Elemental Sulfur and Thiosulfate Disproportionation by Desulfocapsa sulfoexigens sp. nov., a New Anaerobic Bacterium Isolated from Marine Surface Sediment

KAI FINSTER,1,* WERNER LIESACK,2 AND BO THAMDRUP3

Department of Microbial Ecology, Institute of Biological Sciences, University of Aarhus, DK-8000 Aarhus C, Denmark,1 and Max-Planck-Institut für terrestrische Mikrobiologie, D-35043 Marburg,2 and Max-Planck-Institut für marine Mikrobiologie, D-28359 Bremen,3 Germany

Received 25 June 1997/Accepted 10 October 1997

A mesophilic, anaerobic, gram-negative bacterium, strain SB164P1, was enriched and isolated from oxidized marine surface sediment with elemental sulfur as the sole energy substrate in the presence of ferricydrite. Elemental sulfur was disproportionated to hydrogen sulfide and sulfate. Growth was observed exclusively in the presence of a hydrogen sulfide scavenger, e.g., ferricydrite. In the absence of a scavenger, sulfide and sulfate production were observed but no growth occurred. Strain SB164P1 grew also by disproportionation of thiosulfate and sulfate. With thiosulfate, the growth efficiency was higher in ferricydrite-supplemented media than in media without ferricydrite. Growth coupled to sulfate reduction was not observed. However, a slight sulfide production occurred in cultures incubated with formate and sulfate. Strain SB164P1 is the first bacterium described that grows chemolithoautotrophically exclusively by the disproportionation of inorganic sulfur compounds. Comparative 16S rDNA sequencing analysis placed strain SB164P1 into the delta subclass of the class Proteobacteria. Its closest relative is Desulfocapsa thiocymogenes, and slightly more distantly related are Desulfofatisis glycolicus and Desulfhorhalos vacuolatus. This phylogenetic cluster of organisms, together with members of the genus Desulfobulbus, forms one of the main lines of descent within the delta subclass of the Proteobacteria. Due to the common phenotypic characteristics and the phylogenetic relatedness to Desulfocapsa thiocymogenes, we propose that strain SB164P1 be designated the type strain of Desulfocapsa sulfoexigens sp. nov.

Since Bak and Cyponka (2) first described an energy metabolism based on the disproportionation of the inorganic sulfur compounds thiosulfate and sulfate into sulfide and sulfate by a sulfate reducer designated Desulfovibrio sulfodismutans (3), this type of process has been recognized in numerous genera of sulfate-reducing bacteria (15). However, most of the sulfate reducers tested were unable to couple the disproportionation of inorganic sulfur compounds to growth. Disproportionation can be described as an inorganic fermentation in which sulfur compounds with an intermediate oxidation state serve as both electron donors and electron acceptors in an energy-generating redox process (27). In recent years, it was demonstrated that thiosulfate disproportionation is an important metabolism in sulfur transformations in aquatic systems (13) and that microorganisms which are capable of carry out this type of metabolism are numerically abundant (3, 14).

Recently, Thamdrup et al. (32) discovered that enrichment cultures with elemental sulfur and iron or manganese oxides were able to grow by disproportionation of elemental sulfur. This observation was surprising, because under standard conditions the reaction is endergonic with ΔG° = 40.9 kJ reaction−1 (2). The authors stressed that the use of oxidized metals as sulfide scavengers makes the process energetically favorable. Disproportionation of elemental sulfur in pure cultures was first reported by Bak (1). The organism, later named Desulfocapsa thiocymogenes, is a freshwater sulfate reducer that grows either heterotrophically by incomplete oxidation of different alcohols or chemolithoautotrophically by disproportionation of thiosulfate, sulfite, or elemental sulfur (12). Apart from the recently described species Desulfhorhalos vacuolatus (11) and Desulfofatisis glycolicus (9; see below), Desulfocapsa thiocymogenes is most closely related to Desulfobulbus spp. Recently, Lovley and Phillips (18) have demonstrated that Desulfobulbus propionicus 1p3 is also able to disproportionate elemental sulfur. However, this metabolism was observed only when ferric iron was present in the medium as a sulfide scavenger, and growth was very slow.

