNA base composition, the stoichiometric decarboxylation of oxalate to formate, and the assimilation of acetate into cell material. Strain WoOx3 only differs in morphology, the temperature optimum, and the additional use of oxamate as growth substrate. Thus, we propose to attribute strain WoOx3 to the genus *Oxalobacter* as a new species, *O. vibrioformis*.

O. vibrioformis sp. nov., *vibrare* L.v. to vibrate, *vibrio* M.L.masc.n. that which vibrates, e.g. a curved cell, *forma*

L.fem.n. shape, vi.brio.for'mis M.L.masc.adj. shaped like a curved cell.

Motile vibrioid rods, 1.8–2.4 × 0.4 μm in size; single, in pairs, or spiral chains. 1–2 polar flagella; non spore-forming. Gram-reaction negative. No cytochromes. DNA base composition: 51.6 ± 0.6 mol% guanine + cytosine (thermal denaturation).

Strictly anaerobic chemoorganotroph. Oxalate and oxamate (after deamination) decarboxylated to formate. Acetate assimilated for cell carbon synthesis. No reduction of nitrate, sulfate, sulfite, thiosulfate, or sulfur. No growth with malonate, succinate (both 20 mM), glutamate, aspartate, glycine, fumarate + formate, glycerol, ethylene glycol, pyruvate, malate, citrate, acetoin, betaine, 1,2-propanediol, trimethoxybenzoate (all 10 mM), lactate, glycolate, methanol (all 5 mM), glyoxylate, hexamethylenetetramine, glucose, fructose, xylose, arabinose (all 2 mM), yeast extract, casamino acids (both 0.1%), and H_2/CO_2 as substrates. No formation of indole from tryptophan, no hydrolysis of urea, gelatin, or esculin. No catalase activity.

Growth in freshwater or brackish water mineral medium. Temperature range: 18–35 °C, optimum at 30–32 °C; pH range: 5.6–8.3, optimum at 6.8–7.0. Habitat: anoxic freshwater and marine sediments. Type strain: WoOx3, DSM ...., deposited in the Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG.

The Gram-positive, sporulating strain AltOx1 cannot be affiliated with the genus *Oxalobacter*. Morphology, spore formation, and the guanine-plus-cytosine content of the DNA of 36 mol% implies that it is closely related to the clostridia. It differs from any other known *Clostridium* species by its very restricted range of substrates. Only oxalate and oxamate were fermented, and acetate assimilated for the synthesis of cell material. The newly isolated strain AltOx1 is proposed to be assigned to the genus *Clostridium* as a new species, *C. oxalicum*.

C. oxalicum sp. nov., ox.a'li.cum M.L.n. *acidum oxalicum* oxalic acid, *oxalicum* M.L.neutr.adj. referring to the metabolization of oxalic acid.

Sporulating, straight rods with rounded ends, 2.5–4.8 × 0.7–0.9 μm in size, single or in pairs. Motile by peritrichous flagella. Gram-reaction positive. Spores oval, 1.2–1.4 × 0.9 μm, subterminally to centrally formed, heat resistant. No cytochromes. Guanine + cytosine content of the DNA: 36.3 ± 0.9 mol% (thermal denaturation).

Strictly anaerobic chemoorganotroph. Oxalate and oxamate decarboxylated to formate. Acetate assimilated for cell carbon synthesis. No reduction of nitrate, sulfate, sulfite, thiosulfate, or sulfur. No growth with malonate, succinate (both 20 mM), glutamate, aspartate, glycine, fumarate + formate, glycerol, ethylene glycol, pyruvate, malate, citrate, acetoin, betaine, 1,2-propanediol, trimethoxybenzoate (all 10 mM), lactate, glycolate, methanol (all 5 mM), glyoxylate, hexamethylenetetramine, glucose, fructose, xylose, arabinose (all 2 mM), yeast extract, casamino acids (both 0.1%), and H_2/CO_2 as substrates. No formation of indole; no hydrolysis of urea, gelatin, or esculin; no catalase.

Growth in freshwater and brackish water mineral medium. Temperature range: 16–34 °C, optimum at 28–30 °C; pH range: 5.3–8.5, optimum at 7.0. Habitat: anoxic freshwater sediments. Enrichment with pasteurized samples. Type strain: AltOx1, DSM ...., deposited in the Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG.

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