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EXPERIMENTAL ARTICLES

The New Facultatively Chemolithoautotrophic, Moderately Halophilic, Sulfate-Reducing Bacterium *Desulfovermiculus halophilus* gen. nov., sp. nov., Isolated from an Oil Field

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Abstract—The new mesophilic, chemolithoautotrophic, moderately halophilic, sulfate-reducing bacterium strain 11-6, could grow at a NaCl concentration in the medium of 30–230 g/l, with an optimum at 80–100 g/l. Cells were vibrios motile at the early stages of growth. Lactate, pyruvate, malate, fumarate, succinate, propionate, butyrate, crotonate, ethanol, alanine, formate, and H_2/CO_2 were used in sulfate reduction. Butyrate was degraded completely, without acetate accumulation. In butyrate-grown cells, a high activity of CO dehydrogenase was detected. Additional growth factors were not required. Autotrophic growth occurred, in the presence of sulfate, on H_2/CO_2 or formate without other electron donors. Fermentation of pyruvate and fumarate was possible in the absence of sulfate. Apart from sulfate, sulfite, thiosulfate, and elemental sulfur were able to serve as electron acceptors. The optimal growth temperature was 37°C; the optimum pH was 7.2. Desulfoviridin was not detected. Menaquinone MK-7 was present. The DNA G+C content was 55.2 mol %. Phylogenetically, the bacterium represented a separate branch within the cluster formed by representatives of the family *Desulfohalobiaceae* in the class *Deltaproteobacteria.* The bacterium was assigned to a new genus and species, *Desulfovermiculus halophilus* gen. nov., sp. nov. The type strain is 11-6^T (= VKM B-2364), isolated from the highly mineralized formation water of an oil field.

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Key words: a new moderately halophilic sulfate-reducing bacterium, hypersaline environments, complete oxidation of butyrate, CO dehydrogenase activity, chemolithoautotrophy.

In connection with the efforts undertaken to understand the reasons for acetate accumulation in saline ecosystems, including stratal brines of oil fields, a question arose as to the existence of sulfate-reducing bacteria (SRB) that can completely oxidize organic compounds under these conditions [1]. In waters with a mineralization of 150 g/l and above, numerous fermentative bacteria are known to thrive that decompose complex organic compounds with the formation of lower fatty acids, alcohols, H_2, and CO_2. The microorganisms known to be responsible for the terminal stage of organic matter decomposition in these habitats are, on the contrary, not numerous. They include methanogens utilizing one-carbon compounds and sulfate-reducing bacteria that bring about incomplete oxidation of organic compounds with the formation of acetate or acetate and propionate [1]. Some of these SRB can use H_2/CO_2 in the sulfate reduction process under chemolithoheterotrophic conditions. Among SRB that can develop at a mineralization above 130–150 g/l are *Desulfohalobium retbaense* [2], *Desulfovibrio halophilus* [3], *Desulfotomaculum halophilum* [4], and *Desulfocella halophila* [5]. However, the question remains unanswered as to the existence in hypersaline habitats of SRB that completely oxidize organic compounds and SRB that can develop under chemolithoautotrophic conditions. Theoretically speaking, there are no factors forbidding the existence of such organisms. The energetics of complete oxidation of organic compounds by SRB is more favorable than that of incomplete oxidation [6]. This circumstance seems to favor the development of complete oxidizers at high medium mineralization, since synthesis of osmoprotectants requires additional energy, and, in sulfate-reducing eubacteria, they are apparently involved in the mechanism of adaptation to high salt concentrations (e.g., in cells of *Desulfovibrio halophilus*, glycine betaine and trehalose were found [1]). On the other hand, SRB that bring about incomplete oxidation are characterized by higher growth rates than SRB carrying out complete oxidation.

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The aim of the present work was to study a halophilic SRB isolated from the stratal brine of an oil field that was able to completely oxidize organic compounds and grow under chemolithoautotrophic conditions at a salinity higher than 130–150 g/l. The main objectives of the work were to obtain a pure culture of a moderately halophilic SRB, to study its morphology, growth parameters, and metabolic peculiarities, as well as to establish its taxonomic affiliation.

MATERIALS AND METHODS

Subject of the study. Strain 11-6 was isolated from the stratal brine of an oil field where oil is deposited in an anticlinal fold of tertiary terrigenous sediments and is underlain by stratal brine. A mixture of stratal water and washing liquids is injected into the stratum. The stratal brine extracted from the production well is of the calcium chloride type and contains 217 g/l of salts; the content of sulfates is more than 100 mg/l; the contents of calcium and magnesium are 14.0 and 1.10 g/l, respectively; the pH value is 6.2.

Isolation and cultivation. The mineral base of the medium used for the isolation of the halophilic sulfate-reducing bacterium was Widdel marine medium no. 2 modified with respect to the contents of sodium, calcium, and magnesium and having the following composition (g/l): NaCl, 150; $MgCl_2 \cdot 6H_2O$, 3; $CaCl_2 \cdot 2H_2O$, 10; KCl, 0.5; NH_4Cl, 0.3; KH_2PO_4, 0.2; Na_2SO_4, 2.8; $NaHCO_3$, 1.5; sodium butyrate, 1. The medium was supplemented with two reducing agents ($Na_2S \cdot 9H_2O$, 0.5 g/l, and $Na_2S_2O_4$, 50 mg/l), a solution of sodium selenite (1×10^{-5} mol/l), and trace elements [7]. No vitamins or yeast extract were added to the medium.

Samples of stratal water were introduced into 18-ml Hungate tubes containing 10 ml of the medium of the above-specified composition; the gas phase contained $H_2 + CO_2$ (80 : 20 vol/vol); pH 6.4. Inoculated tubes were incubated at 37°C. A pure culture was obtained after a large number of repeated transfers of separate colonies grown in Vinial tubes on solid (1.5% agar) medium of the same composition to liquid medium containing 110 g/l NaCl. Absence of methanogens was verified by inoculation of Widdel marine medium no. 2 devoid of sulfate; the gas phase was $H_2 + CO_2$ (80 : 20 vol/vol).

