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EXPERIMENTAL ARTICLES

Halomonas campisalis, an Obligatorily Alkaliphilic, Nitrous Oxide–Reducing Denitrifier with a Molybdenum Cofactor–Lacking Nitrate Reductase

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Abstract—We isolated eight strains of denitrifying bacteria that reduce nitrate and nitrous oxide at pH 10 from Lake Magadi (Kenya). Despite certain differences between the strains, they are similar in terms of G+C content (66.1–68.1 mol %) and DNA–DNA homology (75–92%) and represent different morphotypes of the same species. On the basis of results of partial 16S rRNA sequencing, strain Z-7398-2 was found to be most closely related to the *Halomonas campisalis* isolate from the Alkali Lake (USA). The DNA–DNA homology level between the tested strain and the type strain of *H. campisalis* 4A was 88%. These two strains were also similar phenotypically. However, the culture isolated by us was characterized by peculiar properties, such as obligate alkaliphily, which manifested itself in the culture dependence on carbonates and lack of growth at pH values below 7, a nitrous oxide–reducing capacity, and an unusual nitrate reductase that lacked molybdenum and a molybdenum cofactor.

Key words: soda lakes, alkaliphily, denitrification, nitrous oxide, nitrate reductase.

Denitrification is carried out by a number of taxonomically diverse facultatively anaerobic microorganisms [1]. Along with nitrate-dependent respiration, denitrification is widely considered an adaptive response of aerobic bacteria to anaerobic conditions. Denitrification has been detected in representatives of a large number of taxonomic groups [1–3]. However, the first alkaliphilic denitrifiers have been isolated relatively recently [4–8], and only scanty data have been obtained concerning the mechanism of the denitrification process at extremely high pH values.

Studies on the microbial communities of soda-containing lakes have revealed that they are rich in nitrate, which is chiefly due to the activities of numerous nitrogen-fixing cyanobacteria and to the combined effects of first-phase [9] and second-phase [10] nitrifiers. Prerequisite for a nitrogen cycle to be completed is the presence of nitrate-reducing microorganisms in the system. It was assumed that this stage can be accomplished by hydrogenotrophic denitrifiers of the *Paracoccus* type, but this assumption was not supported by the isolation of any pure cultures that could carry out the process involved [11]. Recent research has demonstrated that nitrate reduction in soda lakes can be performed by the sulfur-oxidizing bacteria *Thioalkalivibrio denitrificans* [8] and the representatives of the genus *Halomonas*, e.g., *H. magadiensis* [7]. In addition to the earlier studies on the functional and phylogenetic diversity of bacteria in alkaliphilic microbial communities [12], T.N. Zhilina isolated pure cultures of eight strains of denitrifiers from the soda-depositing Lake Magadi (Kenya) in 1998. The denitrifiers were capable of reducing nitrates at pH 10.

The research team headed by N.P. L'vov (Bach Institute of Biochemistry, Russian Academy of Sciences) established that the nitrate reductases of the above strains lack molybdenum and molybdenum cofactor, suggesting that these bacteria possess peculiar enzymes [13]. It was the peculiar nitrate reductase of alkaliphilic denitrifiers that encouraged us to identify and characterize these organisms before conducting detailed enzymological studies.

MATERIALS AND METHODS

Strains and sources. We used strains that were initially isolated from ground sediment samples (with dead purple bacteria on their surface) collected by G.A. Zavarzin in September 1998 at a depth of 30 cm in a lagoon north of the dam built across Lake Magadi. The water temperature in the lagoon was 38°C (mineralization level 260 g/l, pH 10.5). The type strain *Halomonas campisalis* 4A was a generous gift of B.M. Peyton (Washington State University, USA).

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Media and cultivation conditions. Enrichment cultures were obtained under strictly anaerobic conditions on a mineral medium containing (g/l) Na_2CO_3, 90; $NaHCO_3$, 10; NaCl, 50; KH_2PO_4, 0.2; $MgCl_2 \cdot 6H_2O$, 0.05; $Na_2S \cdot 9H_2O$, 0.2; yeast extract, 0.1; and trace element solution [14], 1 ml (pH 10.2). Sodium acetate trihydrate (2 g/l) was used as the energy source, and sodium nitrate (1 g/l) or nitrous oxide (at a pressure of 0.5 atm; the gas phase volume was 60% of the cultivation vessel volume) served as electron acceptors. Oxygen was removed from the medium by evacuation of air with subsequent passing of argon through the medium.

