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ORIGINAL PAPER

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A sulfate-reducing bacterium from the oxic layer of a microbial mat from Solar Lake (Sinai), *Desulfovibrio oxyclinae* sp. nov.

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Abstract In an investigation on the oxygen tolerance of sulfate-reducing bacteria, a strain was isolated from a 10^7-fold dilution of the upper 3-mm layer of a hypersaline cyanobacterial mat (transferred from Solar Lake, Sinai). The isolate, designated P1B, appeared to be well-adapted to the varying concentrations of oxygen and sulfide that occur in this environment. In the presence of oxygen strain P1B respired aerobically with the highest rates [260 nmol O_2 min^{-1} (mg protein)$^{-1}$] found so far among marine sulfate-reducing bacteria. Besides H_2 and lactate, even sulfide or sulfite could be oxidized with oxygen. The sulfur compounds were completely oxidized to sulfate. Under anoxic conditions, it grew with sulfate, sulfite, or thiosulfate as the electron acceptor using H_2, lactate, pyruvate, ethanol, propanol, or butanol as the electron donor. Furthermore, in the absence of electron donors the isolate grew by disproportionation of sulfite or thiosulfate to sulfate and sulfide. The highest respiration rates with oxygen were obtained with H_2 at low oxygen concentrations. Aerobic growth of homogeneous suspensions was not obtained. Additions of 1% oxygen to the gas phase of a continuous culture resulted in the formation of cell clumps wherein the cells remained viable for at least 200 h. It is concluded that strain P1B is oxygen-tolerant but does not carry out sulfate reduction in the presence of oxygen under the conditions tested. Analysis of the 16S rDNA sequence indicated that strain P1B belongs to the genus *Desulfovibrio*, with *Desulfovibrio halophilus* as its closest relative. Based on physiological properties strain P1B could not be assigned to this species. Therefore, a new species, *Desulfovibrio oxyclinae*, is proposed.

Key words Desulfovibrio · 16S rRNA · Cyanobacterial mat · Aerobic respiration · Oxygen-indifferent sulfate reduction

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Introduction

The photic zone of cyanobacterial mats is characterized by varying oxygen and sulfide concentrations. During the light period, oxygenic photosynthesis results in super-saturating oxygen concentrations, whereas in the dark, oxygen is consumed and sulfide accumulates. These changes are a challenge for organisms living in such a habitat. Adaptations to the oxygen-sulfide regimens have been found in cyanobacteria, which can switch from oxygenic to anoxygenic sulfide-dependent photosynthesis (Cohen et al. 1975a, b; De Wit and Van Gemerden 1987a; De Wit et al. 1988; Stal 1991), and in purple sulfur bacteria, which can grow chemolithotrophically in the presence of molecular oxygen (De Wit and Van Gemerden 1987b, 1990; Herbert and Welsh 1994). A switch from anaerobic to aerobic growth of sulfate reducers in microbial mats has not been demonstrated. However, high numbers of sulfate-reducing bacteria in oxic zones of microbial mats (Visscher et al. 1992; Risatti et al. 1994) indicate that they can deal with high oxygen concentrations. Furthermore, physiological studies have shown that sulfate-reducing bacteria are able to use oxygen for aerobic respiration, resulting in ATP formation (Dilling and Cypionka 1990; Dannenberg et al. 1992; Marschall et al. 1993). However, growth of sulfate-reducing bacteria in aerated, shaken cultures was limited to less than two doublings (Marschall et al. 1993; Sass et al. 1996). The strains tested revealed in-

creasing rates of aerobic respiration with decreasing concentrations of oxygen. Prolonged periods of exposure to oxygen caused harmful effects (Fukui and Takii 1990; Marschall et al. 1993).