Subsequently, cultivation was performed in Hungate tubes at 37°C in a medium optimized with respect to the concentrations of NaCl (100 g/l), $CaCl_2 \cdot 2H_2O$ (1 g/l), and $MgCl_2 \cdot 6H_2O$ (3 g/l); pH 7.2. The concentrations of $CaCl_2$ and $MgCl_2$ optimal for growth were established on the medium used for the isolation of strain 11-6. When the concentration of $CaCl_2$ was varied, the $MgCl_2$ content was 3 g/l; when the concentration of $MgCl_2$ was varied, the concentration of $CaCl_2$ was 10 g/l. To determine the growth ranges and optima of temperature, pH, and salinity, an optimized medium was used that contained sodium malate; the gas phase was either nitrogen or argon.

To study the utilization of electron donors and carbon sources, organic compounds were added to the medium in an amount of 1 g/l; the exceptions were alcohol (1 ml/l) and calcium palmitate (3 g/l) [7].

To test the capacity for chemolithoautotrophic growth, an optimized mineral medium without an organic carbon source was used; the gas phase was $H_2 + CO_2$ (80 : 20 vol/vol).

Possible electron acceptors were tested on the above-specified medium with sodium malate (1 g/l); however, in this medium, sulfate was replaced with thiosulfate (2.0 g/l), sulfite (0.5 g/l), or elemental sulfur (amorphous form, 1.5 g/l).

Microscopy. Cell morphology was studied under a Jenaval light microscope (Carl Zeiss, Germany) at a magnification of 1000×. Cell ultrastructure was studied as described earlier [8] under a JEM-100C electron microscope (Jeol, Japan).

Analytical methods. Culture growth was estimated from the optical density of the bacterial suspension at 420 nm, which was measured on a Specol 21 spectrophotometer. The sulfide content in the medium was determined colorimetrically, using N,N'-dimethyl-*p*-phenylenediamine (Truper and Schlegel, cited in [9]). The protein content in the bacterial suspension was determined colorimetrically by the Lowry method (Lowry et al., 1951, cited in [10]).

The rate of sulfate reduction was determined in cultures grown at different concentrations of NaCl by radioisotopic method, using $Na_2{}^{35}SO_4$ and a Rack Beta scintillation counter [11].

The menaquinone type was determined by the Collins method [12] after acetone extraction of lipids from cells disrupted ultrasonically. The lipid extract, thickened by evaporation, was subjected to chromatography on Silufol plates in a diethyl ether–hexane (15 : 18, vol/vol) system. A band with $R_f = 0.55$, showing weak absorption in UV light, was eluted with methanol, thickened by evaporation, and analyzed on a Finnigan MAT 8430 mass spectrometer under standard conditions (sample evaporation temperature, 120–130°C; accelerating voltage, 3 kV; ionization voltage, 70 eV).

The activity of CO dehydrogenase was determined in cells grown on a medium with butyrate (1 g/l) and yeast extract (0.5 g/l). Sedimented cells were resuspended in 10 ml of 0.05 M phosphate buffer, pH 7, supplemented with dithionite. Cellular extracts were obtained by ultrasonic treatment of cells in UZD1-0.063/22 at 22 kHz for 6 min with cooling. Disintegrated cell were centrifuged at 10000 *g* for 15 min. The activity of CO dehydrogenase was assayed in supernatant by measuring the rate of benzyl viologen reduction with CO in anaerobic thermostated (37°C) cuvettes at 600 nm on a Specord (Germany) recording spectrophotometer, as described in [13].

The presence in cells of a specific sulfite reductase—desulfoviridin—was determined on a Hitachi fluorescence spectrophotometer using the Postgate method [10].

Quantitative analysis of fatty acids in the culture liquid was performed by gas–liquid chromatography. Methane production was determined by gas chromatography [9].

For genosystematic and molecular-biological studies, strain 11-6 was grown on mineral medium devoid of sulfates and supplemented with sodium fumarate (1 g/l).

Analysis of DNA. Isolation of DNA from bacterial biomass was performed by the Marmur method (cited in [9]). The DNA G+C content was determined from the DNA melting temperature as described in [9], using *Escherichia coli* K-12 DNA as a standard.

Sequencing of the 16S rRNA gene and phylogenetic analysis. Amplification and sequencing of the 16S rRNA gene of strain 11-6 was performed with universal bacterial primers [14]. Sequencing of amplification products was performed by the Sanger method with the use of a Big Dye Terminator v.3.1 kit on an ABI 3730 automatic sequencer (Applied Biosystems, United States) according to the manufacturer's recommendations.

Preliminary analysis of the nucleotide sequence of the 16S rRNA gene was carried out using data and software available at the site of the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu). Editing of sequences was performed using the BioEdit software package (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). The determined sequences were aligned with the most closely related sequences using the CLUSALW v 1.75 software package. Unrooted phylogenetic trees were constructed using the methods implemented in the TREECONW software package (http://bioc-www.uia.ac.be/u/yvdp/treeconw.html).

The nucleotide sequence of the 16S rRNA gene of strain 11-6 was deposited in GenBank under the accession number DQ139408.

RESULTS AND DISCUSSION

Morphology. In a young culture on medium with propionate, the cells of strain 11-6 were vibrios motile by means of one polar flagellum; they occurred singly or in pairs. In the stationary phase, cells were elongated and had a wormlike shape or, occasionally, were spiral-shaped (Figs. 1A, 1B). The width of cells was 0.5–0.6 μm, and their length varied from 1 to 15 μm. Upon prolonged cultivation on medium with lactate, the length of cells reached 20 μm. The microorganism did not form spores. The cells stained Gram negative. In ultrathin sections, one can clearly see the outer membrane, the cytoplasmic membrane, the periplasmic space, the nucleoid zone, and polyribosomes (Fig. 1C).