In this communication, we report on the enrichment, isolation, and phylogenetic affiliation of a marine microorganism that grows exclusively chemolithoautotrophically by elemental sulfur, thiosulfate, and sulfite disproportionation.

MATERIALS AND METHODS

Sources of inoculum, media, enrichment, and isolation. Oxidized surface sediment from an eelgrass (Zostera marina)-covered mud flat in the basin of Arcachon (France) served as the inoculum for a dilution series. Aliquots (9 ml) of carbonate-buffered marine minimal medium supplemented with trace metals and vitamins (for details, see reference 32; the only modification was the use of 20 g of NaCl liter−1 instead of 15 g liter−1) were dispensed into sterile 16-ml glass test tubes. The test tubes had been supplied in advance with 1 ml of a 300 mM heat-sterilized suspension of ferricydrite (Fe(OH)3) and 200 to 300 mg of sterile sulfur flowers. A 1-cm2 portion of oxidized surface mud was added via a tip-cut plastic syringe to the first test-tube in a series of seven. The medium and the sediment was thoroughly mixed, and 1 ml of the homogenized slurry was transferred to the next test tube. The procedure was repeated until all the tubes had been inoculated. The gas phase of each tube was exchanged for a gas mixture of 90% N2–10% CO2, and the tubes were sealed with black rubber stoppers. The inoculated test tubes were incubated in the dark at 22°C for up to 12 weeks. Positive enrichments were identified by the formation of iron sulfides produced during the disproportionation of elemental sulfur (32). Sulfur-disproportionating bacteria were isolated by deep agar dilutions (23) prepared as described by Finster et al. (7). The isolation tubes contained two separated agar layers, one consisting of 1 ml of ferricydrite suspension in 3 ml of 3% agar and the second containing inoculum and elemental sulfur in 6 ml of 1% agar. Colonies were withdrawn from the agar deeps with drawn Pasteur pipettes and transferred to
liquid medium containing ferrihydrite and elemental sulfur. Tubes containing sulfur-disproportionating bacteria were identified by iron sulfide formation. The purification procedure was repeated by deep agar dilutions in basal marine medium supplemented with ferrous iron (0.52 g of FeCl$_2$·H$_2$O liter$^{-1}$) instead of the iron sulfide layer and thiosulfate (10 mM) instead of elemental sulfur. Growing colonies were directly identifiable as black spots in the agar as a result of iron sulfide precipitation. The colonies were transferred to liquid medium containing thiosulfate in ferrous iron medium, and grown cultures were examined microscopically to compare the morphology with that of the original isolate from elemental sulfur-containing medium. Also, the capability of elemental sulfur disproportionation was tested. Cultures were routinely grown with elemental sulfur in the presence of ferrihydrite.

**Substrate tests.** The spectrum of electron donors and electron acceptors used by the isolate was determined by using 20-ml screw-cap test tubes filled to the rim. The tubes were filled with only 10 ml of medium when oxygen was supplied as the electron acceptor. The bacteria were precultivated with elemental sulfur and ferrihydrite. The inoculum was 1% (vol/vol) of a pregrown culture. In electron donor tests, either sulfur or thiosulfate (10 mM), sulfate (10 mM), dimethyl sulfide (10 mM), nitrate (5 mM), or oxygen (0.02 atm) served as the electron acceptor. Test medium containing elemental sulfur or thiosulfate and ferrihydrite served as positive controls. In electron donor tests when sulfate served as electron acceptor, ferrihydrite was added to visualize the production of hydrogen sulfide. The sulfate-reducing capacity of strain SB164P1 was also assessed in ferric iron-free incubations with sulfate (10 mM), formate (10 mM), acetate, propionate, pyruvate, lactate, fumarate, or hydrogen (90%) as the electron acceptor, ferrihydrite was added to visualize the production of hydrogen sulfide. The temperature range for growth was determined by incubating inoculated 20-ml screw-cap test tubes with elemental sulfur and ferrihydrite. Activity was determined by incubating inoculated test tubes in an insulated aluminum temperature gradient block with a minimum temperature of 0°C and a maximum temperature of 50°C in 5°C steps. All experiments were carried out in duplicate and stopped after a maximum of 6 weeks.