While high sulfate reduction rates have been measured in the oxic layers of microbial mats or sediments (Cohen 1984; Canfield and DesMarais 1991; Fründ and Cohen 1992; Visscher et al. 1992; Jørgensen 1994), no sulfate reduction has been obtained in pure culture studies in assays with oxygen concentrations above 15 μM. Only low rates of sulfate reduction have been observed in assays with low oxygen concentrations and electron donor in excess (Marschall et al. 1993). Inhibition by oxygen could be explained by oxidation or inactivation of enzymes involved in the reduction of sulfate (Postgate 1979; Dijk et al. 1983) or by preference of oxygen as the electron acceptor (Marschall et al. 1993; Krekeler and Cypionka 1995). The occurrence of anoxic microniches within the oxic layer appears unlikely since such microenvironments have not been detected by microelectrode studies. In a microscopic study of the distribution of sulfate-reducing bacteria using oligonucleotide probes for the 16S rRNA, no aggregation of sulfate reducers within the oxic layer resulting in anaerobic microniches was observed (Ramsing et al. 1993).

In the present study, we searched for sulfate-reducing bacteria able to carry out oxygen-indifferent sulfate reduction, i.e., sulfate reduction independent of the presence of oxygen. Therefore, we isolated an abundant sulfate reducer from the oxic zone of a microbial mat (Solar Lake, Sinai), where high sulfate reduction rates in the presence of oxygen have been measured (Fründ and Cohen 1992; Jørgensen 1994). The physiology and the phylogenetic classification of this sulfate-reducing bacterium are presented.

Materials and methods

Source of organism

Strain P1B was isolated from the 3-mm thick surface layer of a cyanobacterial mat that had been transferred from the sediment surface of Solar Lake (Sinai, Egypt) to an experimental hypersaline pond of the Interuniversity Institute of Eilat. These mats developed there under evaporated Red Sea seawater at a total dissolved salinity of 9 ± 0.5% for five years prior to this research.

Cultivation and isolation

The medium used for the gradient enrichments contained per liter of filtered hypersaline pond water: 1.0 g NH_4Cl, 0.5 g K_2HPO_4, 1.0 g sodium citrate dihydrate, 1.0 g yeast extract, 1.0 ml mineral solution SL9 (Tschech and Pfennig 1984), 1.0 ml vitamin solution (Pfennig 1978), 1.0 g ascorbic acid, 0.1 g sodium thioglycolate 5 ml 5% (w/v) $FeSO_4 \cdot 7\ H_2O$ solution, and 20.0 g agar.

The synthetic medium used for growth experiments was based on Postgate's medium C as described by Widdel and Bak (1992) with 50 g/l of NaCl; yeast extract was omitted, and vitamin solution (1 ml/l) and mineral solution SL7 (1 ml/l) were added. For diagnostic purposes, 5 ml/l of 5% $FeSO_4 \cdot 7\ H_2O$ solution was added.

Strain P1B was isolated from sulfide gradient cultures that were prepared as follows: 1 ml of agar solution (2% agar in filtered pond water) with 1 mM Na_2S was placed at the bottom of 18-ml test tubes. Then 10 ml of a dilution series with homogenized surface layer of the mat was added. The dilutions were prepared in medium supplemented with 5 mM ethanol as the carbon and energy source and 2% agar. The test tubes were closed loosely so that exchange of air was possible; they were then incubated at 35°C for up to 14 days.

The upper layers of the gradient enrichments were cut aseptically in 2-mm slices and placed on agar plates containing 20 mM lactate as the carbon and energy source under anoxic conditions in the presence of Fe^{2+}. Sulfide-forming colonies were resuspended and streaked on agar plates that were incubated under N_2/CO_2 (80:20, v/v) at room temperature.

Continuous culture experiments under oxic and anoxic conditions were carried out in an apparatus manufactured at the Biological Center of the University of Groningen (Haren, The Netherlands); the apparatus includes controls for temperature, pH, and dissolved oxygen. The growth temperature was maintained at 35°C, the pH at 7.0–7.5. Dissolved oxygen was measured using a 900 series New Brunswick (Edison, N.J., USA) oxygen electrode.