In order to determine the substrate spectrum utilized anaerobically, substrates were added at a concentration of 2 g/l in the presence of 0.1 g/l yeast extract and 1 g/l sodium nitrate. To find out whether the cultures can grow under aerobic conditions, we replaced sodium nitrate with ammonium chloride (0.5 g/l), thereby preventing nitrate reduction upon depletion of the available oxygen quantity. Sugars in the form of concentrated aqueous solutions were added to a sterile alkaline medium immediately before inoculation in order to avoid medium caramelization.

The composition of the optimized cultivation medium was as follows (g/l): Na_2CO_3, 30; $NaHCO_3$, 50; and NaCl, 0 (pH 9.25). No sodium sulfide was added because the culture was very sensitive to it. No changes were made in the other medium components.

Acceptors were added in the form of concentrated solutions to attain the following concentrations (mM): $NaNO_3$, 10; $NaNO_2$, 2; fumarate, 10; Na_2SO_4, 10; Na_2SO_3, 2 and 10; $Na_2S_2O_3$, 10; S^0, 2% (w/v); and N_2O at a pressure of 0.5 atm. The utilization of sulfur- and nitrogen-containing acceptors was determined from sulfide formation and gaseous nitrogen evolution, respectively.

In order to determine the dependence of growth on medium pH, we adjusted the pH value with 6 M HCl and 12 M NaOH. In these experiments, sodium carbonate was replaced with sodium bicarbonate (at a concentration decreased 10-fold); the optimum sodium molarity was maintained by the addition of sodium chloride. The carbonate dependence of growth was investigated in a system that contained, instead of sodium carbonate, an equimolar amount of NaCl. Serine buffer (50 mM; pH 9) was used to stabilize the pH value of this system. The chloride requirement of the culture was tested by replacing NaCl with an equimolar (in terms of the sodium amount contained) quantity of carbonate and bicarbonate at a ratio that corresponded to the optimum pH value. Magnesium chloride and the chlorides contained in the trace element solution were substituted by sulfates. To assess the Na^+ requirement, all sodium salts were replaced with potassium salts. To determine the dependence of growth rate on NaCl concentration, we decreased the total carbonate content of the medium 10-fold; NaCl at a concentration of 0–30% (w/v) was added to each test tube separately. The temperature dependence was determined at the optimum pH values and mineralization levels. The thermal resistance of a culture was determined by incubating the culture (at the stationary growth phase) for 10 and 50 min at 70°C with subsequent inoculation into optimized liquid media.

Analytical procedures. Growth rate was determined from the optical density of a cell suspension at 600 nm with a Specol (Jena) spectrophotometer either directly in Hungate tubes or in cuvettes with an optical path length of 10 mm. Acetate was quantitatively determined on a model 3700 chromatograph (Russia) with a flame-ionization detector. Nitrogen, oxygen, and hydrogen were quantitatively determined using an LKhM-80 gas chromatograph with a katharometer. Separation of the substances was performed on a molecular sieve 5Å-filled column at room temperature. The nitrous oxide quantity was measured with a model 3700 chromatograph equipped with a katharometer on a Chromosorb 101-filled column (the column temperature was 50°C). Nitrite was determined spectrophotometrically at a wavelength of 548 nm from the formation of a colored azocomplex with sulfanilic acid and 1-*N*-naphthylethylenediamine. Sulfide was determined by monitoring the methylene blue formation process [15]. The thiosulfate content in the medium was controlled by iodometric titration. Total cell protein was determined by the method of Lowry *et al.* The catalase and oxidase tests were carried out by the standard methods using 3% H_2O_2 and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine · 2HCl, respectively. The molybdenum cofactor was detected using the *nit-1* mutant of the fungus *Neurospora crassa* that contained a molybdenum cofactor–deficient nitrate reductase. Complexation of the molybdenum cofactor with the nitrate reductase apoenzyme of the *nit-1* mutant resulted in the assemblage of a functionally active enzyme [16].

