

Fermentation of tartrate enantiomers by anaerobic bacteria, and description of two new species of strict anaerobes, *Ruminococcus pasteurii* and *Ilyobacter tartaricus**

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Abstract. Enumerations of tartrate-fermenting anaerobic bacteria with L-, D-, and m-tartrate as substrates revealed that L-tartrate fermenters outnumbered D- and m-tartrate fermenters by one to three orders of magnitude in all three anoxic environments studied.

Highest numbers of tartrate-fermenting bacteria were found in freshwater creek sediments, less in polluted marine channels, and lowest numbers in anoxic sewage digester sludge. Prevailing bacteria were isolated on every tartrate enantiomer. They all degraded tartrates via oxaloacetate.

D- and m-tartrate-fermenting anaerobes were able to ferment L-tartrate as well, and were assigned to the genera *Bacteroides*, *Acetivibrio*, and *Ilyobacter*. L-Tartrate-fermenting anaerobes only utilized this enantiomer, and were characterized in more detail. Fermentation products on tartrate, citrate, pyruvate, and oxaloacetate were acetate, formate, and carbon dioxide. On fructose and glucose, also ethanol was formed. Freshwater isolates were Gram-positive cocci with large slime capsules, and were described as a new species, *Ruminococcus pasteurii*. Saltwater isolates were Gram-negative short rods, and were also described as a new species, *Ilyobacter tartaricus*. The guanosine-plus-cytosine content of the DNA was 45.2% and 33.1%, respectively.

Key words: Tartrate enantiomers — *Ruminococcus pasteurii* — *Ilyobacter tartaricus* — Tartrate dehydratase — Stereospecificity — Anaerobic degradation

L-(+)-Tartaric acid is widely distributed in nature and occurs in many fruits, free and combined with potassium, calcium, or magnesium (Windholz et al. 1976). It is a byproduct of wine fermentation and is used for industrial purposes. In contrary, the other tartrate isomers only occur very rarely in nature.

Already in 1858, Pasteur observed selective anaerobic degradation of L-tartrate in a racemic mixture of D- and L-ammonium-tartrate. On this observation, he based his theory of biological handedness (Pasteur 1858). Later, other authors reported anaerobic L-tartrate degradation by *Aerobacter aerogenes* (Barker 1936), *Clostridium* sp. (Tabachnick and Vaughn 1948; Mercer and Vaughn 1951), *Bacillus* (Krampitz and Lynen 1956) and "*Pseudomonas*" strains (Nomura and Sakaguchi 1955; LaRivière 1956) as well as *Veillonella alcalescens* (De Vries et al. 1977). Fer-

mentation products were acetate, ethanol, lactate, propionate, succinate, carbon dioxide and hydrogen. Phototrophic utilization of L-tartrate was reported for *Rhodospseudomonas sphaeroides* (van Niel 1944). Aerobically, L-tartrate is decomposed by coliform bacteria (Vaughn et al. 1946) and *Pseudomonas* species (Shilo 1957; Dagley and Trudgill 1963). Some *Salmonella* species and some *Pseudomonas* strains degrade meso- or D-(–)-tartrate (Shilo and Stanier 1957; Rode and Giffhorn 1982). Degradation usually proceeds via dehydration to oxaloacetate (Barker 1936; Nomura and Sakaguchi 1955; Hurlbert and Jakoby 1965); alternative pathways are decarboxylation to glycerate as with a *Pseudomonas* strain (Dagley and Trudgill 1963) or oxidation to oxalglycolate as found in mitochondria (Kun 1956). The present investigation was initiated in order to compare the potential of anaerobic microbial degradation of the different tartrate isomers in natural and man-made anoxic ecosystems.

Materials and methods

The following strains were isolated in pure culture from enrichment cultures inoculated with mud samples:

Strains KoTa1, KoTa2 from anoxic digester sludge of the municipal sewage plant at Konstanz, FRG.

Strains WoTa1, WomTa1, ASDTa1 from black anoxic mud of polluted freshwater creeks near Konstanz, FRG.

Strains GraTa1, GraTa2 and CumTa1 from anoxic marine sediment of Canal Grande and Rio Marin in Venice, Italy, as well as marine sediment sampled at Cuxhaven, FRG.

Methanospirillum hungatei strain M1h was isolated from digested sludge of the sewage plant at Göttingen, FRG.

