Desulfonatronovibrio hydrogenovorans gen. nov., sp. nov.,
an Alkaliphilic, Sulfate-Reducing Bacterium

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A new alkaliphilic, sulfate-reducing bacterium, strain Z-7935T (T = type strain), was isolated from a
soda-depositing lake, Lake Magadi in Kenya. This organism is a motile vibrio which grows in sodium
bicarbonate medium and does not grow at pH 7; the maximum pH for growth is more than pH 10, and
the optimum pH is 9.5 to 9.7. The optimum NaCl concentration for growth is 3% (wt/vol). The optimum temperature
for growth is 37°C. The G+C content of the DNA is 48.6 mol%. 16S ribosomal DNA sequence analysis revealed that strain Z-7935T represents a new lineage with genus status in the delta subclass of the Proteobacteri-
ta. The name Desulfonatronovibrio hydrogenovorans gen. nov., sp. nov., is proposed for this organism; the type
strain of D. hydrogenovorans is strain Z-7935 (= DSM 2929).

The development of alkalinity is often ascribed to sulfate-reducing bacteria, as suggested by Abd-el-Malek and Rizk for the Wadi-el-Natrun (1). Sulfate-reducing bacteria are thought to be responsible for the disappearance of sulfate in Lake Magadi. Direct evidence of sulfate reduction is found in the black muds interbedded with trona (Na2CO3, NaHCO3, 2H2O), which indicate that H2S was formed and sulfate was depleted in the groundwater and that subsequent binding of sulfide by iron occurred in trachitic lava rocks in Lake Magadi (2). However, as pointed out by Tindall (17), this theory has not been supported by the successful isolation of alkaliphilic sulfate reducers which can grow at pH values greater than 9 from soda lakes, in which the pH is often greater than 10. Nevertheless, it has been shown for a group of Central Asian soda lakes with pH values greater than 9 that in these alkali-
philic environments, in which the salinity is as high as 15%, sulfidogenesis constitutes a dominant hydrogen sink, compet-
ing with acetogenesis but not with methanogenesis, which occurs via the methyloxythropic pathway with the utilization of methanol and/or methylamines (26). Thus, the search for and isolation of alkaliphilic, sulfate-reducing bacteria is important for our understanding of (i) the bacterial diversity which em-
loys bioenergetics that are currently unknown or considered impossible, (ii) the role of these bacteria in community metab-
omolism as the main electron sink for hydrogen-producing pri-
mary anaerobes, and (iii) the geochemical processes that are influenced by producers of extreme alkalinity in such environ-
ments. The processes for which causative agent(s) have yet to be isolated remain unelucidated from a microbiological point of view.

Isolation of alkaliphilic, hydrogen-consuming, sulfate-reducing bacteria from the alkaliphilic anaerobic community in Lake Magadi was first reported in 1994 (27). Here, we describe the isolation from Lake Magadi of an alkaliphilic, sulfate-reducing bacterium which grows in an extremely alkaline environment. The phylogenetic affiliation of this organism with representa-
tives of the sulfate-reducing bacteria is described.

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ology, Louisiana State University, Baton Rouge, LA 70803-1715.

MATERIALS AND METHODS

Sampling. A sample was collected from sediments of an alkaline lake, Lake Magadi (East African Rift, Kenya). The sample was taken during the dry season of 1991 from a trench dug into the trona; it was assumed that the liquor at the sample site came from a depth of 8 to 10 ft under the surface of the trona and was enriched with NaCl. Subsamples of the mud were taken anaerobically, and these subsamples were transported to Moscow at the ambient temperature; in the laboratory they were stored at 4°C.