For experiments with cell suspensions, strain P1B was cultivated in a chemostat as described by Cypionka and Pfennig (1986). The culture was supplied with H_2/CO_2 (80:20, v/v) and sulfate at a limiting concentration of 20 mM. The marine medium was supplemented by acetate (2 mM) and yeast extract (1 g/l). The pH was adjusted to 7.4. The cells were harvested by centrifugation and resuspended in a salt solution (850 mM NaCl, 13 mM KCl, and 12.5 mM $MgCl_2$) under N_2.

Physiological tests

Utilization of energy sources, carbon sources, and electron acceptors was tested using a concentration of 20 mM for electron donors and 10 mM for electron acceptors. These tests were performed in completely filled screw-cap tubes (16 ml). Addition of yeast extract was used only for isolation but not for any growth experiments. Tests for growth by disproportionation of sulfite or thiosulfate were carried out in media containing acetate (2 mM) and CO_2 as carbon sources. The turbidity of cultures, the substrates consumed (sulfite and thiosulfate), and the products formed (sulfate and sulfide) were measured. Sulfite was determined as described by Pachmayr (1960), and sulfide as described by Widdel (1980). Sulfate and thiosulfate were measured by ion chromatography as described by Fuseler and Cypionka (1995).

The reduction rates of oxygen and sulfur compounds were determined in a reaction chamber equipped with four different electrodes (oxygen-, pH-, sulfide-, and platinum electrode) as described by Cypionka (1994). These measurements were performed at 30°C in N_2- or H_2-saturated salt solution (see above).

Isolation of nucleic acids, PCR, and sequencing

Nucleic acids were isolated by lysozyme/proteinase K/SDS digest followed by phenol extraction and ethanol precipitation as previously described (Sambrook et al. 1989). The 16S rRNA gene was amplified by PCR using primers GM3 [5′-AGAGTTTGATC(A/C)TGGCTCAG-3′, corresponding to *Escherichia coli* positions 8–23] and GM4 (5′-TACCTTGTTACGACTTG-3′, corresponding to *E. coli* positions 1492–1507) (Muyzer et al. 1995). PCR amplification, purification, and sequencing of the PCR product were performed as previously described (Oren et al. 1995). The sequence is available from Genbank under accession no. U33316.

Phylogenetic tree inference

16S rRNA sequences related to strain P1B were searched and identified in the RDP database (Maidak et al. 1996) using the database's SIMILARITY_RANK tool. Other relevant sequences have been published by Tardy-Jacquenod et al. (1996). The 16S rRNA sequences of strain P1B and the following *Desulfovibrio (Dsv.)*

and *Desulfomicrobium* strains and species were included in the phylogenetic tree: *Dsv.salexigens* (DSM 2638^T), *Dsv. halophilus* strain SL8903 (DSM 5663^T), *Dsv.desulfuricans* strain El Agheila Z (DSM 1926), *Dsv. longus* strain SEBR 2582 (DSM 6739^T), *Dsv. africanus* strain Benghazi (DSM 2603^T), *Dsv. gigas* (DSM 1382^T), *Dsv.gabonensis* strain SEBR 2840^T, *Dsv. piger* (DSM 749^T), *Dsv. desulfuricans* (DSM 6949), *Dsv. vulgaris* strain Hildenborough (DSM 644^T), *Desulfovibrio* species strain PT-2, *Dsv. longreachensis* strain AB16910a (ACM 3958^T), *Desulfomicrobium baculatum* (DSM 1743), and *Desulfomicrobium escambium* strain esc1 (ATCC 51164^T). The phylogenetic tree was inferred using the distance matrix programs DISTANCE and FITCH as implemented in the PHYLIP program package, version 3.5c (Felsenstein 1993). In the program DNADIST, a matrix of evolutionary distances was computed from the sequence alignment using the Jukes-Cantor model (Jukes and Cantor 1969), which assumes independent change at all sites with equal probability. The distance matrix was transformed into the phylogenetic tree with the least-squares algorithm of Fitch and Margoliash (1967) using the program FITCH. Bootstrap resampling of the sequence data was performed with the program SEQBOOT as implemented in PHYLIP. Bootstrapping tests the stability of a branching pattern by introducing random variations into the sequence data set and calculating the tree numerous times to identify stable and unstable features of the tree topology. Here, 100 bootstrap reruns were performed.