Growth characteristics. To determine NaCl concentrations in the medium that favor sulfate reduction, experiments with $^{35}S/SO_4^{2-}$ were carried out. Upon 7-day incubation of the culture in the medium with sodium malate, incorporation of the radioactive label into H_2S was observed beginning with 40 g/l NaCl, and the maximum rate was recorded at 80 g/l NaCl. At NaCl concentrations of 140 g/l and higher, the incorporation rates were very low. However, if the sulfate reduction rate was calculated after a 15-day incubation, its values in media with 140, 180, and 220 g/l of NaCl were noticeable (Fig. 2). These results were confirmed in experiments in which we determined the optical density of the cultures and the production of H_2S on a medium with sodium malate at different concentrations of NaCl. The lowest NaCl concentration at which H_2S production was recorded was 30 g/l, the optimal concentration was 80–100 g/l, and the maximum one was 230 g/l (Fig. 3). In an 11-day culture at a NaCl concentration of 80 g/l, 1.72 μmol/l H_2S was formed and the increase in the optical density was 0.101. In a 30-day culture grown at 80 g/l of NaCl, the production of H_2S was 6.78 μmol/l. The addition of yeast extract to the medium with malate increased the H_2S production rate; however, the NaCl dependence pattern remained the same. The results allow strain 11-6 to be assigned to moderately halophilic sulfate-reducing bacteria.

The concentrations of magnesium and calcium salts optimal for sulfate reduction were 1.38 and 0.37–0.75 g/l as calculated for anhydrous $MgCl_2$ and $CaCl_2 \cdot H_2S$ production was also observed at $MgCl_2$ concentrations of 0.13 and 2.30 g/l and $CaCl_2$ concentrations of 0.07 and 7.54 g/l (Fig. 4).

The microorganism grew in a pH range of 6.0–8.5. The largest increase in the optical density was recorded at pH 7.2. These results show that strain 11-6 is a neutrophile.

Strain 11-6 is a mesophile: an increase in the culture optical density and H_2S production were observed at 25–47°C; the optimal temperature was 37°C. Weak sulfate reduction was observed up to 50°C (H_2S production of 0.87 μmol/l without optical density increase).

Metabolism of organic compounds and hydrogen, CO dehydrogenase activity, and electron acceptors. Strain 11-6 proved to be an obligate anaerobe. It did not require vitamins. It grew in media containing sulfate and sodium salts of lactic, pyruvic, malic, fumaric, succinic, crotonic, propionic, or butyric acids or ethanol, alanine, or formate. The organism also grew under autotrophic conditions at the expense of $H_2 + CO_2$. Acetate, valerate, palmitate, glucose, fructose, benzoate, methanol, butanol, propanol, glycerol, serine, glycine, betaine, and yeast extract did not support growth.

Decomposition of alanine (11 mM), propionate (10 mM), malate (5 mM), fumarate (6 mM), and succinate (6 mM) was accompanied by accumulation of acetate. Butyrate (11 mM) was oxidized completely, without acetate accumulation. The metabolism of strain 11-6 on media with propionate and butyrate is considered in detail below.