Media and growth conditions. Selective alkaline medium II (27) containing (per liter) 15 g of NaHCO3, 10 g of Na2CO3, 10 g of NaCl, 1 g of NH4Cl, 0.2 g of KCl, 0.2 g of K2HPO4, 3 g of Na2SO4, 0.5 g of Na2S · 9H2O, 10 ml of a vitamin solution (24), 1 ml of a trace element solution (21), 0.5 g of yeast extract, and H2O or formate as the substrate was used for enrichment and isolation. A pure culture was maintained in an optimized medium formulated after the study of the physiology of the new isolate was completed and was cultivated under strictly anaerobic conditions in rubber membrane-sealed glass vessels with H2 as the gas phase. The optimized medium contained (per liter) 3.9 g of Na2CO3, 24 g of NaHCO3 (added after cooling), 20 g of NaCl, 5 g of Na2SO4, 0.2 g of K2HPO4, 0.1 g of MgCl2 · 7H2O, 0.5 g of NH4Cl, 0.2 g of KCl, 0.12 g of sodium acetate or 0.5 g of yeast extract, 10 ml of a vitamin solution (24), 1 ml of a trace element solution (21), and 0.5 g of Na2S · 9H2O or thiosulfate (final pH 9.5). Cultures were incubated at 37°C. The substrates (electron donors) used were H2 in the gas phase and sodium formate (5 gliter, with N2 in gas phase). Electron acceptors were added at the following concentrations: Na2S2O3, 30 mM; Na2S2O5, 5 mM; Na2S2O7, 10 mM; Na2S2O3, 10 mM; Na2S2O4, 10 mM; sodium fumarate, 10 mM; S2O32-, 2 gliter; and dimethylsulfide, 0.2 mliter. Twenty-milliliter portions of the me-
dium were distributed into 100-ml rubber-stoppered screw-cap flasks by using a standard strictly anaerobic technique. In the case of roll tube cultivation, 2% (wt/vol) agar (Difco Laboratories, Detroit, Mich.) was added to 4-ml portions of carbonate-free medium. The carbonate solution was injected into Hungate tubes after sterilization. NaCl requirements were studied with optimized medium in which the Na2CO3 and NaHCO3 were replaced by 5 g of K2CO3 per liter and the Na2S was replaced by K2S. Growth, detected as visible turbidity, was measured by determining the optical density at 600 nm in Hungate tubes with a spectro-
photometer (Spekol, Jena, Germany) equipped with a type ER tube adapter. Microscopic counts were also used to determine growth. The pH limits for growth and sulfidogenesis were determined in the carbonate medium containing 10 g of Na2CO3 per liter and 15 g of NaHCO3 per liter; the pH was adjusted to the appropriate value by adding 6 N NaOH or 6 N HCl to an anaerobic flask with a built-in pH electrode under a stream of N2. After the dispensed medium had been autoclaved and the pH had been verified, the flasks were inoculated with 1% (vol/vol) inoculum and incubated for 9 days at 37°C. The growth response was measured by determining sulfide formation and turbidity. Sulfidogenesis at different pH values was measured by monitoring H2S formation from radiola-
belled sulfate at 37°C during incubation for 24 h (5, 20). All of the chemicals used in this study were obtained from Russian suppliers.

Analytical procedures. The presence of desulfoviridin was checked fluoro-
metrically by using a spectrofluorimeter (Hitachi, Tokyo, Japan) (10). H2S was quantified by the methylene blue reaction and colorimetric detection (18). Sulfate consumption (or formation of sulfate from Na2S2O3) was measured by a
nephelometric reaction with BaCl₂ (4). Hydrogen consumption was monitored by measuring pressure and/or by gas chromatography with a type 5A molecular sieve column. Lipids were extracted from cell biomass that was dried in a stream of helium and then under a vacuum. To 30 mg of dry biomass, 200 µl of a 5.4 N solution of anhydrous HCl in methanol was added, and the mixture was heated at 70°C for 2 h. The methyl esters of fatty acids and aldehyde derivatives obtained were extracted twice with 100 µl of hexane. The extract was dried and silylated in 20 µl of N,O-bis(trimethylsilyl)trifluoroacetamide for 15 min at 65°C. A 1-µl portion of the reaction mixture was analyzed with a model HP-5985B gas chromatography-mass spectrometry system (Hewlett-Packard, Palo Alto, Calif.) equipped with a capillary column (25 by 0.25 mm) consisting of fused quartz containing an Ultra-1 nonpolar methylsilicone phase. The temperature profile included a 2-min isotherm at 150°C and subsequent programmed temperature increases (the temperature was increased at a rate of 5°C/min to 250°C and then at a rate of 10°C/min to 300°C). Data processing was carried out with an HP-1000 computer by using the standard programs of the gas chromatography-mass spectrometry system (Hewlett-Packard).