DNA assay. DNA isolation and purification was performed as described earlier [17]. The G+C content was determined from thermal denaturation curves using a Pye Unicum SP 1800 spectrophotometer and the formula G+C (mol %) = $T_{melting}$ – 106.4. The DNA homology level was determined by the method described in [18].

16S rRNA gene determination and sequencing was carried out as described earlier [17].

RESULTS AND DISCUSSION

Pure culture isolation. Enrichment cultures of denitrifying bacteria were obtained on a mineral-rich alkaline medium with acetate and nitrate at pH 10.2 and 37°C. On the sixth day of cultivation, the culture was dominated by (i) large (up to 1 μm in size) motile cocci, including solitary cells, cell pairs, or cluster-shaped aggregates that were embedded in mucilage and (ii) motile rods (up to 1 μm in diameter) that represented solitary cells, cell pairs, or short chains. Con-

comitant microflora consisted of motile spirilla and nonmotile short thin rods. Pure cultures were isolated under strictly anaerobic conditions by diluting the enrichment cultures 10-fold and thereupon inoculating them on a medium containing 3% agar; a suitable sodium molarity was maintained by adding NaCl. The isolates were somewhat different in shape, colony color and size, cell morphology, and adhesion degree. When growing aerobically in a liquid medium, some of the strains formed a surface film. Under anaerobic conditions, most strains formed nitrous oxide as an intermediate denitrification product (Table 1). Nevertheless, the strains were similar in terms of their G+C content (66.1–68.1 mol %, Table 1) and DNA–DNA homology degree (75–92%), and this indicates that these morphotypes belong to the same species.

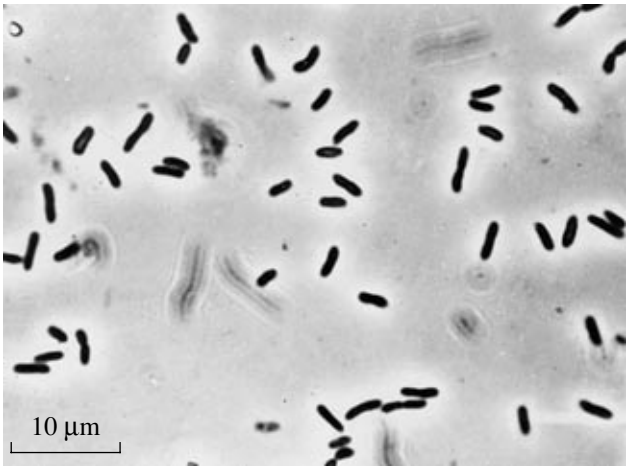

Fig. 1. Exponential-phase cells under a phase-contrast light microscope.

Nitrate reductase. In a culture grown on a medium with 1 mM Na_2WO_4, the denitrification process was not repressed. As a molybdenum analog, tungsten causes the expression of the gene encoding nitrate reductase, the key enzyme of denitrification. However, the resulting enzyme is inactive because tungsten is incorporated into the active center instead of molybdenum. Because denitrification is not inhibited in a culture grown in a tungsten-containing medium, we can suggest that either the cells of the tested strains are tungsten-resistant or they synthesize an alternative nitrate reductase that does not contain a molybdenum cofactor. Taking into account the negative results of the test using the

Table 1. Comparative characterization of the strains of denitrifying bacteria isolated from Lake Magadi