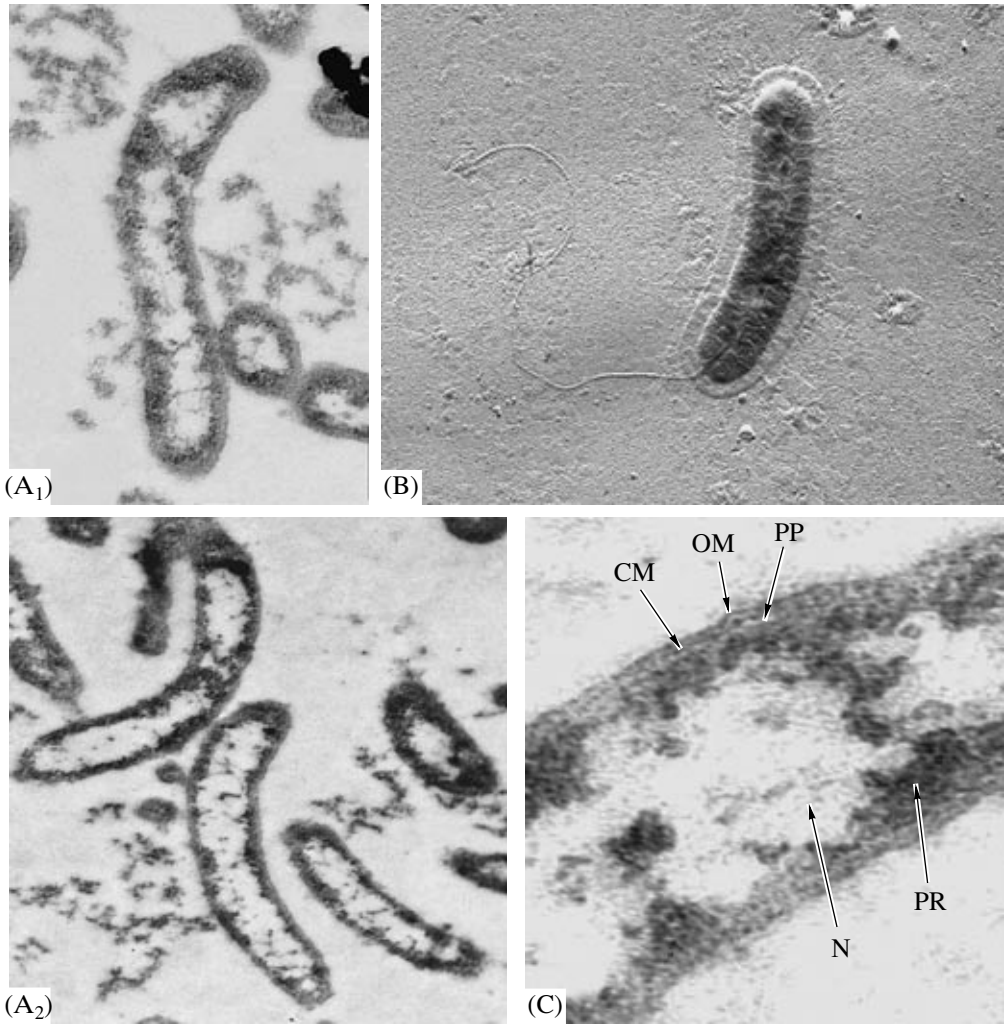

Fig. 1. (B) Flagellated cell of a 15-day culture grown on a medium with malate and 100 g/l of NaCl (whole-cell specimen; magnification, 61308×) and ultrathin sections of cells of the same culture at magnifications of (A_1) 43650×, (A_2) 25146×, and (C) 225000×. OM, outer membrane; CM, cytoplasmic membrane; PP, periplasmic space; N, nucleoid; PR, polyribosomes.

At a propionate content in the medium of 9.86 mmol/l (100 g/l NaCl), the amount of acetate accumulated per unit of oxidized propionate approximated the theoretical value calculated from the equation of incomplete oxidation characteristic of representatives of the genus *Desulfobulbus* [6] (Table 1, variant 1, equation (1)). With a decrease in the initial propionate concentration, the amount of accumulated acetate decreased (Table 1, variants 3 and 4). At a propionate content in the medium of 2.36 mmol/l (variant 4), the acetate/oxidized propionate ratio was almost two times lower than the theoretical value. It can be assumed that, in this case, the acetyl-CoA that formed from propionate was partially converted to acetate and partially oxidized to CO_2, i.e., the substrate oxidation was more complete than in variant 1. During complete oxidation of propionate, acetate is not formed (2):

$$4CH_3CH_2COO^- + 7SO_4^{2-} \longrightarrow 12HCO_3^- + 7HS^- + H^+ \quad (2)$$

(this equation is derived from the general formula of oxidation of fatty acid with an odd number of carbon atoms [6]).

The variations in the amount of acetate accumulated are characteristic of sulfate-reducing bacteria capable of complete oxidation. For example, *Desulfotomaculum acetoxidans* oxidizes butyrate with acetate accumulation. Complete oxidation of butyrate is observed only when it is introduced into the medium in limited amounts [15]. Another SRB capable of complete oxidation, *Desulfotomaculum* sp. strain 435, accumulates less acetate as the lactate concentration in the medium is decreased [9]. It is thought that the accumulation of acetate at high substrate concentrations by SRB capable of complete oxidation is related to an increased

accumulation rate of acetyl-CoA, a part of which is converted to acetate [6].

In experimental variants 1, 3, and 4 (Table 1), the decrease in the propionate content in the medium led to a decrease in the amount of acetate formed per unit of H_2S produced.

The addition of yeast extract (0.5 g/l) to the medium containing propionate (12.57 mmol/l) stimulated the oxidation process. Propionate was completely consumed, and the accumulation of acetate per unit of substrate oxidized decreased (Table 1, variant 2) compared to the variant without yeast extract (Table 1, variant 1), indicating more complete substrate oxidation in variant 2. The stimulatory effect of yeast extract observed at high medium salinity (100 g/l of NaCl) is apparently connected to utilization by the bacteria of yeast extract components: purines, pyrimidines, vitamins, amino acids, etc. A special role is played by certain amino acids, which provide for an osmoprotective effect. In addition, yeast extract is known to contain glycine betaine [1]. During propionate oxidation, yeast extract seems to promote activation of the enzymes catalyzing acetyl-CoA decomposition, which leads to more complete oxidation of the substrate.

An increase in the NaCl concentration in the medium to 200 g/l did not significantly influence propionate decomposition; acetate was also accumulated. In variant 5 (Table 1), approximately the same amount of propionate was oxidized as in variant 1, where NaCl concentration was 100 g/l; the acetate/oxidized propionate ratios were close in these two variants. It should be mentioned that an increase in the NaCl concentration in the medium influenced the duration of substrate decomposition, and this resulted in the extension of the cultivation period from 75 to 103 days.

The decomposition of butyrate, at its relatively high concentration in the medium (11.25 mM) and a NaCl concentration of 100 g/l, proceeded without accumulation of acetate (Table 2, variant 1). The amount of butyrate oxidized in this experiment was 2.55 mmol/l. Complete oxidation of butyrate occurs according to the following equation (3), derived from the general formula of oxidation of fatty acids with an even number of carbon atoms [6]:

$$2CH_3(CH_2)_2COO^- + 5SO_4^{2-} \longrightarrow 8HCO_3^- + 5HS^- + H^+. \quad (3)$$

An increase in the NaCl concentration in the medium to 200 g/l resulted in low butyrate consumption (0.57 mmol/l) and acetate accumulation in a 1 : 1 ratio to the substrate oxidized (Table 2, variant 2). The same ratio between the product and the substrate oxidized was observed during butyrate utilization by *Desulfotomaculum thermobenzoicum* ([16]; Table 2, equation (4)). It should be mentioned that, under hypersaline conditions (Table 2, variant 2), a considerable amount of hydrogen sulfide was formed (1.28 mmol/l), in spite of the weak

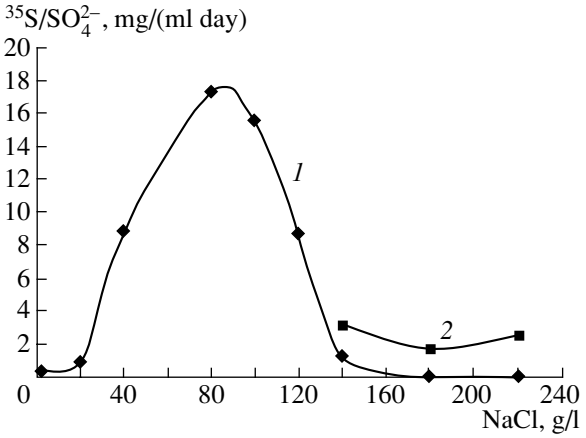

Fig. 2. Rates of sulfate reduction as determined by cultivation with $^{35}S/SO_4^{2-}$ in a medium with malate at different contents of NaCl for (*1*) 7 and (*2*) 15 days.