Microscopy. The morphology of cultures was observed with an anoptral Zeiss microscope (Reichert, Vienna, Austria). Negative staining of whole cells by phosphotungstic acid and fixation for preparation of thin sections were carried out as described previously (28). Microscopy was performed with a model JEM-100C electron microscope (JEOL, Tokyo, Japan).

G+C content. The guanine-plus-cytosine (G+C) content of the genomic DNA was determined by a thermal denaturation method (9). *Escherichia coli* K-12 DNA was used as the standard.

16S rDNA sequence determination and data analysis. Genomic DNA was extracted and the gene coding for 16S rRNA (16S rDNA) was amplified as described previously (12). Purified PCR products were directly sequenced by using Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). Sequence reaction mixtures were electrophoresed with an Applied Biosystems model 373A DNA sequencer. The 16S rDNA sequence of strain Z-7935T (T = type strain) was manually aligned with sequences of representatives of the delta subclass of the Proteobacteria. Pairwise evolutionary distances were computed by using the correction of Jukes and Cantor (6). The neighbor-joining method was used to reconstruct a phylogenetic tree from the distance matrices (14).

Nucleotide sequence accession numbers. The 16S rDNA sequences determined in this study are available from EMBL under accession numbers X99234 to X99237.

RESULTS

Enrichment and isolation. The sediment sample used in this study had an oily black appearance, and a white mineral film formed on the surface. The purple bacteria usually found in the lagoons of Lake Magadi were absent at the sampling site. At the moment of sampling, the pH of the water was 10.2 and the temperature was 50°C. Samples of the sediment from Lake Magadi were incubated in 100-ml screw-cap bottles containing selective alkaline medium II (pH 9.7) with H₂ as the substrate; after several weeks of incubation, dominant vibrioid bacteria were observed. A pure culture was isolated by the serial dilution method in liquid selective alkaline medium II containing 0.5 g of yeast extract per liter and H₂ as the substrates. Colonies of the sulfate reducer were obtained in roll tubes with sodium formate as the substrate. The colonies were yellowish, translucent, lens shaped, and less than 0.2 mm in diameter. The purity of the culture was indicated by the absence of growth on glucose-peptone sulfate-free medium containing excessive quantities of yeast extract. The absence of growth in media containing various substrates utilized by sulfate-reducing bacteria provided additional proof of purity. Several single colonies were isolated. One of these colonies, designated strain Z-7935T, was chosen for further characterization.

Morphology. Strain Z-7935T is a highly motile vibrio with a polar flagellum and filamentous appendages (Fig. 1). Motility was observed in young cultures but drastically diminished during 1 year of subcultivation. At pH 10 short spirilla were observed. The cells, which occurred singly or in pairs, were 0.5 µm in diameter and 1.5 to 2.5 µm long. Multiplication was by binary fission with the formation of two sister cells (Fig. 1b). The cell wall had a typical gram-negative structure in ultrathin sections, and the periplasmic space was well-developed (Fig. 2). Spores were never observed.

Metabolic properties. Strain Z-7935T is very restricted with respect to the range of electron donors utilized. A total of 28 substrates were tested as electron donors, and only H₂ and formate were utilized. No growth occurred on the following compounds (each at a concentration of 5 g/liter): acetate, propionate, butyrate, pyruvate, lactate, malate, fumarate, succinate, methanol, ethanol, glycerol, glycine, cysteine, cystine, serine, alanine, glutamate, aspartate, Casamino Acids, yeast extract, choline, betaine, glucose, fructose, rhamnose, and mannose. Sulfate, sulfite, and thiosulfate were utilized as elec-
tron acceptors. Formation of sulfide was observed in the presence of dimethyl sulfoxide; however, there was no growth in subcultures. Elemental sulfur inhibited growth. Fumarate and nitrate did not support growth. Strain Z-7935T was strictly anaerobic, and its growth was inhibited by air. Thiosulfate was determined with various pH values and salt contents. In growth experiments sulfidogenesis occurred without a lag phase at pH 8.0 to 10.2. In short-time experiments with radiactive sulfate, sulfidogenesis increased linearly to a maximal value at pH 10. The pH of the medium did not change during growth. At pH values higher than 10 there was a decrease in the rate of sulfidogenesis, while at pH 11.5 to 12 formation of H2S did not occur. Strain Z-7935T is an obligate alkaliphile which does not grow at pH 7; carbonate ions are required for growth. When Na2CO3-NaHCO3 was replaced by NaCl and the pH values were maintained with 50 mM serine buffer (pK, 9.4), no growth occurred. Dependence on sodium carbonate ions is not detected. Strain Z-7935T is a weak halophile that is obligately dependent on sodium ions. There was no growth or sulfidogenesis without sodium ions. Maximal sulfidogenesis was observed in the presence of 3% (wt/vol) NaCl, and at higher NaCl concentrations sulfidogenesis decreased. Strain Z-7935T is a weak halophile that is obligately dependent on sodium ions. Dependence on chloride ions was not detected. Strain Z-7935T is a mesophilic. The optimal temperature for growth was 37°C, while at 40°C growth stopped after a fast start. At 45 and 15°C there was no growth, and at 22 or 26°C growth began after a lag phase. Desulfoviridin, a pigment common to Desulfovibrio species, was not found in strain Z-7935T.