Cultivation and analyses

All procedures for cultivation and isolation as well as all methods for analysis of metabolic products were as described in earlier papers (Widdel and Pfennig 1981; Schink and Pfennig 1982). For isolation of pure cultures, the agar shake culture method (Pfennig 1978) was applied. Formate was quantified by a colorimetric method (Lang and Lang 1972). Protein was determined after Hartree (1972). *Tartrate dehydratase* was assayed in crude cell extracts obtained by French pressure cell treatment according to Bergmeyer (1974). All *chemicals* were of reagent grade quality and obtained from Merck, Darmstadt; Serva, Heidelberg; Fluka, Neu-Ulm, and Sigma, München, FRG.

* Dedicated to Prof. Dr. H. G. Schlegel on occasion of his 60. birthday

Table 1. Enumeration of tartrate-degrading anaerobes in three different anoxic environments (cells/ml)

Environments	Substrates		
	L-Tartrate	D-Tartrate	m-Tartrate
Anoxic sewage sludge (Konstanz)	93	4	< 1
Anoxic freshwater creek sediments (near Konstanz)	$1.5-11 \times 10^4$	1.5×10^3	460
Anoxic marine sediment (Rio Marin, Venice)	$2.4-4.6 \times 10^3$	43	240

Table 2. Substrates utilized by tartrate-degrading isolates

Substrate	Strains					
	Concentration (mM)	ASDTa1	WomTa1	CumTa1	KoTa2	GraTa2
L-Tartrate	10	+	+	+	+	+
D-Tartrate	10	+	±	-	-	-
m-Tartrate	10	-	±	+	-	-
Citrate	10	+	+	+	+	+
Pyruvate	10	-	+	+	+	+
Oxaloacetate	10	-	+	+	+	+
Malate	10	±	-	-	-	-
Fumarate	10	±	-	-	-	-
Glycerate	10	+	-	-	-	-
Glycerol	10	-	-	-	-	+
Glucose	2	+	-	+	+	+
Fructose	2	+	-	+	+	+
Xylose	5	±	-	-	+	-
Arabinose	5	±	-	-	+	-

(+) Good growth; (-) no growth; (±) weak growth

Table 3. Dehydratase activities for tartrate enantiomers in crude cell extracts of isolated strains (mkat per kg protein)

Tartrate enantiomer	Strains				
	ASDTa1	WomTa1	CumTa1	KoTa2	GraTa2
L-Tartrate	2.17	0.85	0.71	19.1	8.0
D-Tartrate	11.18	0.38	<0.1	<0.1	<0.1
m-Tartrate	<0.1	5.17	4.0	<0.1	<0.1

Strains were grown in 1 l cultures with 20 mmol/l of the respective tartrate enantiomer used for their isolation. Cell extracts used for enzyme assays contained 5.1–17.8 mg protein per ml

Results

Enumeration and isolation experiments

Enumerations of anaerobic tartrate-fermenting bacteria were carried out in mineral medium with 10 mM L-, D-, and m-tartrate as substrate using the three tube most-probable-number technique. After 4 weeks of incubation, tubes were checked for growth and product formation, and numbers of tartrate-fermenting anaerobes were calculated from positive tubes according to standard tables (American Public Health Association 1969). Acetate and methane were the only fermentation products detected. Numbers of L-tartrate-fermenting anaerobes were highest in freshwater creek sedi-

ments, smaller in marine sediments of a channel in Venice, and very small in anoxic sewage sludge (Table 1). In every single sample examined, numbers of D- and m-tartrate-degrading anaerobes were lower than those of L-tartrate-degrading anaerobes. Subcultures were inoculated from final dilution tubes on every enantiomer, and were purified in agar shake dilution series after two further transfers. The isolates were characterized with respect to specificity for their isolation substrate, to physiology, and cell morphology.

General characterization of isolated strains

The substrates utilized for growth by tartrate-degrading isolates are listed in Table 2. The strains isolated with L-tartrate did not utilize any other tartrate enantiomer whereas those strains isolated with D- or m-tartrate were also able to grow on L-tartrate; one freshwater isolate grew on all three tartrates. All strains exhibited tartrate dehydratase activities for their respective isolation enantiomer in the range of 0.4–19.1 mkat per kg protein (Table 3). Strains ASDTa1, WomTa1, and CumTa1 also showed weak dehydratase activities for the other tartrate enantiomers used for growth. Strain ASDTa1 was isolated as prevalent D-tartrate-degrading anaerobe from freshwater creek sediments. Cells were Gram-negative straight to slightly curved slender rods motile by probably polar flagella. Fermentation products were acetate, propionate and formate. The organism behaved aerotolerant in agar shake gradient cultures, and reduced nitrate to nitrite. Sulfur

