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Sporomusa malonica sp. nov., a homoacetogenic bacterium growing by decarboxylation of malonate or succinate

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Abstract. A new strictly anaerobic bacterium was isolated from an enrichment culture with glutarate as sole substrate and freshwater sediment as inoculum, however, glutarate was not metabolized by the pure culture. The isolate was a mesophilic, spore-forming, Gram-negative, motile curved rod. It fermented various organic acids, alcohols, fructose, acetoin, and H_2/CO_2 to acetate, usually as the only product. Other acids were fermented to acetate and propionate or acetate and butyrate. Succinate and malonate were decarboxylated to propionate or acetate, respectively, and served as sole sources of carbon and energy for growth. No inorganic electron acceptors except CO_2 were reduced. Yeast extract (0.05% w/v) was required for growth. Small amounts of cytochrome b were detected in membrane fractions. The guanine-plus-cytosine content of the DNA was 44.1 ± 2 mol%. The isolate is described as a new species of the genus *Sporomusa*, *S. malonica*.

Key words: *Sporomusa malonica* sp. nov. – Gram-negative sporeformer – Homoacetogenesis – Malonic acid decarboxylation – Bioenergetics

Several species of acetogenic bacteria have been described which gain their growth energy by the reduction of carbon dioxide to acetic acid. *Clostridium aceticum* was the first homoacetogenic bacterium isolated from mud by Wieringa (1940). Other spore-forming Gram-positive acetogens were discovered: *C. thermoaceticum* (Fontaine et al. 1942), *C. formicoaceticum* (Andreesen et al. 1970), *C. thermoautotrophicum* (Wiegel et al. 1981), and *C. magnum* (Schink 1984).

Also non spore-forming Gram-positive acetogenic bacteria were isolated: *Acetobacterium woodii* (Balch et al. 1977), *A. wieringae* (Braun and Gottschalk 1982), *A. carbinolicum* (Eichler and Schink 1984), and the thermophilic *Acetogenium kivui* (Leigh et al. 1981). Other strains such as *Eubacterium limosum* (Sharak Genthner et al. 1981) or *Butyribacterium methylotrophicum* (Zeikus et al. 1980) produce acetate and butyrate during growth with H_2/CO_2 or methanol. Recently, Gram-negative spore-forming acetogens were described which were assigned to the new genus *Sporomusa* (Möller et al. 1984).

We report here on the isolation and characterization of a new Gram-negative spore-forming homoacetogen which oxidizes a broad variety of organic substrates and can also grow by decarboxylation of malonate or succinate as sole energy and carbon sources.

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Materials and methods

Sources of microorganisms

Strain WoGl2 was isolated from enrichment cultures inoculated with anoxic mud of a polluted freshwater creek near Konstanz, FRG. *Sporomusa acidovorans* DSM 3132 was obtained from the German collection of Microorganisms (DSM), Braunschweig, FRG.

Media and growth conditions

All procedures for isolation and cultivation were the same as described in earlier papers (Pfennig and Trüper 1981; Widdel and Pfennig 1981). The freshwater mineral medium (Schink and Pfennig 1982) which was carbonate-buffered (30 mM) and sulfide-reduced (1 mM) contained 7-vitamin solution (Widdel and Pfennig 1981), selenite-tungstate solution, and the trace element solution SL10 (Widdel et al. 1983). The pH was 7.2–7.4 and the growth temperature 28–30°C. Growth was determined by measuring turbidity in a Spectronic 70 spectrophotometer (Bausch and Lomb, Rochester, NY, USA) in 20 ml screw-cap tubes. Substrates were added from sterile, neutralized stock solutions.

For further characterization, also a commercial media system (API 20A, BioMérieux, Nürtingen, FRG) was applied.

Cytological characterization

Gram-staining was carried out according to Magee et al. (1975) with *Acetobacterium woodii* and *Klebsiella* sp. as reference organisms.

Cytochromes were assayed in French press cell extracts as well as in membrane preparations ($150000 \times g$, 1 h) by

redox difference spectroscopy (dithionite-reduced minus air-oxidized) in an Uvikon 860 photometer (Kontron, Zürich, Switzerland).

The DNA base composition was determined by thermal denaturation according to De Ley (1970) after extraction as described by Marmur (1961). *Escherichia coli* strain K12, DSM 498, was used as reference strain.

Chemical analyses

Sulfide formation from sulfate or sulfur was analyzed by the methylene blue method (Cline 1969), nitrite formation from nitrate with sulfanilic acid and α-naphthylamine (Procházková 1959).