Growth conditions were evaluated by comparing the time passed until iron sulfide formation was observed. The fastest iron sulfide formation was interpreted as optimal conditions.

**Molybdate addition.** Thiosulfate-ferrihydrite-grown cultures of strain SB164P1 were inoculated into marine medium with thiosulfate plus ferrihydrite or elemental sulfur plus ferrihydrite (0.52 g of FeCl$_2$·H$_2$O liter$^{-1}$) instead of the iron sulfide layer and thiosulfate (10 mM) instead of elemental sulfur. Growing colonies were directly identifiable as black spots in the agar as a result of iron sulfide precipitation. The colonies were transferred to liquid medium containing thiosulfate in ferrous iron medium, and grown cultures were examined microscopically to compare the morphology with that of the original isolate from elemental sulfur-containing medium. Also, the capability of elemental sulfur disproportionation was tested. Cultures were routinely grown with elemental sulfur in the presence of ferrihydrite.

**Analytical procedures.** Sulfide was determined spectrophotometrically by the methylene blue method (5). Sulfate and thiosulfate were quantified by nontagged anion-exchange chromatography (pump model 510, I.Pak anion column, conductivity detector model 430 (Waters). HCl-extracted Fe$^{2+}$ was measured spectrophotometrically by the ferrozine method (6).

**Stoichiometry of the disproportionations.** The transformations of thiosulfate and elemental sulfur were quantified in 250-mI batch cultures in medium with and without ferrihydrite. Cultures were inoculated with 2% (vol/vol) of a pregrown culture, incubated at 28°C in the dark, and sampled regularly for 11 days when thiosulfate was the substrate and for 24 days when elemental sulfur was the substrate. Aliquots for sulfate, dissolved sulfide, and thiosulfate determination were filtered and preserved unfilled in zinc acetate (final concentration, 5%). While aliquots for iron sulfides were preserved unfilled in zinc acetate (final concentration, 5%). Aliquots for cell counts were fixed in glutaraldehyde (final concentration, 2.5%). Aliquots of 100 μl for Fe(II) measurements were extracted in 5 ml of 5.5 M nitric acid. Immediately after acidifying, the headspace of the incubation bottles was flushed with a gas mixture consisting of 90% N$_2$–10% CO$_2$, and the bottles were sealed with rubber stoppers.

**Substrate tests.** The spectrum of electron donors and electron acceptors used by the isolate was determined by using 20-ml screw-cap test tubes filled to the rim. The tubes were filled with only 10 ml of medium when oxygen was supplied as the electron acceptor. The bacteria were precultivated with elemental sulfur and ferrihydrite. The inoculum was 1% (vol/vol) of a pregrown culture. In electron donor tests, either sulfur or thiosulfate (10 mM), sulfate (10 mM), dimethyl sulfide (10 mM), nitrate (5 mM), or oxygen (0.02 atm) served as the electron acceptor. Test medium containing elemental sulfur or thiosulfate and ferrihydrite served as positive controls. In electron donor tests when sulfate served as electron acceptor, ferrihydrite was added to visualize the production of hydrogen sulfide. The sulfate-reducing capacity of strain SB164P1 was also assessed in ferric iron-free incubations with sulfate (10 mM), formate (10 mM), acetate, propionate, pyruvate, lactate, fumarate, or hydrogen (90%) as the electron donor (concentrations, 5 mM if not otherwise indicated). The test tubes were incubated at 26°C in the dark. All tests were carried out in duplicate, and the tubes were incubated for 4 weeks and periodically examined for sulfide production and/or growth.

**Growth conditions.** Growth parameters. The temperature tolerance of the isolate, were studied by incubating the isolate in marine medium supplemented with elemental sulfur and ferrihydrite. Cultures were routinely grown with elemental sulfur in the presence of ferrihydrite. Growth parameters.