Strain	G+C, mol %	Homology with Z-7498-2	Colony properties (on the third day of anaerobic cultivation)	Cell morphology	N_2O evolution under anaerobic conditions	Film formation under aerobic conditions
Z-7198	66.5	91	Colonies are transparent, light pink, iridescent, with smooth edges, 1 mm in diameter; submerged colonies are lens-shaped	Short thick rods with pronounced adhesive properties; 1–1.2 × 1.5–3 μm in size. Cells solitary or arranged in pairs or chains ("strings of beads"). Not all cells are motile	+	–
Z-7198-2-1	66.2	81		Short oval rods, very motile ("swarming"), 0.8–1 × 1.5–3 μm in size; weakly adhesive; lateral sides of cells occasionally join together	+	–
Z-7398-2	66.4	92	Colonies are transparent, light pink in color, iridescent, 1–2 mm in diameter; submerged colonies are angular in shape; surface colonies have uneven edges	Cocci or short rods, pear-shaped, 0.8–1 × 1–3 μm in size. Not all cells are motile	+	+
Z-7398-2-3	66.6	80			–	–
Z-7498-1-1	68.1	75	Colonies are yellowish and dotlike, with uneven edges and a dense center	Short motile rods, 1.1–1.2 × 2–3 μm in size, slightly curved, with a transparent zone at the end or in the middle of the cell; weakly adhesive	+	+
Z-7498-2	66.1	100	Colonies are transparent, light pink, iridescent, with even edges, 1–2 mm in diameter	Short thick rods (solitary cells or cell pairs), or, occasionally, cocci; 1–1.2 × 1–1.5 μm; cells with pronounced adhesive properties	+	+
Z-7498-2-1	67.3	90			+	+
Z-7498-5	66.7	89	Colonies are dark coral with even edges, 1 mm in diameter; submerged colonies are lens-shaped	Oval thick rods, 0.8–1 × 1.2–2 μm. Not all cells are motile	+	–

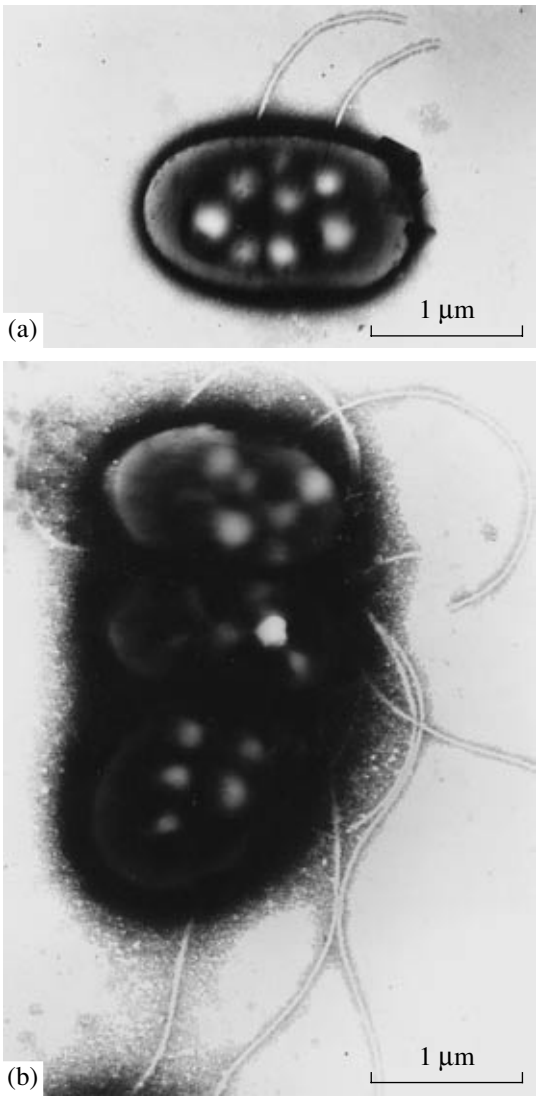

Fig. 2. Cells negatively stained with phosphotungstic acid under an electron microscope. (a) The attachment site of two lateral flagella can be seen. (b) An aggregate consisting of several cells. It is evident that flagella are rather long provided that they have not been damaged during the preparation procedure.

nit-1 mutant of *Neurospora crassa*, we conclude that the second suggestion is valid. Further research aimed at identifying and investigating the nitrate reductase system was done with strain Z-7398-2. It was one of the first strains in which a peculiar molybdenum-lacking nitrate reductase was detected; this enzyme was subsequently found in other strains as well [13].