Fatty acids were assayed with a Carlo Erba Vega 6000 gas chromatograph (Milano, Italy) with flame ionization detector and a glass column (2 m × 2 mm) packed with 60/80 Carbopak C/0.3% Carbowax 20 M/0.1% H_3PO_4 (Supelco, Inc., USA). Injector and detector temperatures were 180° C, column temperature 120° C, carrier gas nitrogen, 45 ml/min, injection volume 2 µl. Samples and standards were acidified prior to injection with formic acid to 0.5 M final concentration. Chromatograms were recorded with a Merck-Hitachi D-2000 integrator (Tokyo, Japan).

Malonate was quantified as dimethyl ester by capillary gas chromatography as described earlier (Dehning and Schink 1989).

All chemicals (analytical grade) were obtained from Fluka, Neu-Ulm, or Merck, Darmstadt, FRG.

Results

Isolation

Enrichment cultures in freshwater medium with 10 mM glutarate as sole energy and carbon source were inoculated with freshwater sediment samples. Gas production started after 3 – 4 weeks, and the glutarate-degrading bacterial community could be transferred at 1:10 ratio into subcultures which grew up again within 1 – 2 weeks. After the fifth transfer, the culture was diluted in agar shake series with various substrates.

In the dilution series with glutarate as sole substrate, growing colonies were observed only in the first two tubes indicating that the glutarate-degrading bacteria depended on a cooperation with other bacteria. The glutarate-fermenting bacteria could not be isolated, no matter whether yeast extract or hydrogen-scavenging methanogens or sulfate reducers were added to the medium.

In agar medium with 10 mM crotonate, brown, lens-shaped colonies developed within 2 weeks up to the sixth dilution tube. Colonies were picked with sterile Pasteur pipettes, resuspended in small amounts of anoxic medium, and again diluted in shake series with the same substrate. The resulting isolates were checked for purity by microscopic control and by inoculation into complex AC-medium (Difco, Detroit, MI, USA) with and without added crotonate. The isolated strain WoGl2 required yeast extract (at least 0.01%) for growth with crotonate in pure culture, and did not utilize glutarate.

Morphology and cytological characterization

Cells of strain WoGl2 were slightly curved, spore-forming rods with rounded ends, 0.7 × 2.6 – 4.8 µm in size, occurring mostly single and sometimes in pairs (Fig. 1). Growing cells exhibited tumbling movement. Flagella staining revealed 2 – 5 flagella per cell which were inserted on the concave cell side. Oval spores (1.2 × 1.5 µm) were sporadically formed subterminally and were released into the medium (Fig. 1). Spores survived pasteurization at 80° C for 15 min.

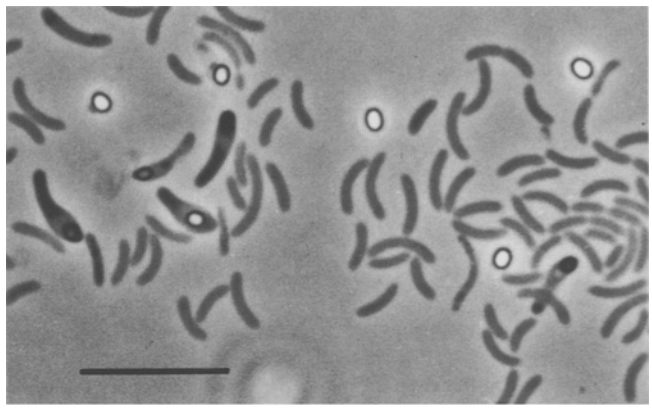

Fig. 1. Phase contrast photomicrograph of the isolate WoGl2 showing vegetative cells, free spores and subterminal spore-formation. Bar equals 10 µm

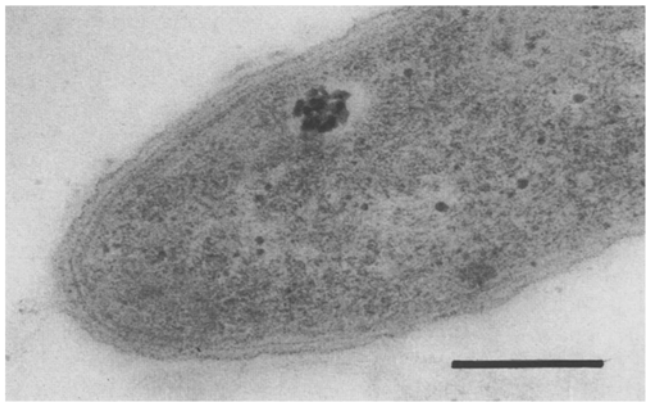

Fig. 2. Electron micrograph of an ultrathin section of strain WoGl2 showing a multilayered cell wall. Bar = 0.2 µm

The Gram staining reaction was negative. Electron micrographs of ultrathin sections showed a typical Gram-negative, multilayered cell wall, consisting of a convoluted outer membrane, a thin peptidoglycan layer, and an inner cytoplasmic membrane (Fig. 2).