Results

Enrichment and isolation

The cyanobacterial mat (originating from Solar Lake, Sinai) used for enrichment revealed diurnal changes between oxic and sulfidic milieu (up to 1,200 µM oxygen and no detectable sulfide at noon, and no oxygen and up to 100 µM sulfide at midnight). Sulfide gradient medium was inoculated with serial dilutions (up to 10^{-7}) of the 3-mm thick surface layer. After two weeks, bacterial colonies of various morphological types had developed in the upper layer of these gradients. Strain P1B was isolated from one of these colonies out of a 10^{-7} dilution.

Physiological characteristics

Cells of strain P1B were motile and vibrio- to rod-shaped. They possessed desulfoviridin. The amounts of sulfide formed per substrate oxidized indicated that the strain carried out incomplete oxidation of some organic acids and alcohols. Acetate was required for lithotrophic growth with H_2 (plus CO_2) or formate (Table 1). Strain P1B had a salt requirement of 2.5–22.5% NaCl with a growth optimum at 5–10% NaCl. Strain P1B grew by reduction of sulfur compounds and fumarate, and also by fermentation of organic substrates (pyruvate) and disproportionation of sulfite and thiosulfate.

Phylogenetic position

By 16S rRNA sequence, strain P1B falls into the genus *Desulfovibrio* within the phylogenetically defined family Desulfovibrionaceae (Devereux et al. 1990). Strain P1B clusters with the closely related halophilic species *Dsv. halophilus*, which also has been isolated from Solar Lake (Caumette et al. 1991), and with the salt-requiring species *Dsv. salexigens* (Fig. 1). This clustering is supported by 76% bootstrap. Other salt-tolerant or salt-requiring *Desulfovibrio* species, e.g., *Dsv. gabonensis* and *Dsv. africanus*, are not specifically related to strain P1B (Tardy-Jacquenod et al. 1996). The 16S rRNA sequences of strain

Table 1 Characteristics of halophilic *Desulfovibrio* species (*nd* not determined)

	Desulfovibrio strain P1B	*Desulfovibrio halophilus*[a]	*Desulfovibrio salexigens*[b]
Shape	Rods to curved rods	Vibrio	Vibrio
Size (µm width × length)	0.5 × 2.3	0.6 × 2.5–5	0.5–1 × 3.5
Motility	+	+	+
Salinity range (%)	2.5–22.5	3–18	0.5–12
Salinity optimum (%)	5–10	6–7	2–4
Vitamins required	–	–	–
Electron donors (with sulfate):			
$H_2 + CO_2$	–	–	–
H_2 + acetate	+	+	+
Formate + acetate	+	+	+
Lactate	+	+	+
Pyruvate	+	+	+
Citrate	–	nd	nd
Succinate	–	nd	nd
Fumarate	–	–	–
Malate	+	–	+
Acetate	–	–	–
Propionate	–	–	–
Butyrate	–	nd	–
Methanol	–	nd	nd
Ethanol	+	+	+
Propanol	+	+	–
Butanol	+	–	–
Glucose	–	nd	nd
Fructose	–	nd	nd
Glycolate	–	–	nd
Electron acceptors (with lactate as energy and carbon source):			
Sulfate	+	+	+
Sulfite	+	+	nd
Thiosulfate	+	+	nd
Sulfur	+[c]	+	nd
Oxygen	+[c]	nd	+[c]
Nitrate	–	–	nd
Fumarate	+	–	nd
Fermentation[d]:			
Thiosulfate	+	nd	nd
Sulfite	+	nd	nd
Pyruvate	+	–	–