**Nucleotide sequence accession number.** The 16S rDNA sequence of strain SB164P1 has been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Y13672.
RESULTS

Enrichment cultures. Black spots in the reddish brown ferrihydrite, indicating the formation of iron sulfide, were observed in the first dilution step after 4 days and occurred up to the fifth dilution step. Sulfate accumulated concomitantly with iron sulfide. Cultures from the highest dilution with growth (10^7) were used for further isolation. Microscopic examination revealed two major bacterial morphotypes. The predominant type was rod shaped and slender, and some cells were motile. The other type was coccolid and nonmotile. Both types grew in close proximity to the particle phase, and an increased number of cells were seen after particle disruption. Bacteria were seldom observed in supernatant aliquots. In addition to the dominating cell types, small, highly motile vibrioid cells were observed. The culture used for isolation was purified by repeated application of deep agar dilution series.

Isolation. In low agar dilutions (up to tube 3), large amounts of black iron sulfide developed within 3 weeks of incubation and obscured the bacterial colonies. In the higher dilutions, only a little iron sulfide was produced, allowing the identification of bacterial colonies under the dissection microscope. The colonies were round and whitish and developed in close proximity to sulfur particles. A microscopic examination of an isolated colony revealed only slender rod-shaped cells. After a transfer to liquid medium, five morphologically identical cultures were obtained. The cultures were able to grow with thiosulfate as a substitute for elemental sulfur. An additional purification was conducted with thiosulfate and Fe^{2+}, leaving out the iron agar layer. Thiosulfate-disproportionating colonies were black due to iron precipitates. Well-separated colonies from the highest dilution were transferred to liquid thiosulfate-containing medium. Microscopic examination of the colonies revealed the same structure and cell type as described for the sulfur-ferrihydrite agar shakes. The six isolates were able to grow by elemental sulfur disproportionation. One isolate, designated SB164P1, was chosen for detailed physiological and phylogenetic characterization.

Morphology. Strain SB164P1 cells were 2 to 4 μm long and 0.5 μm wide with slightly pointed ends (Fig. 1). Motile cells were observed. Cells from outgrown cultures stained gram negative.

Growth and temperature requirements. Strain SB164P1 did not require organic carbon or vitamins for growth, but NaCl and MgCl_2 were necessary for growth. Growth was inhibited below 25 mM NaCl and 2 mM MgCl_2 · 6H_2O. The cultures developed equally well within concentration ranges of 170 to 330 mM NaCl and 7 to 15 mM MgCl_2 · 6H_2O, respectively. Higher concentrations were not tested. The pH range for growth was from 6.0 to 8.2 with an optimum between 6.7 and 7.3. In growing cultures, the pH could drop below 6.0 when elemental sulfur served as the substrate. The pH tests were carried out in bicarbonate-buffered medium only. Strain SB164P1 grew within a temperature range from 5 to 35°C. The optimum growth temperature was 30°C.

Metabolic properties. Apart from sulfur, thiosulfate, and sulfite disproportionation, strain SB164P1 reduced small amounts of sulfate in the presence of formate and hydrogen. Within a period of 6 weeks, 0.09 and 0.11 mM sulfide were produced in the presence of formate and hydrogen, respectively. Sulfite reduction did not sustain growth. No sulfide was detectable (detection limit, 1 μM) in the other incubations. Sulfate reduction with formate was also demonstrated in incubations with radiolabeled sulfate. Radiolabeled sulfate accumulated exclusively with formate as the electron donor. A fermentative type of metabolism with pyruvate, lactate, or glucose was not observed. Neither nitrate, dimethyl sulfoxide, nor oxygen (2%) was used as the electron acceptor.

Disproportionation of sulfur compounds. Elemental sulfur and thiosulfate were disproportionated by strain SB164P1 with or without ferric iron present in the growth medium (Fig. 2 and 3). The rate of thiosulfate consumption was similar in the presence and absence of ferrihydrite. In ferric iron-free medium (Fig. 2, top), the ratio of sulfate produced to thiosulfate consumed was 1:1, in agreement with thiosulfate disproportionation:

\[
\text{S}_2\text{O}_3^{2-} + \text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + \text{HS}^- + \text{H}^+ 
\]

Sulfide production was somewhat lower than predicted, which can be explained by degassing of H_2S. When ferrihydrite was present as a sulfide scavenger during thiosulfate disproportionation (Fig. 2, middle), the amount of sulfate plus sulfide produced closely matched the amount of thiosulfate consumed. Under these conditions, sulfide accumulated as FeS and dissolved sulfide was not detectable, so that loss through degassing was excluded. Ferrihydrite reacts rapidly with hydrogen sulfide, producing Fe^{2+}. Ferrous iron may further precipitate sulfide as FeS (24), and elemental sulfur formed may be further disproportionated. This results in a sulfide to sulfate accumulation ratio smaller than 1.0.