Redox difference spectra of cell-free extracts and membrane preparations showed weak absorption bands at about 559, 529, and 428 nm indicating the presence of a cytochrome b at very low concentration (1 – 2 mg/g protein in the cell-free extract). The spectra did not vary qualitatively or quantitatively, no matter whether malonate or fumarate was used as substrate. No cytochrome was detected in the cytoplasmic fraction.

The guanine-plus-cytosine content of the DNA was determined to be 44.1 ± 2 mol%.

Analysis by high-performance liquid chromatography of the corrinoid preparation extracted from this acetogenic bacterium revealed with a high probability a *p*-cresolylcobamide.

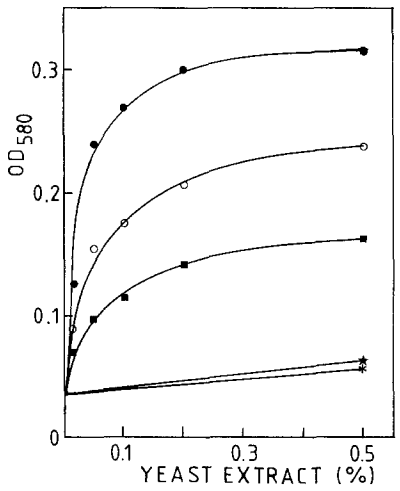

Fig. 3. Dependence of growth of strain WoGl2 on the amount of yeast extract (w/v) added to mineral medium containing malonate at various concentrations. *Symbols:* * no malonate, ★ no malonate, 10 mM acetate, ■ 10 mM malonate, ○ 20 mM malonate, ● 30 mM malonate. OD_{580}: optical density at 580 nm

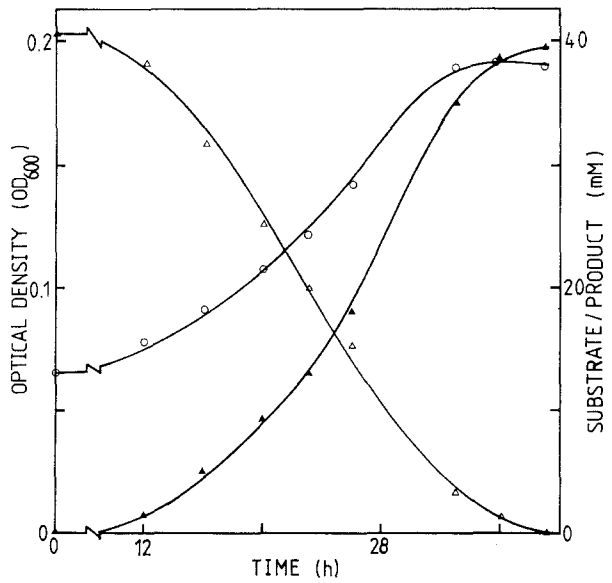

Fig. 4. Fermentation time course of strain WoGl2 in mineral medium containing 40 mM malonate and 0.05% (w/v) yeast extract. Experiments were performed at 30°C in 130 ml serum bottles. Samples were removed with a syringe and the headspace was flushed with N_2/CO_2. *Symbols*: ○ cell density, △ malonate, ▲ acetate formed

Physiology and fermentation stoichiometry

Growth of strain WoGl2 was possible only under strictly anaerobic conditions. The isolate grew in freshwater as well as in brackish water medium containing 10 g NaCl and 1.5 g $MgCl_2 \times 6\ H_2O$ per liter, but not in saltwater medium. Phosphate up to 50 mM concentration did not inhibit growth.