[a] After Caumette et al. (1991)
[b] After Postgate (1979)
[c] Growth was not obtained
[d] Tested but not fermented by strain P1B: formate, lactate, malate, methanol, ethanol, propanol, butanol, and iso-butanol

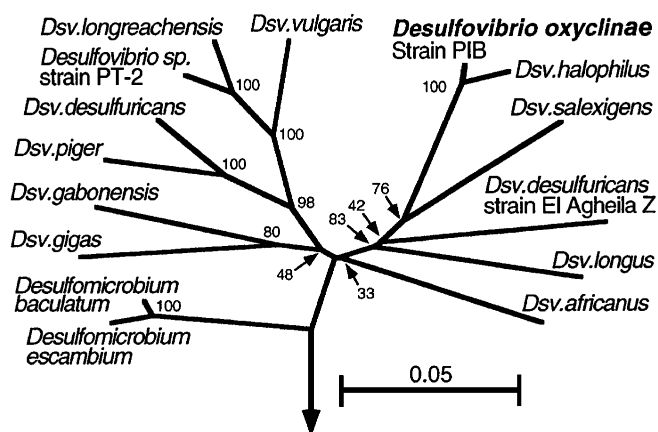

Fig. 1 16S rRNA distance tree of *Desulfovibrio* based on 16S rRNA positions 24–1384 (*Escherichia coli* numbering). The 5′-end of most *Desulfovibrio* 16S rRNA sequences were incomplete and, therefore, were excluded from the analysis. The tree was rooted with *Desulfobacter vulgaris* as outgroup. *Bootstrap values* indicate how often branching patterns of the tree remained stable (of 100 reruns). Sequences used in preparing this figure were obtained from Genbank and from the Ribosomal Database Project (RDP) (Maidak et al. 1996). The sequence of *Desulfovibrio oxyclinae* strain P1B is available under Genbank accession no. U33316 [*bar* 0.05 substitutions per nucleotide position (Jukes Cantor)]

P1B and of its closest relative *Dsv. halophilus* show 98.4% similarity in their known positions. It has been suggested that bacteria with 16S rRNA sequence similarities higher than 97% can possibly be assigned to the same species if additional phenotypic and genomic data support species identity. Still, species definition rests ultimately on phenotypic characteristics and DNA-DNA hybridization (Stackebrandt and Goebel 1994). Due to its capacities for pyruvate fermentation, reduction of fumarate, and use of butanol and malate as electron donors (Table 1), strain P1B is sufficiently different from *Dsv. halophilus* to be considered a distinct species for which the name *Desulfovibrio oxyclinae* is proposed.

Reduction of various electron acceptors

Washed cells of strain P1B catalyzed the reduction of five different electron acceptors with H_2 as the electron donor

Table 2 Reduction of different electron acceptors in H_2-saturated solution by strain P1B (Rates were measured with cell suspensions, *nd* not determined). Rates of reactions [nmol electrons transferred min^{-1} (mg protein)$^{-1}$

Electron acceptors	Concentration of the electron acceptor			
	30 mM	300 μM	30 μM	3 μM
Sulfate	440	20	< 1	< 1
Sulfite	nd	250	< 1	< 1
Thiosulfate	nd	300	nd	125
Sulfur	nd	nd	40	25
Oxygen	nd	160	1,040	≥ 1,040