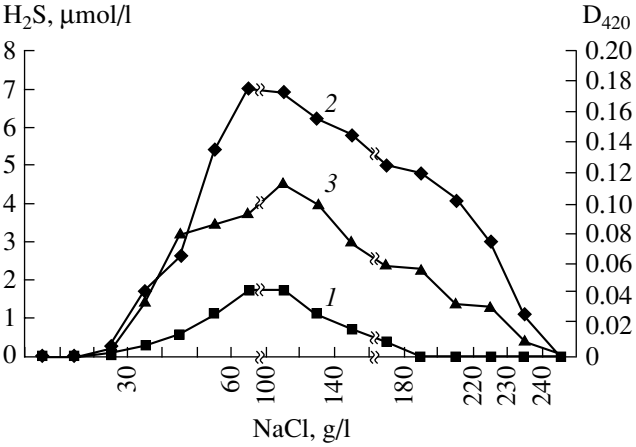

Fig. 3. Production of (*1, 2*) H_2S and (*3*) biomass at different NaCl concentrations in a medium with malate after (*1, 3*) 11 and (*2*) 30 days of cultivation.

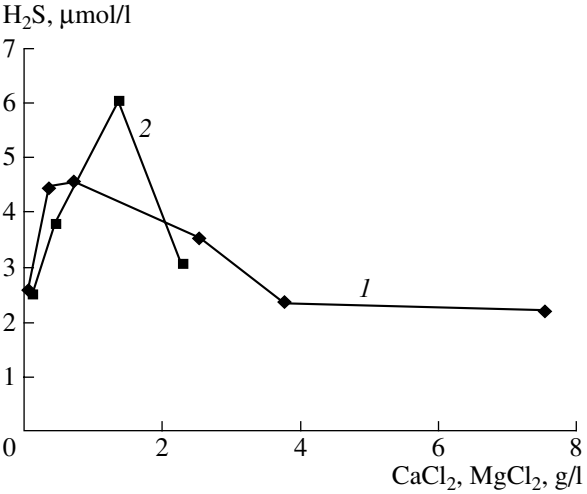

Fig. 4. H_2S production after 9 days of growth on a medium with sodium malate and 100 g/l of NaCl at different contents of (*1*) $CaCl_2$ and (*2*) $MgCl_2$ in the medium.

Table 1. Decomposition of propionate in the process of sulfate reduction under different cultivation conditions

Variant	Cultivation time, days	NaCl content in the medium, g/l	Yeast extract in the medium, g/l	Propionate		Acetate production, mmol/l	H_2S production, mmol/l	Acetate/oxidized propionate		Acetate/H_2S	
				initial, mmol/l	oxidized, mmol/l			in experiment	theoretically*	in experiment	theoretically*
1	75	100	0	9.86	6.88	5.60	5.0	0.81	1.00	1.12	1.33
2	15	100	0.5	12.57	12.57	3.58	9.64	0.28	1.00	0.37	1.33
3	20	100	0	5.81	4.72	2.75	3.74	0.58	1.00	0.73	1.33
4	20	100	0	2.36	2.08	0.95	2.82	0.45	1.00	0.33	1.33
5	103	200	0	10.14	7.35	7.00	5.92	0.93	1.00	1.18	1.33

* Calculated according to the equation of respiration of *Desulfobulbus* representatives during incomplete oxidation of propionate [6]:
$4CH_3CH_2COO^- + 3SO_4^{2-} \longrightarrow 4CH_3COO^- + 4HCO_3^- + 3HS^- + H^+$, $\Delta G^{0'} = -150$ kJ (1).

Table 2. Decomposition of butyrate in the process of sulfate reduction at NaCl contents in the medium of 100 and 200 g/l

Variant	Substrate	NaCl content in the medium, g/l	Cultivation time, days	Butyrate		Acetate production, mmol/l	H_2S production, mmol/l	Acetate/oxidized butyrate		Acetate/H_2S	
				initial, mmol/l	oxidized, mmol/l			in experiment	theoretically*	in experiment	theoretically*
1	Butyrate	100	87	11.25	2.55	0	3.84	–	–	–	–
2		200	103	7.61	0.57	0.58	1.28	1.0	1.0	0.17	0.66

* Calculated according to the equation of respiration of *Desulfotomaculum thermobenzoicum* during incomplete oxidation of butyrate [16]:
$CH_3(CH_2)_2COO^- + + 1.5SO_4^{2-} \longrightarrow CH_3COO^- + 2HCO_3^- + 0.5H^+ + 1.5HS^-$ (4).

substrate oxidation (0.57 mmol/l), and the H_2S/oxidized butyrate ratio was 2.24, whereas, according to the equation of butyrate oxidation by *D. thermobenzoicum*, it is 1.5 (Table 2, equation (4)). To explain the result obtained in our experiment, we may assume changes in the process of sulfate respiration. It is possible that the processes of disproportionation of intermediate products (such as sulfite) was switched on, and this resulted in additional production of H_2S.

Our results on the decomposition of organic compounds by strain 11-6 allowed us to suggest that cells of this strain possess enzymes of the Wood pathway [6] which catalyze acetyl-CoA decomposition to CO_2. CO dehydrogenase is one such enzyme [6]. Our supposition was confirmed by a high CO dehydrogenase activity (6.75 μmol/(min mg protein)), which we determined in cells grown in the medium with butyrate. This value is comparable to the activity of this enzyme in cells of *Desulfotomaculum acetoxidans* which decomposes acetate [17].