Growth occurred only in the presence of at least 0.01% (w/v) yeast extract. Higher concentrations stimulated growth (Fig. 3); a concentration of 0.2% was optimal. However, yeast extract did not serve as energy source. The growth rate with 20 mM malonate and 0.05% yeast extract was 0.124 h^{-1} ($t_d = 5.6$ h). With 0.2% yeast extract, the growth rate was enhanced to 0.182 h^{-1} ($t_d = 3.8$ h) and the cell yield increased considerably (Fig. 3).

The growth curve (Fig. 4) illustrates the correlation of growth, malonate decomposition, and acetate formation by strain WoGl2 in the presence of 0.05% yeast extract. Growth was possible at temperatures between 15 and 38°C; the optimum was at 28–32°C. The optimal pH was around 7.3, the limits were pH 6.0 and 8.5. No inorganic electron acceptors such as nitrate, sulfate, thiosulfate, sulfite, or sulfur were reduced.

Besides crotonate, strain WoGl2 was able to utilize a variety of substrates listed in Table 1, including H_2/CO_2. Slow growth was also observed with methylmalonate + acetate, betaine, choline, and 2,3-butanediol. Oxalate, glutarate, adipate, pimelate, methylsuccinate, 2- or 3-methylglutarate, L-alanine, L-serine, benzoate, indolylacetate, ethylene glycol, glycerol, diacetyl, glucose, xylose, arabinose, lactose, and casamino acids were not metabolized. Growth with the dicarboxylic acids was tested in the presence of 2 mM acetate.

The only fermentation products were acetate, propionate, and butyrate. The stoichiometries of substrate utilization, product formation, and growth yields are presented in Table 1. Crotonate, 3-hydroxybutyrate, and butanol were fermented to acetate and butyrate. Succinate and malonate were decarboxylated to the respective fatty acids. Fumarate, malate, propanol, and 1,2-propanediol were degraded to acetate and propionate. The fermentation of citrate, pyruvate, lactate, acetoin, formate, fructose, glutamate, methanol, ethanol, H_2/CO_2, and the methoxyl groups of trimethoxycinnamic acid led to acetate as sole fermentation product.

Studies with Sporomusa acidovorans

Growth experiments with *S. acidovorans* revealed that also this strain produced propionate during growth with succinate. Malate, fumarate, and 1,2-propanediol were fermented to acetate and propionate (Table 2). Malonate, crotonate, and propanol were not metabolized.

Discussion

Physiology

The new anaerobic bacterium described in this paper was obtained from freshwater enrichment cultures with glutarate and was isolated with crotonate as sole substrate. Strain WoGl2 exhibited a versatile metabolism fermenting a variety of organic compounds as well as H_2/CO_2. Acetate was the main end product; no ethanol or hydrogen were formed. Thus, strain WoGl2 belongs to the physiological group of homoacetogenic bacteria.

Fructose, pyruvate, lactate, citrate, methanol, formate, acetoin, glutamate, the methoxyl groups of trimethoxycinnamic acid, and H_2/CO_2 were fermented to only acetate as this is usual for homoacetogens (e.g. *Acetobacterium woodii*, *Clostridium aceticum*, *C. magnum*). Oxidation of ethanol to acetate was already described for *Clostridium aceticum* (Adamse 1980) and *C. formicoaceticum* (Andreesen et al. 1970), however, these clostridia could not degrade higher alcohols. The fermentation of propanol, butanol, and

Table 1. Growth yields and stoichiometry of fermentation of various substrates by strain WoGl2

Substrate	Amount of substrate added (μmol)	OD_{600}	Cell dry matter formed (mg)[a]	Acetate assimilated (μmol)[b]	Products formed (μmol)			Electron recovery (%)	Growth yield (g/mol)
					Acetate	Propionate	Butyrate		
Crotonate	200	0.28	1.4	29	228		81	113	7.0
3-OH-Butyrate	200	0.26	1.3	27	196		64	96	6.5
Succinate	200	0.09	0.45	10[c]		184		97	2.25
Malonate	200	0.08	0.4	8	183			96	2.0
Malate	200	0.36	1.8	37	144	81		112	9.0
Fumarate	200	0.35	1.75	36	121	90		110	8.75
Citrate	100	0.26	1.3	27	212			106	13.0
Pyruvate	100	0.14	0.7	14.5	110			100	7.0
Lactate	200	0.25	1.25	26	271			99	6.25
Acetoin	200	0.38	1.9	39	477			103	9.5
Formate	100	0.04	0.2	4	26			117	2.0
Fructose	40	0.35	1.75	36	89			104	43.75
Trimethoxycinnamic acid	50	0.21	1.05	22	83			93	21.0
Methanol	100	0.16	0.8	17	62			105	8.0
Ethanol	200	0.11	0.55	11	290			100	2.75
Propanol	200	0.09	0.45	10	113	175		99	2.25
Butanol	200	0.08	0.4	8	114		186	103	2.0
1,2-Propanediol	200	0.24	1.2	24	72	167		105	6.0
Glutamate	200	0.19	0.9	19	202			97	9.4