Denitrification system. While reducing nitrate, strain Z-7398-2 accumulates nitrite as an intermediate denitrification product. However, it was completely consumed at the subsequent stages of the process that yield gaseous products (nitrogen and an insignificant quantity of N_2O). Hence, the organism carries out a complete denitrification process. Nitrite accumulation points to a lower activity of nitrite reductase, the second enzyme of the nitrate reduction pathway, compared to nitrate reductase. This is consistent with biochemical data (Antipov and Boltyanskaya, unpublished).

Morphology. The cells of strain Z-7398-2 are characterized by different morphological patterns depending on the culture age. At the late developmental stages, short chains consisting of several cells or cell aggregates (Fig. 2b) and long curved filaments may form. A mucilaginous capsule surrounds the cells, and, therefore, cells in aggregates are located at a certain distance from one another. The rounded inclusions detectable in these cells probably consist of poly-β-hydroxybutyric acid, because we observed the characteristic luminescence under the microscope. The morphology of the cells during the exponential phase shown in Fig. 1 and Table 1. The cells divide evenly by constriction. The cells are motile, because they have two lateral flagella located on the same side of the cell (Fig. 2a). The flagella are long (Fig. 2b) and are many times longer than the cells themselves. However, these flagella frequently detach during the preparation procedures. A loss of motility can also result from adhesion and, therefore, both highly motile and nonmotile forms can be observed.

To determine catalase and oxidase activities, we obtained colonies under aerobic conditions on a medium with 3% agar. On the third day of cultivation, strain Z-7398-2 forms rounded cream-colored colonies with smooth edges and a homogeneous structure. The colony surface is smooth, glossy, and opaque. The organism is catalase- and oxidase-positive. A lack of spore formation was demonstrated visually and confirmed by the negative result of a test for thermal stability resistance. Gram staining and a test using 3% KOH yielded results that are typical of gram-negative bacteria.

Growth characteristics. Strain Z-7398-2 grows at a medium mineralization level of 0.16–3.1 M Na^+ with a sharp optimum at 1.0 M (Fig. 3a). No growth was detected at 3.5 M Na^+. Therefore, the organism can be considered a moderate halophile. The organism develops within the alkaline pH range (between 7.5 and 10.4) with an optimum of 8.8–9.5. No growth occurred at pH 7 and at pH values above 10.5 (Fig. 3b). At the optimum pH values and mineralization levels, the culture grows within a wide temperature range (from 10 to 55°C) with an optimum of 36–40°C (Fig. 3c). No growth was detected at 7 and 58°C.

Replacing carbonate and bicarbonate with NaCl (added at an Na^+-equimolar concentration) results in a lack of growth. The culture does not grow if potassium salts are substituted for sodium salts. This phenomenon is characteristic of alkaliphilic microorganisms. The growth of the organism after replacement of NaCl with equimolar amounts of $Na_2CO_3 + NaHCO_3$ is consistent with the idea that it does not require chloride.

Although the culture was isolated under strictly anaerobic conditions, the strain, like an overwhelming

majority of other denitrifiers, is also capable of growing aerobically and oxidizing a large number of organic substances. As for anaerobic conditions, we tested nitrate, nitrite, nitrous oxide, and fumarate as electron acceptors. We also attempted to use each of them in combination with acetate, formate, hydrogen, sulfide, and a polysulfide mixture. In the studies using hydrogen and sulfur compounds as electron donors, acetate served as a substrate. Of all tested donor–acceptor pairs, the organism could only grow on acetate with nitrate or nitrous oxide. The growth on N_2O without other nitrogen sources was stable after three consecutive culture transfers. No growth occurred with nitrite, but the organism is capable of reducing it, because the nitrite forming at the first denitrification stage is completely consumed subsequently. In addition, no growth on acetate was detected after elemental sulfur and sulfur compounds (sulfate, sulfite, and thiosulfate) were introduced as electron acceptors.

Apart from its inability to use sulfide, strain Z-7398-2 was also characterized by a low tolerance to sulfur in the medium, which is typical of denitrifiers. An initial Na_2S concentration of 3 mM caused partial (13%) growth inhibition, and no growth occurred at 6 mM Na_2S. This high sensitivity of the culture to sulfide prevents the development of the organism in the anaerobic zone, which is characterized by active sulfidogenesis. These findings confirm the suggestion that the capacity for dissimilatory nitrate reduction is an adaptation of aerobic organisms to anaerobic conditions.