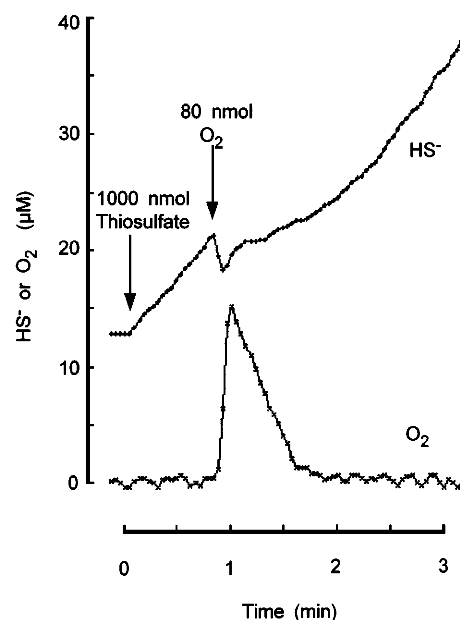

Fig. 2 Influence of oxygen on thiosulfate reduction by strain P1B. Washed cells (2.7 ml, 0.13 mg protein ml^{-1}) were incubated in hydrogen-saturated salt solution (850 mM NaCl, 13 mM KCl, and 12.5 mM $MgCl_2$). Oxygen added to thiosulfate-reducing cells was immediately consumed, while the sulfide concentration was hardly influenced

(Table 2). The highest rates [1,040 nmol electrons transferred min^{-1} (mg protein)$^{-1}$] were obtained with oxygen, followed by the four different sulfur compounds. While sulfite and sulfate were reduced only at concentrations above 30 μM, thiosulfate and oxygen were also reduced at low (3 μM) concentrations. At a sulfate concentration of 30 mM, the rates of sulfate reduction were 20-fold higher than at 0.3 mM.

Oxygen (30 μM) added to cells reducing sulfate, thiosulfate (3.7 mM), or sulfur (37 μM) was consumed immediately, while the sulfide concentration remained almost constant. During oxygen reduction there was no increase of the sulfide concentration when sulfate or sulfur was the alternative electron acceptor. Thiosulfate, however, was reduced when the oxygen concentration became lower than 2 μM (Fig. 2). The high rates of oxygen consumption indicated that under these conditions H_2 was the electron donor, but not sulfide (see below).

Oxidation of various substrates with oxygen

Various compounds were metabolized with oxygen as the electron acceptor. The highest rates [260 nmol O_2 min^{-1} (mg protein)$^{-1}$] were obtained with hydrogen as the electron donor, followed by the sulfur compounds sulfide and sulfite [38 and 14 nmol O_2 min^{-1} (mg protein)$^{-1}$, respectively]. Although formate and ethanol were used for sulfate reduction, these compounds were not oxidized with O_2. The enzymes involved were possibly oxygen-sensitive. Also with lactate only low rates [2 nmol O_2 min^{-1}

(mg protein)$^{-1}$] were observed. No consumption of oxygen was found with acetate, thiosulfate, or elemental sulfur (dissolved in methanol and added in the absence of sulfide). Sulfide and sulfite were oxidized to sulfate as indicated by the stoichiometric ratio of sulfide and sulfite to oxygen and analysis of the products formed. The sulfide oxidized was quantitatively (97%) found as sulfate. As an intermediate product of sulfide oxidation, elemental sulfur was observed as described by Fuseler et al. (1996).

Suspensions of strain P1B without added substrates tolerated exposure to 50, 250, and 1250 µM O_2 for at least 8 h without decrease of the viable cell counts in MPN series.

Aerobic growth of strain P1B was examined in continuous culture experiments. First, strain P1B was grown anaerobically in the chemostat on lactate and sulfate at a dilution rate of 0.05 h^{-1}. Under these conditions, sulfate was completely reduced to sulfide; 2 mol of lactate were oxidized to acetate for 1 mol of sulfate reduced. The yield was 3.4 g protein per mol sulfate consumed. When oxygen (1%) was added to the gas phase, the cells clumped and stuck to the wall. Under these conditions a steady state was never established, although sulfate was reduced and high cell concentrations were maintained for 200 h.

Discussion

In the present investigation, for the first time the physiology and phylogenetic position of a sulfate-reducing bacterium that thrives in high numbers in the upper layer of a cyanobacterial mat was studied. It turned out that strain P1B is well-adapted to this extreme environment since it can change its metabolism according to the varying oxygen-sulfide regimens. The high abundance of this bacterium has recently been confirmed (D. Krekeler, A. Teske, and H. Cypionka, unpublished results) by reisolation and 16S rRNA analysis using denaturing gradient gel electrophoresis [DGGE; see Muyzer et al. (1993)].