Lipid analysis. The fatty acid distribution in the cells of strain Z-7935T is shown in Table 1. Saturated fatty acids accounted for 66.7% of the total fatty acids, with C16:0 and C18:0 acids predominating. Branched saturated fatty acids accounted for only 11% of the total fatty acids. The following monounsaturated fatty acids, which accounted for 12% of the total fatty acids, were also present: C16:1, C18:1 ω-9, C18:1 ω-11, and C16:1 ω-7 fatty acids. The absence in strain Z-7935T of isoheptadecenoic acid (iso C17:1) and the presence of cis-vaccenic acid (C18:1 ω-7) are notable.

DNA base composition. The G+C content of the DNA of strain Z-7935T is 48.6 mol%.
Phylogenetic analysis. An almost complete 16S rDNA sequence comprising 1,503 nucleotides was determined for strain Z-7935T. A comparative analysis of this 16S rDNA sequence data are available. Strain Z-7935T was determined and included in the phylogenetic analysis. The phylogenetic dendrogram in Fig. 4 shows the position of strain Z-7935T within the radiation of the members of the delta subclass of the Proteobacteria for which 16S rDNA sequence data are available. Strain Z-7935T represents a distinct lineage, clustering with Desulfohalobium rebaense and strain TD3 (13). The highest levels of 16S rDNA sequence similarity were the levels of similarity between strain Z-7935T and Desulfohalobium rebaense (88.7%) and between strains Z-7935T and TD3 (86.6%) (Table 2).

DISCUSSION

The role of hydrogen-consuming, sulfate-reducing bacteria in the alkaliphilic community investigated in this study is interesting from an ecological and physiological viewpoint. The type of nutrition displayed by strain Z-7935T is typical of lithoheterotrophic, sulfate-reducing bacteria that utilize hydrogen or formate for the reduction of oxidized sulfur compounds in the catabolic reaction and acetate in the anabolic pathway (16). Strain Z-7935T differs from other sulfate-reducing bacteria by its unusually narrow range of electron donors utilized. Strain Z-7935T is a lithoheterotrophic, sodium-dependent alkaliphile which may be responsible for the consumption of hydrogen in the alkaliphilic community from which it was isolated. This organism is clearly a member of an alkaliphilic anaerobic community in which primary anaerobes (e.g., alkaliphilic spirochetes [27, 28]) produce hydrogen and acetate.

The geographical distribution of alkaliphilic sulfate reducers in continental soda lakes was revealed by the study of anaerobic communities in the lakes of Central Asia. Physiologically similar pure cultures were isolated from four alkaline lakes in Tuva, and these cultures were found to belong to the same species and to have DNA-DNA homology values of more than 90% (7). It was demonstrated that in Asian lakes, which represent a range of pH and salinity combinations and harbor different microbial communities, as in “natural elective cul-