With elemental sulfur, strain SB164P1 produced sulfide and sulfate at a ratio close to 3:1 at the beginning of the incubation (Fig. 3, top), in agreement with thiosulfate disproportionation:

\[
4\text{S}_0 + 4\text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + 3\text{HS}^- + 5\text{H}^+ 
\]

After 10 days, the ratio shifted to lower values. Removal of sulfide by degassing during sampling is again the most likely explanation. About 10 times as much sulfate was produced in the presence of ferrihydrite (Fig. 3, middle). Free sulfide was not detected. Sulfide accumulated as both FeS and pyrite, FeS_2. Pyrite may form through the following reaction (4):

\[
\text{FeS} + \text{S}^0 \rightarrow \text{FeS}_2 
\]

The final ratio of Fe(III) consumed to sulfate produced was 2:1, as expected for disproportionation in the presence of ferrihydrite (32).
Strain SB164P1 also grew by sulfite disproportionation in the absence of ferrihydrite (data not shown).

**Growth by sulfur and thiosulfate disproportionation.** Strain SB164P1 grew exponentially at the beginning of the incubation period except with elemental sulfur in ferrihydrite-free medium (Fig. 2 and 3, bottom). With elemental sulfur and ferrihydrite, growth ceased after 7 days while sulfur disproportionation continued throughout the entire incubation period. However, the rate of sulfur disproportionation decreased from day 7 until the end of the incubation. Growth cessation coincided with the depletion of Fe(III). The doubling times were 1 and 1.5 days with thiosulfate in the presence and absence of Fe(III), respectively, and 2 days with elemental sulfur and ferrihydrite in the medium. Cultures grew without a lag phase when iron was present in the medium, while the iron-free thiosulfate cultures were delayed by 1 day. In the latter culture, growth ceased after 4 days, when 1.2 mM thiosulfate were consumed. The disproportionation of the remaining 3 mM was not accompanied by growth. Thiosulfate-ferrihydrite cultures grew exponentially at the beginning of the incubation. Afterwards, growth was linear with a slow increase throughout the rest of the incubation. The shift in the growth pattern was paralleled by the time courses of thiosulfate degradation and sulfate and ferrous iron production.

**Molybdate addition.** Thiosulfate and elemental sulfur disproportionation were affected by the presence of molybdate. Both thiosulfate and elemental sulfur disproportionation were inhibited completely by 5 mM molybdate. At lower concentra-
DISCUSSION

Strain SB164P1 was routinely cultured in marine medium containing elemental sulfur as a source of energy and reduction equivalents and ferric iron as a sulfide scavenger. It grew exclusively by disproportionation of elemental sulfur, thiosulfate, or sulfite. With thiosulfate and sulfite, a sulfide scavenger was not required, although growth with thiosulfate was stimulated by the presence of ferrihydrite. Growth by elemental sulfur disproportionation has recently been described for marine enrichment cultures (32) and the freshwater isolates Desulfocapsa thiozymogenes (12) and Desulfobulbus propionicus (1), which, however, were both grown with acetate as the carbon source. In the presence of ferric iron, the growth yield was 1.3 g (dry weight) mol of thiosulfate$^{-1}$. Based on the growth yields given above, we can estimate the growth efficiency of strain SB164P1 with thiosulfate or elemental sulfur. Assuming that (i) strain

![Phylogenetic tree](image-url)
SB164P1 grew autotrophically and CO₂ assimilation produced glucose or organic carbon with the same chemical composition and formation energy as those of glucose and that (ii) thiosulfate or elemental sulfur was the only energy and electron source, we calculate a carbon yield of 3.5 g of C mol of thiosulfate⁻¹. The isolate produced 0.94 g (dry weight) mol of thiosulfate⁻¹, corresponding to 0.38 g of C (using a 40% [by weight] C content of glucose), or 10.7% of the theoretically possible amount of carbon. In the presence of elemental sulfur, a growth efficiency of approximately 3% can be determined. These values are in the range of thermodynamic efficiency values of chemolithoautotrophic sulfide-oxidizing bacteria compiled by Nelson and Hagen (22).