Autotrophic growth at the expense of $H_2 + CO_2$ was accompanied by the formation of small amounts of methane (286 nmol/100 ml of culture liquid); this value was higher than that determined for *Desulfotomaculum* strain 435 grown on $H_2 + CO_2$ (122 nmol/100 ml of culture liquid) [9]. This result testifies to the presence in cells of strain 11-6 of enzymes catalyzing synthesis of acetyl-CoA. During the growth of strain 11-6 on propionate, which was oxidized with the production of acetate (Table 1, variant 1), only 13 nmol of CH_4 was formed per 100 ml of culture liquid.

The growth rate of strain 11-6 was higher on the medium with propionate (0.0015 h^{-1}, Table 1, variant 1) than on the medium with butyrate (0.0007 h^{-1}, Table 2, variant 1). Yeast extract (0.5 g/l) accelerated the growth of strain 11-6 on the medium with propionate by a factor of 2.5, to a value of 0.0038 h^{-1}. The growth rate of SRB capable of complete oxidation is considerably lower than that of SRB that bring about incomplete oxidation [6]. This is most probably related to the difference between the rates of acetyl-CoA conversion to acetate and to two CO_2 molecules.

The rate of growth of strain 11-6 on media containing propionate or butyrate and 200 g/l of NaCl was not determined. However, it can be said that oxidation of these substrates took more time at 200 g/l of NaCl than at 100 g/l of NaCl (Table 1, variants 1 and 5; Table 2, variants 1 and 2). The amount of butyrate oxidized over 103 days of cultivation in medium with 200 g/l of NaCl was small. Thus, the process of oxidation of organic compounds is decelerated under hypersaline conditions. This conclusion agrees with the observations of

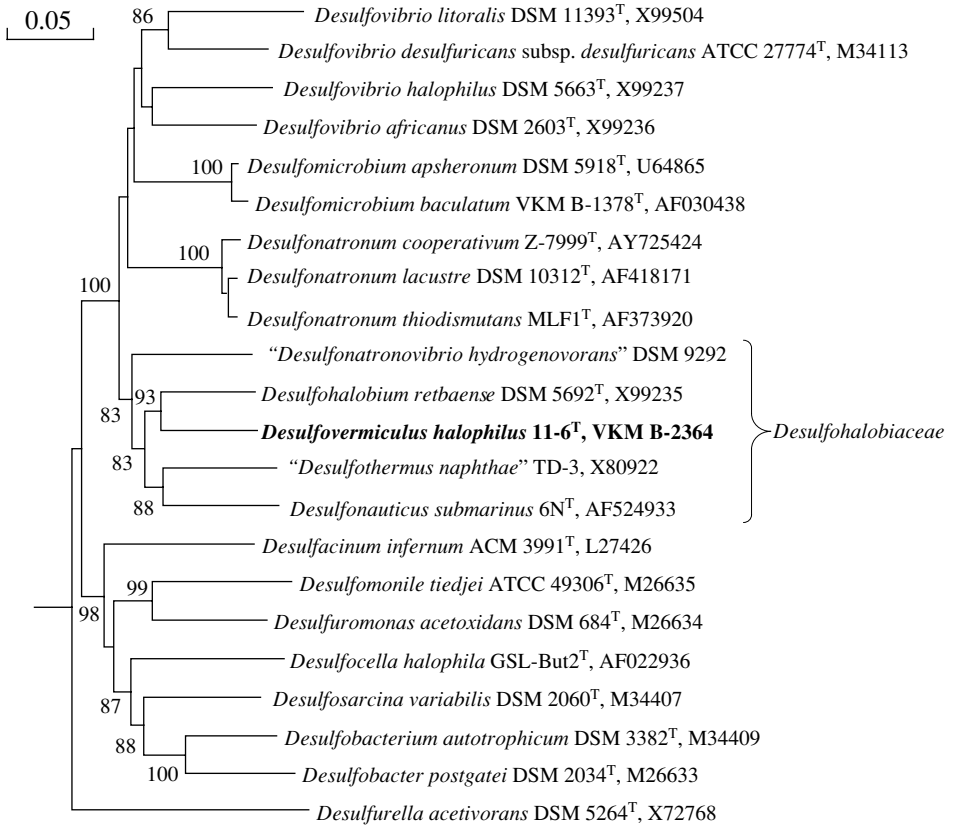

Fig. 5. 16S rRNA–based phylogenetic tree of sulfate-reducing *Deltaproteobacteria.* The root of the tree was found by using the *E. coli* sequence as an outgroup. Scale bar corresponds to 5 nucleotide substitutions per 100 nucleotides. Numerals show the statistic significance of the branching order as determined by bootstrap analysis of 100 alternative trees (values above 80% were considered significant).

Tarasov et al. [18], who noted that mineralization of stratal brines equal to 200 g/l is the boundary after which the sulfate reduction rate in these ecosystems is minimal.

Strain 11-6 fermented fumarate and pyruvate but not lactate. Apart from sulfate, sulfite, thiosulfate, and elemental sulfur could be electron acceptors during growth of the strain on sodium malate–containing medium.

Biochemical characteristics. Desulfoviridin could not be detected in cells of strain 11-6. The electron transport chain included menaquinone MK-7, which is present in many SRB capable of complete oxidation of organic compounds and in representatives of *Desulfotomaculum* (but not of *Desulfovibrio*) [19].