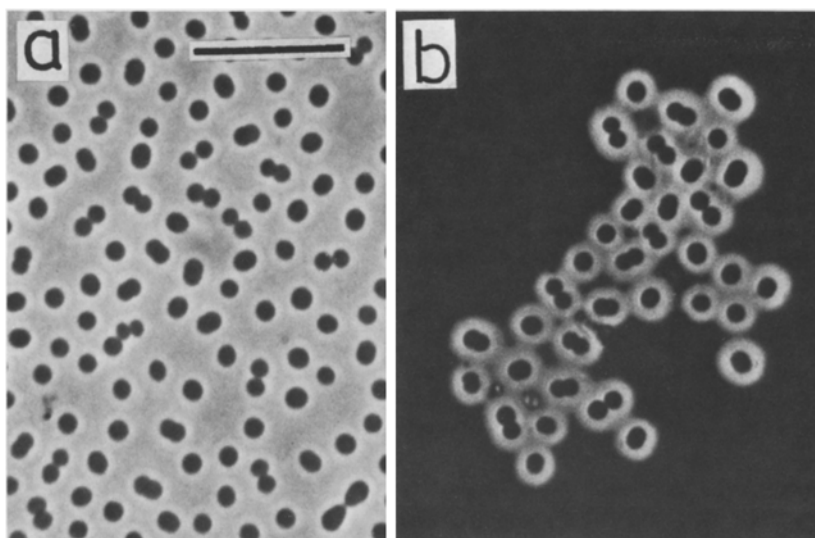


Fig. 1
Phase contrast photomicrograph of strain KoTa2.
a Fresh culture grown on L-tartrate;
b Indian ink preparation. Bar equals 10 μm
for both prints

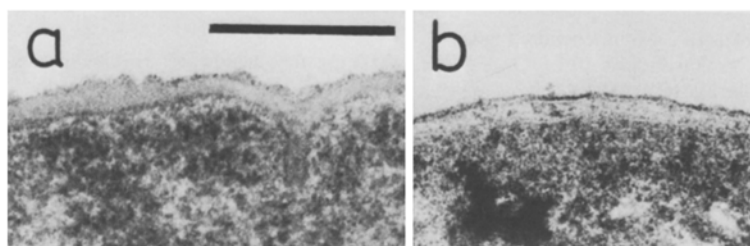


Fig. 2
Electron micrographs of ultrathin sections of
L-tartrate-fermenting isolates. Bar equals 0.2 μm .
a Strain KoTa2; **b** strain GraTa2

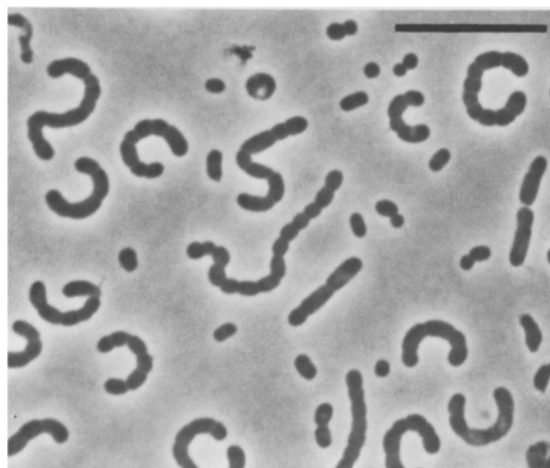


Fig. 3. Phase contrast photomicrographs of marine isolate strain GraTa2. Bar equals 10 μm for both prints

compounds were not reduced. It was assigned to the genus *Bacteroides*.

Strain WomTa1 was the prevalent m-tartrate-fermenting anaerobe in freshwater creeks. Cells were Gram-negative small, very motile vibrioid rods. Acetate and formate were the only detectable fermentation products. No growth was found on sugars. Metabolism was strictly anaerobic; nitrate or oxidized sulfur compounds were not reduced. This strain was assigned to the genus *Acetivibrio*.

Strain CumTa1 was isolated as prevalent m-tartrate-degrading anaerobe from marine sediments. Cells were Gram-negative straight rods. Acetate and formate were the only fermentation products; on glucose and fructose, also ethanol was formed. Neither nitrate nor sulfur compounds were reduced. This strain was classified with the genus *Ilyobacter* (Stieb and Schink 1984).