All growth tests were carried out in 20 ml tubes completely filled with mineral medium in the presence of 0.05% (w/v) yeast extract. The background production with yeast extract alone was $OD_{600} = 0.01$ and was subtracted from the values measured with the above substrates. All figures are means of two independent assays

[a] Cell dry matter was calculated from turbidity at 600 nm using the conversion factor 0.1 OD_{600} = 25.0 mg/l, which was obtained by direct determinations with 500 ml cultures grown with crotonate

[b] Assimilation of acetate into cell material was calculated by the equation: $17\ C_2H_3O_2^- + 11\ H_2O \rightarrow 8\ \langle C_4H_7O_3\rangle + 2\ HCO_3^- + 15\ OH^-$; thus, 20.6 μmol acetate was required for 1.0 mg of cell dry matter

[c] Assimilation of succinate: $17\ C_4H_4O_4^{2-} + 32\ H_2O \rightarrow 14\ \langle C_4H_7O_3\rangle + 12\ HCO_3^- + 22\ OH^-$

Table 2. Stoichiometry of fermentation of succinate, malate, and 1,2-propanediol by *Sporomusa acidovorans*

Substrate	Amount of substrate added (μmol)	Products formed (μmol)	
		Acetate	Propionate
Succinate	200		187
Malate	200	245	11
1,2-Propanediol	200	23	189

Experiments were carried out in mineral medium in the presence of 0.05% yeast extract

1,2-propanediol to acetate and the respective fatty acids was demonstrated with *Acetobacterium carbinolicum* (Eichler and Schink 1984).

Crotonate and 3-hydroxybutyrate were degraded to acetate and butyrate according to the following equation (for crotonate):

$$2\ C_4H_5O_2^- + 2\ H_2O \rightarrow 2\ C_2H_3O_2^- + C_4H_7O_2^- + H^+.$$

Strain WoGl2 formed more acetate than expected from this equation (Table 1). Obviously, the bacteria carried out two different fermentation patterns simultaneously, crotonate dismutation and homoacetogenic fermentation. A similar fermentation was reported for *Clostridium kluyveri* (Thauer et al. 1968) and *Ilyobacter polytropus* (Stieb and Schink 1984). It was shown recently that also obligately syntrophic fatty acid-oxidizing bacteria can grow in pure culture by the same dismutation of crotonate (Beaty and McInerney 1987).

Malate and fumarate were fermented to acetate and propionate. In this case as well (Table 1), more acetate was formed than expected from the classical fermentation equation for propionibacteria, e.g., *Veillonella* sp. (for malate):

$$3\ C_4H_4O_5^{2-} + 3\ H^+ \rightarrow$$
$$2\ C_3H_5O_2^- + C_2H_3O_2^- + 4\ CO_2 + H_2O.$$

Apparently, propionic acid fermentation and homoacetogenic fermentation were performed simultaneously. The presence of small amounts of cytochrome b indicates that energy could perhaps be conserved by electron transport phosphorylation coupled to fumarate reduction. However, the cell yields obtained do not provide conclusive evidence for this hypothesis. The bacteria are obviously not able to carboxylate pyruvate to oxaloacetate as do the classical propionibacteria. This is probably the reason why lactate is not fermented to propionate.

Of special interest is the ability of strain WoGl2 to grow by decarboxylation of malonate and succinate to acetate or propionate, respectively (for malonate):

$$C_3H_2O_4^{2-} + H^+ \rightarrow C_2H_3O_2^- + CO_2.$$

Growth by decarboxylation of a dicarboxylic acid was so far studied with only one bacterium, *Propionigenium modestum*, growing on succinate (Schink and Pfennig 1982). This bacterium forms ATP from the small free energy change of the decarboxylation reaction (around −20 kJ/mol) (Thauer et al. 1977) by coupling the decarboxylation of

methylmalonyl-CoA by a membrane-bound decarboxylase to sodium ion extrusion and sodium ion-dependent ATP synthesis (Hilpert et al. 1984).