Genotypic characteristics and phylogenetic analysis. The DNA G+C content of strain 11-6 was 55.2 mol %. To establish the phylogenetic position of strain 11-6, we determined almost the entire nucleotide sequence of its 16S rRNA gene (1429 nucleotides corresponding to *E. coli* positions 8–1520). Preliminary screening in the RDP database showed that the 16S rRNA gene sequence of strain 11-6 was closest to analogous sequences of representatives of the family *Desulfohalobiaceae* within the class *Deltaproteobacteria.* In the phylogenetic tree that we constructed (Fig. 5), the 16S rRNA of strain 11-6 formed a separate branch within the cluster that united *Desulfohalobiaceae* representatives. The single species of the type genus of this family, *Desulfohalobium retbaense*, was closest to strain 11-6 (90.2% sequence identity). The homology level of the 16S rRNA gene of strain 11-6 with other genera of *Desulfohalobiaceae* was 87.1–88.1%, which corresponded to the intergeneric level in this family (86.7–88.2%).

Table 3 presents a comparison of the characteristics of strain 11-6 and pylogenetically related SRB: *Desulfohalobium retbaense* [2], *Desulfonatronovibrio hydrogenovorans* [20], and *Desulfothermus naphthae* [21]. These three SRB species have been assigned to the new family *Desulfohalobiaceae* [21]. These SRB are obligately dependent on Na^+ and/or Cl^- and are mesophiles (except *Desulfothermus naphthae*). The recently described *Desulfonauticus submarinus* [22] also belonged to the cluster formed by representatives of *Desulfohalobiaceae* (Fig. 5); however, it essentially differs from other members of the cluster in being halotolerant, i.e., in the lack of obligate dependence on the presence of NaCl in the medium.

Strain 11-6 differs from the closest phylogenetic relative, *Desulfohalobium retbaense*, in its capacity for

Table 3. Comparison of the characteristics of strain 11-6 and phylogenetically close sulfate-reducing bacteria of the family Desulfohalobiaceae

Characteristics	Strain 11-6 (VKM B-2364)	*Desulfohalobium retbaense* DSM 5692^T	*Desulfonatronovibrio hydrogenovorans* DSM 9292^T	*Desulfothermus naphthae* DSM 13418	*Desulfonauticus submarinus* DSM 15269
Cell shape	vibrios and curved rod	rods	vibrios and spirilla	curved rods	curved rods
Motility, flagellation	+, one polar	+, 1–2 polar	+, one polar	+, one polar	+, one polar
Cell size, μm	0.5–0.6 × 1–15	0.7–0.9 × 1–20	0.5 × 1.5–2	0.8 – 1.0 × × 2.0–3.5	0.35–0.5 × 5–6
Temperature range (optimum), °C	25–47 (37)	25–43 (37–40)	43 (37–40)	50–69 (60–65)	30–60 (45)
NaCl range (optimum), g/l	30–230 (80–100)	30–240 (100)	10–120 (30)	Up to 21	0–5 (2)
pH optimum	7.2	6.5–7.0	9.0–9.7	6.5–6.8	7.0
DNA G+C content, mol %	55.2	57	47.9–48.5	37.4	34.4
Electron donors with SO_4^{2-}:					
H_2 + acetate + CO_2 (lithoheterotrophic growth)	ND	+	+	–	+
H_2 + CO_2 (lithoautotrophic growth)	+	–	–	–	–
formate	+	–	–	–	–
formate + acetate or yeast extract	+	–	+	–	+
lactate	+	+	–	–	–
pyruvate	+	+	–	–	–
malate	+	–	–	–	–
fumarate	+	–	–	–	–
acetate	–	–	–	–	–
propionate	+	–	–	–	–
butyrate	+	–	–	+	–
ethanol	+	+	–	–	–
alanine	+	–	–	–	–
Oxidation of organic substrates	complete	incomplete	*	complete	*
Fermentative growth (without SO_4^{2-}) with:					
pyruvate	+	(+)	–	–	–
fumarate	+	–	–	–	–
Electron acceptors** S^0	+	+	–	ND	+
Reference	present work	[2]	[19]	[6]	[21]

Note: "+" means that the character is positive, "–" means that the character is negative, (+) signifies weak growth, and ND means "no data".

* No substrates other than formate are used.

** Electron acceptors other than SO_3^{2-}, SO_4^{2-}, and $S_2O_3^{2-}$.

complete oxidation of organic compounds. Unlike *D. retbaense* and other phylogenetically related organisms, in sulfate reduction it can use a wide range of organic compounds, including salts of lactic acid, salts of dicarboxylic acids (malate, fumarate, and succinate), and salts of fatty acids (propionate and butyrate), as well as ethanol and the amino acid alanine.

By its capacity to completely oxidize butyrate (without acetate accumulation) with the involvement of CO dehydrogenase, stain 11-6 is similar to *Desulfothermus naphthae*, which also completely oxidizes butyrate via the CO dehydrogenase pathway; in addition, in the process of sulfate reduction, the latter organism can use C_5–C_{18} fatty acids and C_6–C_{14} alkanes but

no other substrates. Among moderately halophilic SRB, only *Desulfocella halophila* is also known to oxidize fatty acids with a number of carbon atoms above four [5]. However, this bacterium brings about incomplete oxidation of fatty acids: the fatty acids with an even number of carbon atoms are oxidized to acetate, and those with an odd number are oxidized to acetate and propionate. This microorganism is phylogenetically remote from strain 11-6. Another halophilic SRB, *Desulfobacter halotolerance* [23], which is also phylogenetically remote from strain 11-6, decomposes acetate to CO_2 but differs from strain 11-6 in having a low optimal value of the NaCl concentration in the medium (10–20 g/l) and an essentially different type of metabolism (it oxidizes acetate via a complete oxidative tricarboxylic acid cycle).