Characterization of strain KoTa2

Of three similar L-tartrate-fermenting isolates from freshwater creek sediments and sewage sludge, strain KoTa2 was chosen for further characterization. Cells were cocci, 1.0–1.5 μm in diameter, either single or in pairs (Fig. 1 a). Indian ink preparations made large slime capsules visible (Fig. 1 b). Cells were nonmotile and stained weakly Gram-positive. In ultrathin sections, a typical Gram-positive cell wall architecture was visible, and the mode of cell fission was also typical of Gram-positive cells (Fig. 2 a). Spore formation was not observed. The guanine-plus-cytosine content of the DNA was $45.2 \pm 1.0\%$.

Strain KoTa2 grew fast with a minimum doubling time of 1.2 h ($\mu_{\text{max}} = 0.578 \text{ h}^{-1}$) at 35°C. Growth was possible also at low temperatures: Cells grew at 4°C with 24 h and at 0°C with 32 h doubling time (in the presence of 2% ethylene glycol), and no growth was found at –2°C and 45°C. The pH limits of growth were pH 5.5 and 8.8 with an optimum between 7.0 and 8.0. For optimal growth in mineral medium, biotin was the only vitamin required. NaCl inhibited growth at concentrations higher than 1%.

Table 4. Stoichiometry of fermentation and growth yields of isolates obtained on L-tartrate

Substrate	Amount of substrate degraded (μmol)	Cell dry weight formed (mg) ^a	Substrate assimilated (μmol) ^b	Products formed (μmol)			Growth yield mg/mmol substrate utilized	Carbon recovery %
				Acetate	Formate	Ethanol		
Strain KoTa2								
L-Tartrate	200	2.1	34.6	171	195	—	10.6	100.1
Oxaloacetate	200	1.01	16.6	176	214	—	5.06	101.6
Pyruvate	200	1.11	18.3	170	206	—	5.6	98.6
Citrate	200	2.02	22.2	370	192	—	10.1	97.4
Glucose	40	1.11	6.3	44	72	26	27.8	104.8
Fructose	40	1.26	7.0	38	72	25	31.6	100.0
Strain GraTa2								
L-Tartrate	200	2.07	34.1	174	190	—	10.4	101.3
Oxaloacetate	200	1.06	17.5	185	148	—	5.3	92.3
Pyruvate	200	1.16	19.1	186	172	—	5.8	97.0
Citrate	200	2.1	23.1	363	178	—	10.6	95.5
Glucose	40	1.31	7.3	37	71	27	32.7	101.4
Fructose	40	1.34	7.4	41	75	21	33.5	101.7

Experiments were carried out in 20 ml tubes. All figures are means of 2–6 independent assays

^a Cell dry weights were calculated by cell density using conversion factors ($0.1 \text{ OD}_{650} \cong 23.6\text{--}25.3 \text{ mg dry weight per l}$) which were obtained by direct determination in 500 ml cultures for each strain separate

^b Substrate assimilated was calculated using the formula ($\text{C}_4\text{H}_7\text{O}_3$) for cell material

Characterization of strain GraTa2

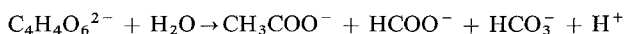
Of three similar L-tartrate-fermenting isolates from marine sediments, strain GraTa2 was further characterized. Cells were short rods, $1.0 \times 1.2\text{--}2.5 \mu\text{m}$ in size, often forming irregular, odd-shaped chains (Fig. 3). Slime capsules were small (not shown). Cells were nonmotile and stained Gram-negative. Ultrathin sections revealed a typical Gram-negative cell wall architecture (Fig. 2b). Spore formation was never observed. The guanine-plus-cytosine content of the DNA was $33.1 \pm 1.0\%$. Strain GraTa2 grew optimally at 28°C with a minimum doubling time of 1.6 h ($\mu = 0.433 \text{ h}^{-1}$); the temperature limits were 12 and 40°C . The pH limits were pH 5.5 and 8.0; the optimum was between pH 6.5 and 7.2. Growth was possible in mineral medium with at least 1% NaCl; no growth occurred in freshwater medium. No vitamins were required for growth on tartrate or glucose.