Compared to the marine sulfate reducers studied so far (Dannenberg et al. 1992), strain P1B is remarkable in several aspects. It has the highest rate of aerobic respiration found to date among marine sulfate-reducing bacteria. The maximum rates [260 nmol O_2 min^{-1} (mg protein)$^{-1}$] are similar to those of most aerobic bacteria. Additionally, the number of electron donors that can be oxidized with O_2 is higher than that in other marine sulfate reducers. However, strain P1B has some properties typical of microaerophilic bacteria. The highest respiration rates were obtained at very low oxygen concentrations. While the bacteria possessed catalase activity and survived oxygen exposure, we did not obtain continuous aerobic growth.

Strain P1B also has notable features with respect to its sulfur metabolism. So far only sulfate reducers of freshwater origin have been found to oxidize sulfur compounds completely (Dannenberg et al. 1992; Fuseler and Cypionka 1995). The mechanism of sulfide oxidation appears to be the same as that found in freshwater species (Fuseler et al. 1996). Polysulfide is probably an intermediate since sulfur was not oxidized in the absence of sulfide. Furthermore, strain P1B is the first marine sulfate reducer found to grow by disproportionation of thiosulfate or sulfite (Bak and Cypionka 1987; Krämer and Cypionka 1989). That thiosulfate might be an important natural substrate for this bacterium was indicated by the fact that even traces were metabolized, while sulfate was reduced only at concentrations above 30 µM.

With respect to the situation in the microbial mat, we conclude that strain P1B is well-adapted to survive oxygen exposure. It can respire with oxygen at high rates and with various substrates. However, sulfate reduction in the presence of oxygen as found in the mat (Fründ and Cohen 1992) cannot be explained by the activity of this strain since oxygen blocked the reduction of sulfur compounds. The same holds true for thiosulfate disproportionation, which accounted for up to 50% of the thiosulfate transformations in this zone (Jørgensen 1994). Additional mechanisms and/or organisms must be involved in these processes.

Strain P1B is clearly a member of the genus *Desulfovibrio*. Its closest relative is *Desulfovibrio halophilus* (Fig. 1, Table 1). The difference in 16S rRNA alone may not be sufficient to assign strain P1B and *Dsv. halophilus* to two different species. However, physiologically strain P1B can be differentiated from *Dsv. halophilus* by at least four features. Therefore, it is necessary to assign strain P1B to a new *Desulfovibrio* species, as was the case with *Desulfomicrobium escambium* (Sharak Genthner et al. 1994), which differs from *Desulfomicrobium baculatus* in the same order of magnitude. Therefore, the new species *Desulfovibrio oxyclinae* is suggested.

Description of *Desulfovibrio oxyclinae* sp. nov.

O.xy.'cli.ae. Gr. adj. *oxys*, acidic; Gr. v. *clinein*, decline; *oxyclinae* referring to the oxycline as habitat. Straight or curved rods, 0.5 µm wide and 2–3 µm long. Gram-negative. Motile. Salinity range: 2.5–22.5% NaCl; optimum at 5–10% NaCl. Catalase is present. Reduces oxygen, sulfate, sulfite, thiosulfate, sulfur, and fumarate. Grows only anaerobically. Substrates used for aerobic respiration: hydrogen, lactate, sulfide, and sulfite. Sulfide and sulfite are oxidized to sulfate. Substrates oxidized by sulfate reduction: hydrogen, formate, lactate, pyruvate, malate, ethanol, propanol, and butanol. Ferments pyruvate; grows also by disproportionation of sulfite or thiosulfate. Desulfoviridin is present. Vitamins and growth factors are not required but may result in increased growth yields. Habitats: oxic zone of hypersaline cyanobacterial mats. Type strain: P1B, DSM (deposited in the Deutsche Sammlung von Mikroorganismen, Göttingen, Germany).

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