The culture did not carry out anaerobic fermentation processes in the absence of nitrate; however, in the presence of oxygen or nitrate, it utilized a wide variety of substrates (Table 2).

Phylogeny. An analysis of the incomplete 16S rRNA gene sequence (500 nucleotides in terms of the *E. coli* nomenclature) revealed that the organism isolated by us is characterized by a high degree of similarity to *Halomonas campisalis* (98.5%). The DNA–DNA homology between strain Z-7398-2 and the type strain *Halomonas campisalis* 4A was 88%. Accordingly, the organism is a new strain of the formerly monotypic *H. campisalis* strain. The 16S rRNA gene sequence of strain Z-7398-2 was deposited with the GenBank collection and denoted as AY 466451. The properties of the two *H. campisalis* strains are compared in Table 2.

Despite their geographically remote areas, strains 4A and Z-7398-2 are similar in not only genotypic but also phenotypic terms. The ability to grow within a wide range of pH, mineralization, and temperature values implies their resistance to seasonal changes in these variables under natural conditions. The organisms cannot ferment, but they oxidize a large number of substrates, including sugars, alcohols, fatty acids, and nitrogen-containing compounds, both under aerobic and anaerobic conditions. The differences between the strains in terms of their substrate spectra are insignificant. In general, such metabolic flexibility is characteristic of the genus *Halomonas*. It enables them to use both the pepto- and saccharolytic pathways of organic substance degradation and provides for their high adaptive potential. Nevertheless, it is this flexibility that does not allow us to place them in a particular niche within the trophic chain of the alkaliphilic microbial community. Due to their capacity to utilize various types of organic compounds, representatives of the genus *Halomonas* are of considerable biotechnological interest, particularly in terms of nitrate-containing alkaline wastewater treatment in industry [19].

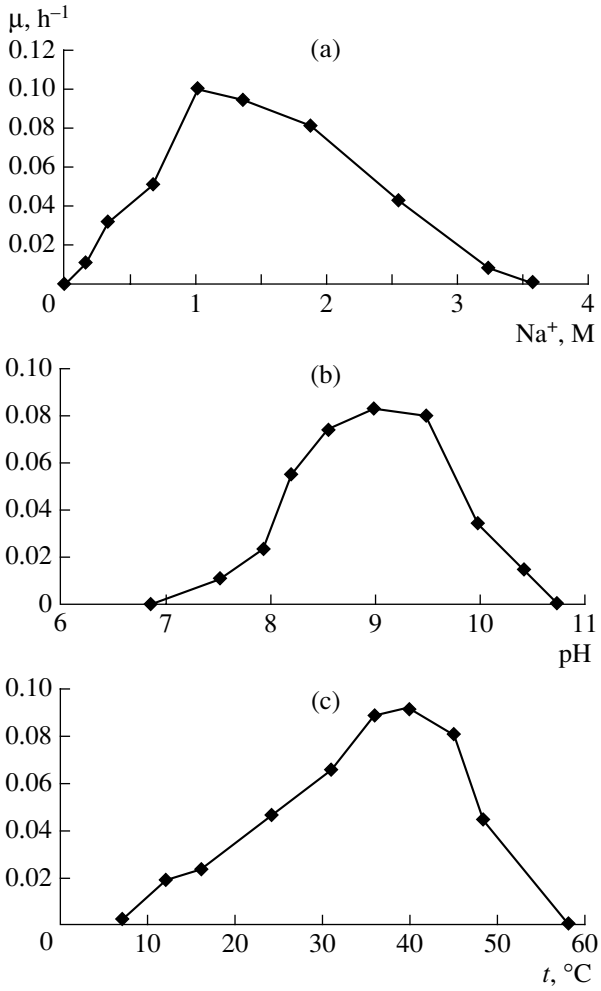

Fig. 3. Dependence of the specific growth rate of strain Z-7398-2 on (a) mineralization level, (b) pH, and (c) temperature.