Physiology of strains KoTa2 and GraTa2

Strains KoTa2 and GraTa2 were very similar with respect to physiological properties. Besides L-tartrate, they both degraded citrate, pyruvate, oxaloacetate, glucose, and fructose. Strain KoTa2 grew on several other carbohydrates (listed at the end of the "Discussion" section), whereas strain GraTa1 grew on glycerol in the presence of acetate. Yeast extract was not used as a growth substrate, however, enhanced growth yields by 15–20%.

Fermentation products of both strains on tartrate, citrate, pyruvate and oxaloacetate were formate, acetate and probably carbon dioxide, according to the following fermentation equations (Table 4):

Tartrate:



Citrate:



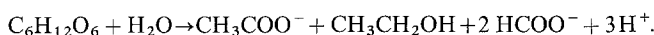
Oxaloacetate:



Pyruvate:



Fructose and glucose were degraded to acetate, ethanol, and formate according to the equation:



No cytochromes were detectable on cell-free extracts or membrane preparations of glucose- or tartrate-grown cells. In cocultures with the hydrogen- and formate-degrading methanogen *Methanospirillum hungatei*, acetate and methane were the only products of tartrate degradation, according to the following equation:



Discussion

Ecology and physiology of tartrate fermentation

The present study demonstrates that all three tartrate isomers can be fermented anaerobically. Since L-tartrate is by far the most important enantiomer in nature, it is not astonishing that numbers of L-tartrate-fermenting anaerobes exceeded those of D- or m-tartrate-fermenters by one to three orders of magnitude. Highest numbers of

tartrate-degrading anaerobes were found in freshwater creek sediments in which L-tartrate as a component of fresh plant matter probably plays a more important role as a substrate than in a highly polluted marine-channel or a sewage digester. The fact that potassium tartrate is not degraded in wine is probably due to its low pH (pH 3.2–3.7) and high ethanol content rather than to low temperature as the properties of our and earlier isolates suggest. Pasteur's early observation that only L-tartrate was fermented in a racemic mixture of both enantiomers was probably based on the fact that he used an inoculum enriched for several generations on L-tartrate. He described the bacteria involved as "little rods or granules of very small diameter, organized in clumps, and welded together by a sticky substance" (Pasteur 1858). To my knowledge, there is no slime-forming coccoid bacterium among the tartrate-fermenting anaerobes described so far. The organism described by Pasteur very well resembles strain KoTa2 presented in the present study.

All strains degraded tartrate via oxaloacetate, no matter if L-, D-, or m-tartrate was the substrate. The data at hand do not allow conclusions on whether the different tartrate dehydratase activities found in strains ASDTa1, WomTa1, and CumTa1 are due to different dehydratase enzyme proteins, or if they all are functions of one single enzyme protein with low substrate specificity, or if a racemase is involved. With one exception, all strains isolated converted tartrate to acetate, formate, and carbon dioxide. Degradation of tartrate and citrate via oxaloacetate and pyruvate would allow ATP synthesis only by acetate kinase. The cell yields obtained with all strains ranged around 10 g cell dry weight per mol of substrate, and thus agree with an energy yield of 1 mol ATP per mol of substrate degraded (Stouthamer 1979). With *Veillonella alcalescens*, a slightly higher cell yield was obtained on L-tartrate (13.1 g/mol; De Vries et al. 1977), however, this bacterium can gain energy also during fumarate reduction to succinate, and was grown in the presence of yeast extract. On pyruvate and oxaloacetate, cell dry weights obtained only accounted for about half of those expected. The cell yields obtained during conversion of glucose and fructose to acetate, ethanol, and formate agree with an assumed formation of 3 mol ATP per mol of sugar degraded. Similar values were obtained with *Ilyobacter polytropus* which exhibits the same fermentation pattern on sugars (Stieb and Schink 1984).

Taxonomy

The D- and m-tartrate-fermenting isolates described in the present study were tentatively assigned to existing genera. The L-tartrate-fermenting strains were characterized in more detail. Although they both were very similar with respect to physiological properties, they appeared to be very different with respect to cytological characters, e.g. Gram reaction, cell shape, guanine plus cytosine content of the DNA. Strain KoTa2 is a strictly anaerobic, Gram-positive coccus. Its fermentation pattern as well as the guanine plus cytosine content of the DNA do not allow affiliation with the genera *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Peptococcus*, or *Sarcina* (Buchanan and Gibbons 1974). The formation of acetate, formate, and ethanol from glucose is common in the genus *Ruminococcus*, and the guanine plus cytosine content of the DNA of reported strains (39.8–45.5%) is close to that of strain KoTa2. Although strain KoTa2 lacks some typical properties of the genus

Ruminococcus, e.g. cellulose degradation or fastidious growth requirements, it appears justified to assign this strain to the genus *Ruminococcus* as a new species, *R. pasteurii* sp. nov.