![phylogenetic dendrogram showing the position of alkaliphilic, sulfate-reducing bacterial strain Z-7935T within the radiation of sulfate-reducing bacteria belonging to the delta subclass of the Proteobacteria. The position of the root was determined by including Escherichia coli as an outgroup organism. Scale bar = 5 inferred substitutions per 100 nucleotides.](image)
n-Alkanes (C6-C16)
Fatty acids (C14-C18)
Fermentation of pyruvate
Utilization of electron donors (with SO₄²⁻)
H₂ + CO₂
H₂ + acetate
Formate + acetate
Lactate
Pyruvate
Malate
Fumarate
Acetate
Propionate
Ethanol
Glycerol
Serine
Fatty acids (C₁₄-C₁₈)
Fatty acids (C₆-C₁₈)
Fermentation of pyruvate (no SO₄²⁻)
Utilization of electron acceptors
Sulfate
Sulfite
Thiosulfate
Sulfur

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<th>Desulfonatronovibrio hydrogenovorans Z-7935ᵀ</th>
<th>Desulfovibrio halophilus SL 8003ᵃ</th>
<th>Desulfovibrio salexigenesᵇ</th>
<th>Desulfohalobium rethbaense HR100ᶜ</th>
<th>Strain TD3ᵈ</th>
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<td>Vibrio</td>
<td>Vibrio</td>
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<td>+</td>
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<td>NR⁻</td>
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<td>Cell size (µm)</td>
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<td>0.7-0.9 × 1.0-3.0</td>
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<td>pH range (optimal pH)</td>
<td>8.0-10.2 (9.5-9.7)</td>
<td>5.5-8.5 (6.5)</td>
<td>NR (7.0)</td>
<td>5.5-8.0 (6.5-7.0)</td>
<td>NR (6.8)</td>
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<td>Salinity range (optimal salinity)</td>
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<td>3.0-18.0 (6.0-7.0)</td>
<td>0.5-12.0 (2.0-4.0)</td>
<td>1.0-24.0 (10.0)</td>
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<td>55-65</td>
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<td>NR</td>
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ᵃ Data from reference 3.
ᵇ Data from references 11 and 23.
ᶜ Data from reference 8.
ᵈ Data from reference 13.
⁻ One or two flagella are present.
⁻⁻ NR, not reported.
⁻⁻⁻ ND, not determined.
⁻⁻⁻⁻ +/−, substrates are used as donors, but growth is slow.

Hydrogen-consum ing sulfate-reducing bacteria are dominant at levels of salinity up to 15% (26). Organisms such as strain Z-7935ᵀ represent the putative alkaliphilic sulfate reducers that may be responsible for the formation of alkaline conditions up to pH 10. The complete breakdown of organic matter by anaerobes requires the removal of hydrogen; this is achieved by conversion of the hydrogen to H₂S. The H₂S in turn is oxidized anaerobically by purple bacteria (e.g., in the alkaliphilic community in Lake Magadi by Ectothiorhodospira species) or aerobically by alkaliphilic thionic bacteria, as in Asian Lake Khadyn (15). In accordance with the assumption that athalassic soda lakes may harbor novel terrestrial communities (25), the new alkaliphilic isolate represents a distinct phylogenetic lineage whose closest relative is the moderately halophilic organism Desulfohalobium rethbaense. A comparison of the characteristics of strain Z-7935ᵀ with those of halophilic, sulfate-reducing bacteria is shown in Table 3. Strain Z-7935ᵀ clearly represents a new taxon of sulfate-reducing bacteria, since it has a unique combination of characteristics and grows in a habitat from which pure cultures of sulfate-reducing bacteria have not been isolated. Morphologically, strain Z-7935ᵀ resembles typical desulfovibrios. This typical morphology is found in species of the genera Desulfovibrio, Desulfofobacter, and Desulfohalobacter (23). Desulfoviridin is not present in strain Z-7935ᵀ, and the G+C content of this organism is 48.6 mol%, which is similar to the values reported for Desulfovibrio salexigenes and Desulfovibrio simplex, which are phenotypically and phylogenetically distinct from strain Z-7935ᵀ. The halophilic species Desulfofobacter hydrogenophilus can utilize H₂ in addition to acetate (22). The halophilic organism Desulfohalobium rethbaense (8) could be considered a halassic counterpart of alkaliphilic strain Z-7935ᵀ. The fatty acid profile of strain Z-7935ᵀ contains compounds that are typical of the membranes of members of the bacterial domain and are associated with sulfate-reducing bacterial species (8, 19). The lipid profile of sulfate-reducing bacteria is quite characteristic, and the profiles of strain Z-7935ᵀ and 72 sulfate-reducing bacteria, including previously studied Desulfovibrio species (19), were compared. The lipid profile of strain Z-7935ᵀ lacks iso-C₁₇:₁ fatty acids, which are biomarkers of species of the genus Desulfo-
vibrio. On the basis of its fatty acid profile, strain Z-7935<sup>T</sup> cannot be affiliated with any species except Desulfohalobium retbaense and Desulfomicrobium baculatus [sic] (correlation coefficient, 0.23). On the basis of the phylogenetic analysis it can be concluded that strain Z-7935<sup>T</sup> is a member of a recently discovered new lineage within the delta branch of the Proteobacteria that comprises hydrocarbon-utilizing strain TD3 (13) and Desulfohalobium retbaense (Fig. 4 and Table 2). With its distinct branch point and 165 rDNA sequence divergence of >11% compared with its nearest relative, as well as its distinct phenotypic characteristics (Table 3), strain Z-7935<sup>T</sup> clearly represents a new genus. We propose that the alkaliphilic, sulfate-reducing strain Z-7935<sup>T</sup> should be placed in a new genus and species, Desulfonatronovibrio hydrogenovorans; the name of this organism describes its ecophysiology.