The strains differed in terms of their behavior towards mineral ions. The type strain (4A) grows quite well on a carbonate-lacking medium, whereas Z-7398-2 is obligatorily carbonate-dependent but does not require chlorides.

The strain isolated by us does not grow at pH values below 7. It should, therefore, be regarded as a true alkaliphile. Since its growth does not depend on chlorides, the organism is in all likelihood of athalassic origin. In

Table 2. Comparative characterization of strains *Halomonas campisalis* Z-7398-2 and 4A

Property	*H. campisalis* Z-7398-2	*H. campisalis* $4A^T$
Morphology	Rods	Rods
Cell size, μm	1 × 1–3	1 × 3–5
Gram staining	–	–
Motility	+	+
Endospores	–	–
Reaction to O_2	Facultatively anaerobic	Facultatively anaerobic
Catalase	+	+
Oxidase	+	+
Na^+ dependence	+	+
Carbonate dependence	+	–
Cl^- dependence	–	No data
Na^+ concentration, M (growth range/optimum)	0.16–3.1/1.0	0.2–4.5/1.5
pH (growth range/optimum)	7.5–10.4/8.8–9.5	6–11/9.5
T, °C (growth range/optimum)	10–55/36–40	4–50/30
Fermentation capacity	–	–
With acetate as electron donor:		
N_2 formation from NO_3^-	+	+
NO_2^- formation from NO_3^-	+	+
growth with nitrite	–	+
nitrite reduction	+	+
growth with N_2O	+	–[2]
Substrates used:		
formate	–	–[2]
acetate	+	+
propionate	+	+[2]
butyrate	+	+[2]
pyruvate	+	+
succinate	+[1]	+[2]
oxalate	–	–[2]
lactate	+	+
fumarate	+	+[2]
citrate	+	+[2]
benzoate	No data	–
glycolate	+[1]	+[2]
betaine	+[1]	–[2]
methanol	–	–
ethanol	+	+
peptone	+	No data
casamino acids	+	No data
yeast extract	+	+
D-ribose	+	–
D-xylose	–	–
D-arabinose	–	–
D-fructose	+	+
D-sorbose	–	–

Table 2. (Contd.)

Property	*H. campisalis* Z-7398-2	*H. campisalis* 4A^T
D-glucose	+	+
D-mannose	+/–	–
D-galactose	–	–
fucose	–	No data
glycerol	–	+
mannitol	–	No data
L-inositol	–	No data
L-sorbitol	–	No data
sucrose	+	+
D-maltose	+	+
D-lactose	–	–
trehalose	+	+
N-acetyl-D-glucosamine	–	+
alanine	+	+[2]
arginine	+	–[2]
aspartate	+	+[2]
histidine	+	+[2]
glutamate	+	+[2]
proline	+	No data
Tween 80	+	No data
G+C, mol %	66.4	66
Isolation site	Lake Magadi, Kenya	Alkali Lake, USA

Note: The symbol "+/–" signifies that the property manifests itself to an insignificant extent.

[1] The substrate is used only under aerobic conditions.

[2] The data on substrate utilization by *H. campisalis* 4A were obtained by us.

contrast, strain 4A is more likely to belong to the halophiles that secondarily adapted to extremely high pH values. The fact that strain 4A does not need carbonates can be due to the low carbonate content of the Alkali Lake [20] from which the strain was isolated [6]. Both strains depend on Na^+, which is a common property of alkalo- and halophiles.

Under anaerobic conditions, strain 4A grows on both nitrate and nitrite but not N_2O, which distinguishes it from the culture isolated by us. The dissimilatory nitrous oxide reduction capacity of strain Z-7398-2 and the formation of nitrous oxide as an intermediate denitrification product is of considerable ecological importance. The strains isolated from Lake Magadi can serve as both sources and sinks of this greenhouse gas in intracontinental soda-containing lakes.

Another interesting point about the new *H. campisalis* strains is the fact that they possess peculiar nitrate reductases. They do not contain molybdenum and molybdenum cofactors. For this reason, these organisms are of particular enzymological and, more generally, biochemical interest.

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