Ruminococcus pasteurii sp. nov. pa. steu'ri.i.M.L. gen. attr. referring to *Louis Pasteur* who probably first enriched and observed this bacterium during studies on tartrate fermentation. Cocci, 1.0–1.5 µm in diameter, single, in pairs or small packages, surrounded by thick slime capsules. Gram-positive, non-motile, non-sporeforming. Strictly anaerobic chemoorganotroph. L-Tartrate, citrate, oxaloacetate, pyruvate utilized for growth as well as a variety of sugars including glucose, fructose, lactose, sucrose, cellobiose, maltose, mannose, raffinose, sorbose, rhamnose, trehalose. No growth on D-tartrate, m-tartrate, glycerol, glycerate, formate, acetate, lactate, glyoxylate, glycolate, fumarate, malate, ethanol, ethylene glycol, 2,3-butanediol, 3-hydroxybutyrate, xylose, arabinose, cellulose, xylane, peptone, yeast extract. Fermentation products include acetate, formate, ethanol, and carbon dioxide. Growth in freshwater medium with less than 1% sodium chloride. Sulfate, sulfur, thiosulfate, sulfite, or nitrate not reduced. Indole not formed, gelatin or urea not hydrolyzed. No catalase activity, no cytochromes. Biotin required for growth in mineral medium.

Selective enrichment from freshwater sediments with L-tartrate as sole carbon and energy source.

pH range: 5.5–8.8; optimum pH 7.0–7.5. Temperature range: 0–42°C; optimum temperature 35°C.

DNA base ratio: 45.2 ± 1.0% G + C (thermal denaturation). Habitats: anoxic muds in freshwater lakes and creeks. Type strain: KoTa2, DSM 2381, deposited in Deutsche Sammlung von Mikroorganismen, Göttingen.

The marine L-tartrate-fermenting isolate, strain GraTa2, is a Gram-negative, non-sporeforming strictly anaerobic rod which has to be assigned to the family Bacteroidaceae (Krieg and Holt 1984). Its simple growth requirements, fermentation pattern, cell shape, and guanine plus cytosine content of the DNA do not allow affiliation to any of the 12 so far accepted genera of this family. The isolate shares several important properties, especially the fermentation pattern on sugars, with the recently described *Ilyobacter polytropus* (Stieb and Schink 1984). Therefore, it is affiliated to this genus as a new species, *Ilyobacter tartaricus* sp. nov. Strain CumTa1 was affiliated with this species, too.

Ilyobacter tartaricus sp. nov. tar. ta'ri. cus.M.L.n. *acidum tartaricum* tartaric acid; *tartaricus* referring to tartaric acid as isolation substrate. Short, straight rods, 1.0 × 1.2–1.2–2.5 µm in size, often in chains, surrounded by slime capsules, non-motile, Gram-negative, non-sporeforming.

Strictly anaerobic chemoorganotroph. Grows on L-tartrate, citrate, pyruvate, oxaloacetate, glucose, fructose, raffinose, glycerol. Fermentation products include acetate, formate, and ethanol. No growth on formate, acetate, lactate, methanol, ethanol, ethylene glycol, 2,3-butanediol, glycerate, malate, fumarate, glyoxylate, glycolate, mannose, maltose, lactose, sucrose, cellobiose, sorbose, rhamnose, trehalose, xylose, arabinose, peptone, yeast extract. Grows in saltwater medium with at least 1% sodium chloride. Oxidized sulfur compounds or nitrate not reduced.

Indole not formed, gelatin or urea not hydrolyzed. No catalase activity, no cytochromes. No vitamin requirements. Selective enrichment from marine sediments with L-tartrate as sole carbon and energy source.

pH range: 5.5–8.0; optimum pH 6.5–7.2. Temperature range: 10–40°C; optimum temperature 28–34°C. DNA base ratio: $33.1 \pm 1.0\%$ G + C (thermal denaturation). Habitats: anoxic marine sediments. Type strain: GraTa2, DSM 2382, deposited in Deutsche Sammlung von Mikroorganismen, Göttingen.

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