The low growth yields of 2.4 g per mol succinate with *P. modestum*, 2.0 g per mol malonate with the newly isolated bacterium *Malonomonas rubra* (Dehning and Schink 1989), and 2.25 g per mol succinate (2.0 with malonate) with strain WoGl2 (Table 1) correlate with this small change in free energy. It will be a subject of further research to examine whether succinate and malonate fermentation by strain WoGl2 use a similar energy coupling system as does succinate fermentation by *P. modestum*.

The cell yields of strain WoGl2 obtained after growth with various substrates were in the same range as those of *A. carbinolicum* (Eichler and Schink 1984) growing with the same substrates. However, all growth yields with the exception of pyruvate were higher than those of *I. polytropus* (Stieb and Schink 1984), probably because strain WoGl2 can gain additional ATP by acetogenic CO_2 reduction. The reduction of bicarbonate as an external electron acceptor allows the concomitant oxidation of the respective organic substrate to acetate, and enhances the yield of conserved metabolic energy.

Taxonomy

The new acetogenic spore-forming bacterium showed a typical Gram-negative cell wall. This feature excludes it from the Gram-positive clostridia. Other autotrophically growing acetogens like *Acetobacterium* sp. (e.g. Balch et al. 1977), *Acetogenium kivui* (Leigh et al. 1981), *Eubacterium limosum* (Sharak Genthner et al. 1981), or *Butyribacterium methylotrophicum* (Zeikus et al. 1980) have similar physiological properties, however, they do not form spores and are Gram-positive.

The fermentation of various organic compounds as well as H_2/CO_2 to mainly acetate, the banana-shaped cell form, the Gram-negative cell wall architecture, spore-formation, unilaterally inserted flagella, the G + C content of the DNA of 44 mol%, and the likely presence of *p*-cresolylcobamide (Stupperich et al. 1988) indicate that strain WoGl2 is closely related to the genus *Sporomusa*. Within this genus, five species have been described. Our isolate differs from *S. ovata*, *S. sphaeroides* (Möller et al. 1984), and *S. paucivorans* (Hermann et al. 1987) by using fumarate, malate, citrate, succinate, and malonate as substrates. *S. acidovorans* (Ollivier et al. 1985) is able to grow with various organic acids, but does not utilize crotonate, citrate, lactate, malonate, ethanol, and propanol. Unlike the new isolate, *S. acidovorans* could grow with glycerol and serine, and the pH optimum for growth was around 6.5. The recently described species *S. termitida* (Breznak et al. 1988) isolated from gut contents of termites, can also decarboxylate succinate and malonate. However, this bacterium did not use fructose, propanol, fumarate, or malate, and fermentation of lactate or mannitol led to acetate and ethanol as end products. Thus, we propose to assign the described strain WoGl2 to the genus *Sporomusa* as a new species, *S. malonica*.

Species description

Sporomusa malonica sp. nov., ma.lo'ni.ca, M.L.n. *acidum malonicum* malonic acid, *malonica* M.L.fem.adj., referring to metabolization of malonic acid.

Sporulating, curved rods, $0.7 \times 2.6-4.8$ µm in size, Gram-negative. Motile by laterally inserted flagella, occurring singly or in pairs. Spores oval, 1.2×1.5 µm, subterminal, heat-resistant.

Strictly anaerobic chemoorganotroph or autotroph. Crotonate and 3-hydroxybutyrate, as well as butanol fermented to acetate and butyrate. Succinate and malonate degraded to propionate or acetate, respectively. Malate and fumarate, as well as propanol and 1,2-propanediol fermented to acetate and propionate. Citrate, pyruvate, lactate, fructose, methanol, ethanol, glutamate, acetoin, formate, H_2/CO_2, and methoxyl groups of trimethoxycinnamic acid metabolized to acetate. No other organic acids, sugars, or alcohols metabolized.

Growth in freshwater and brackish water mineral salts medium with small amounts of yeast extract (0.05% w/v). Sulfate, thiosulfate, sulfite, sulfur, or nitrate not reduced. Indole not formed, gelatin or urea not hydrolyzed; no catalase activity. Mesophilic; temperature range: 15–38° C, optimum around 30° C. pH range: 6.0–8.5, optimum at 7.3. Membrane-bound cytochrome b. DNA base composition: 44.1 ± 2 mol% G + C (thermal denaturation). Habitat: anaerobic sediment of freshwater origin. Type strain: WoGl2, DSM 5090, deposited with the Deutsche Sammlung von Mikroorganismen (DSM), Braunschweig.

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