The capacity of strain 11-6 to grow chemolithoautotrophically at the expense of $H_2 + CO_2$ or formate in the absence of organic compounds, i.e., its ability to synthesize acetyl-CoA from one-carbon units, is a significant distinction of this strain from the representatives of *Desulfohalobiaceae* described in Table 3 and from all other currently recognized moderately halophilic SRB. Thus, *Desulfothermus naphthae* does not use $H_2 + CO_2$ or formate, whereas *Desulfohalobium retbaense* and others grow on $H_2 + CO_2$ under chemolithoheterotrophic conditions. *D. retbaense* does not grow at the expense of formate, and *Desulfonatronovibrio hydrogenovorans* and *Desulfonauticus submarinus* can grow on formate only in the presence of other organic compounds needed for their anabolism.

As far as the sulfur compounds used as electron acceptors are concerned, all the closely related microorganisms listed in Table 3 can, apart from sulfate, reduce sulfite and thiosulfate in the course of their growth; strain 11-6, *Desulfohalobium retbaense*, and *Desulfonauticus submarinus* can also reduce elemental sulfur.

Strain 11-6 and *D. retbaense* are characterized by high growth maxima and optima of the NaCl concentration in the medium; for *D. hydrogenovorans* and *D. submarinus*, these values are relatively low.

As distinct from strain 11-6, which is a neutrophile, *Desulfonatronovibrio hydrogenoformans* is an alkaliphile.

Strain 11-6 is a mesophile and is thus essentially different from the thermophilic *D. naphthae* and *D. submarinus* in its growth temperature parameters.

The last point to be mentioned is that all of the microorganisms listed in Table 3 had different values of the DNA G+C content.

Thus, our study was the first to reveal a moderately halophilic SRB that can develop at a high mineralization of the medium and can grow chemoautotrophically at the expense of $H_2 + CO_2$ or formate in the absence of organic compounds. The microorganism oxidizes butyrate without acetate accumulation and exhibits a high activity of CO dehydrogenase. This is the first SRB that is capable of complete oxidation of organic compounds and at the same time possesses the Wood pathway enzymes and can grow at a NaCl concentration in the medium of 100 g/l and higher. Under conditions of hypersalinity, strain 11-6 oxidizes butyrate with the production of acetate. This fact, together with the inability of the organism to oxidize acetate as substrate, may explain the accumulation of acetate in hypersaline ecosystems, oil field brines in particular. It should be mentioned, however, that under natural conditions, fermentation products are consumed by SRB in parallel with their formation. It is possible that, under conditions of limitation by the substrates, their oxidation in hypersaline ecosystems occurs without accumulation of acetate.

Based on the results of physiological and molecular-biological investigations, we suggest that strain 11-6 should be assigned to a new genus and species, *Desulfovermiculus halophilus* gen. nov., sp. nov. within the family *Desulfohalobiaceae* [21].

Description of *Desulfovermiculus* gen. nov. (De.sul.fo.ver.mic.ul.us: L. prefix *de*; L. n. *sulfur,* sulfur; L. n. *vermiculus,* diminutive of L. n. *vermis*, worm; vermiform sulfate-reducing bacterium).

Cells are vibrioid, with a gram-negative cell wall structure, obligately anaerobic. In media with sulfate or some other oxidized sulfur compounds, metabolism is respiratory. Chemoorganotrophic growth at the expense of anaerobic respiration occurs with various carbon and energy sources, including sodium salts of certain fatty acids. Organic compounds are decomposed completely (to CO_2) or incompletely, with the formation of larger or lesser amounts of acetate in dependence on the substrate concentration in the medium. Chemolithoautotrophic growth at the expense of $H_2 + CO_2$ or formate is possible in the absence of organic compounds. Certain organic substrates can be fermented. The bacteria are neutrophilic, moderately halophilic, and mesophilic. Cells do not contain desulfoviridin. Menaquinone MK-7 is present. The type species of the genus is *Desulfovermiculus halophilus.*

Description of *Desulfovermiculus halophilus* sp. nov. (ha.lo.phil.us: Gr. n. *halos*, salt; Gr. adj. *philus,* loving; L. adj. *halophilus,* salt-loving).

Cells are non-spore-forming vibrios or spirilla; they measure 0.5–0.6 × 1.0–20 μm and are motile at the early growth stage by means of one polar flagellum. Cells stain Gram negative; the cell wall structure is typical of gram-negative bacteria. Cell division occurs by constriction. Growth occurs in a temperature range of 25–47°C, with an optimum at 37°C, a pH range of 6.4–8.2 with an optimum at 7.2, and a NaCl concentration range of 30–230 g/l with an optimum at 80–100 g/l. Strictly anaerobic. Sulfates are reduced with the production of H_2S on media with lactate, malate, fumarate, pyruvate, propionate, butyrate, formate, $H_2 + CO_2$, crotonate, succinate, ethanol, and alanine. Butyrate is decomposed completely, without acetate accumulation.

Cells grown on butyrate exhibit a high activity of CO dehydrogenase. Other substrates are decomposed with the production of larger or lesser amounts of acetate. Growth factors are not required; however, the addition of yeast extract to the medium increases growth rate. Chemolithoautotrophic growth occurs at the expense of $H_2 + CO_2$ or formate in the presence of sulfate; in the $H_2 + CO_2$ gas phase, minimethane can be detected. Serine, glycine, betaine, yeast extract, glucose, fructose, citrate, glycerol, butanol, propanol, methanol, benzoate, acetate, valerate, and palmitate are not utilized. In the absence of sulfates, pyruvate and fumarate can be fermented. Apart from sulfate, sulfite, thiosulfate, and elemental sulfur can be used as electron acceptors. Cells do not contain desulfoviridin. Menaquinone MK-7 is present. The DNA G+C content is 55.2 mol %. Phylogenetically, the species forms a separate branch within the cluster of *Desulfohalobiaceae* members in the class *Deltaproteobacteria.* The identity level of the 16S rRNA gene with that of *Desulfohalobium retbaense* is 90.2%. The type strain is 11-6^T (= VKM B-2364), isolated from highly mineralized stratal water of an oil field.

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