**Description of Desulfonatronovibrio gen. nov. Desulfonatronovibrio** (De.sul.f.o.na.to.ni.o.vi.’b.rio. M. L. pref. de-, negative; M.L.n. sulfo-, sulfite; M.L.n. natron, soda; M.L.n. vibrio, curved rod; M.L.n. Desulfonatronovibrio, sulfate-reducing curved rod from a soda environment). Alkaliphilic sulfate-reducing eubacterium. Cells are motile, asporogenous, gram-negative vibrios with polar flagella. Lithoheterotrophic, utilizing hydrogen for the reduction of sulfur compounds. Strictly anaerobic. Obligately dependent on sodium ions. Vitamins and acetate as a carbon source are necessary for growth on hydrogen or formate and can be replaced by yeast extract. Phylogenetically a member of the delta subclass of the Proteobacteria. The type species is *Desulfonatronovibrio hydrogenovorans*.

**Description of Desulfonatronovibrio hydrogenovorans sp. nov.** *Desulfonatronovibrio hydrogenovorans* (hy.dro.ge.no.vo’rans. M.L.n. hydrogen, hydrogen; M.L. part. vorans, utilizing; M.L. part. hydrogenovorans, hydrogen utilizing). Motile vibrio with one polar flagellum. Cells are 0.5 by 1.5 to 2 µm, occur singly or in pairs, and develop short spirilla under suboptimal conditions. Multiplication is by binary fission. Gram-negative cell wall structure. Strictly anaerobic and lithoheterotrophic. Utilizes only hydrogen and formate as electron donors and sulfate, sulfite, and thiosulfate as electron acceptors. Sulfur is not reduced. Sulfide is the only product of catabolism. Yeast extract and acetate are utilized for anabolism. Obligate alkaliphile which does not grow at pH 7; the maximum pH for growth is about 10.2, and the optimal pH for growth and sulfidogenesis in sodium carbonate medium is 9.5 to 9.7. Sodium ions are required for growth; no growth occurs in the presence of NaCl concentrations less than 1% (wt/vol) or more than 12% (wt/vol). Growth occurs if NaCl is replaced by equimolar (2 mmoles) of Na<sub>2</sub>CO<sub>3</sub> and NaHCO<sub>3</sub>. Requires carbonate anion. The optimum temperature for growth is 37°C, and the temperature range for growth is 15 to 43°C. Slow growth occurs at 22 to 26°C after a long lag phase. The G+C content of the DNA is 48.6 mol% (as determined by the thermal denaturation method). Habitat: bottom deposits of alkaline athalassic soda lakes. The type strain is strain Z-7935, which was isolated from the sediments of an equatorial soda lake, Lake Magadi. This strain has been deposited in the Deutsche Sammlung von Mikroorganismen as strain DSM 9292<sup>T</sup>.

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**References**