Effect of molybdate. The inhibitory effect of molybdate on sulfate reduction is well understood (31). By formation of adenosine-5-phosphomolybdate, molybdate depletes the internal ATP pool of the cell. Interestingly, molybdate also inhibited elemental sulfur and thiosulfate disproportionation. Recently, Krämer and Cypionka (15) and Fusetier and Cypionka (10) proposed that ATP synthesis by disproportionation results from the inversion of the initial steps of sulfate reduction, including the formation of adenosine-5-phosphosulfate through substrate-level phosphorylation. Molybdate may interfere with the formation of APS and consequently may inhibit disproportionation reactions. We have no explanation why sulfur disproportionation was more sensitive to molybdate than was thiosulfate disproportionation.

Ecological considerations. Strain SB164P1 is the first marine bacterium found to be able to thrive by disproportionation of oxidized inorganic sulfur compounds alone. Since it was isolated from the fifth step in a series of 10-fold dilutions, it appears to be abundant in the sediment. Up to 10⁶ cells per cm² of sulfur-disproportionating bacteria have previously been counted in marine sediments with a medium similar to that used in the present study (32). Most-probable-number counts of sulfate reducers in sediments from Arcachon Bay range from 10⁶ to 10⁷ cells per cm² (35). The occurrence of obligate sulfur disproportionators in numbers comparable to those of sulfate reducers is a strong indication that disproportionation is a common way of living in these environments. This is further accentuated when the relatively low growth yield of the process is considered. Jørgensen and Bak (13) argued on the basis of most-probable-number counts that thiosulfate disproportionation in marine environments is mainly a cometabolism executed by sulfate reducers but not a major type of metabolism. These authors, however, did not include a sulfide scavenger in their medium. In natural sediments, the scavenging of hydrogen sulfide by iron oxides is a very important process, which often maintains sulfide concentrations at low levels in spite of intense sulfide production through sulfate reduction. We conclude from the results of our studies that the presence of a sulfide scavenger is essential for counting, enrichment, and isolation of SB164P1-like bacteria and, consequently, that life by disproportionation is more common than has so far been assumed.

Phylogenetic position. The closest relative of strain SB164P1 is Desulfooccus thiyozymogenes. Desulforhopalus vacuolatus and Desulfobulbus propionicus are slightly more distantly related (Fig. 4). From the phylogenetic point of view, the dissimilarity value of 8.5% between the 16S rRNA sequences of Desulfooccus thiyozymogenes and strain SB164P1 would allow us to propose the taxonomic rank of a new genus for the latter organism. However, considering the very similar phenotypic and genotypic properties, including the G+C content of the genomic DNA (47.2% versus 50.7%), we favour the inclusion of strain SB164P1 into the genus Desulfoocpus and suggest the species name D. sulfoexigens.

Description of Desulfoocpus sulfoexigens sp. nov. Desulfococcus sulfoexigens (sul.fo.ex.i.gens. L. n. sulfurum, sulfur; L. v. exigo, to demand; M. L. part. adj. sulfoexigens, demanding sulfur for growth). Gram-negative cells that are elongated with slightly pointed ends, 0.5 μm wide and 2 to 4 μm long. Multiplication occurs by binary fission. The pH range is from 6.0 to 8.2 with an optimum of 6.7 to 7.3. The temperature range is 4 to 35°C with an optimum of 30°C. The habitat is oxidized marine surface sediment. The type strain was isolated from marine sediment covered by the eelgrass, Zostera noltii, in the basin of Arcachon, France.

The G+C content of the genomic DNA is 47.2 ± 0.2 mol%.

The type strain is strain SB164P1, DSM 10523, deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

ACKNOWLEDGMENTS

K.F. is indebted to Niels Peter Revsbech for support. Sonja Fleissner and Swantje Fleischer are acknowledged for technical assistance. We thank three anonymous reviewers for constructive criticism of the manuscript.

The study was financed by a European Union project on Coastal Lagoon Eutrophication and Anaerobic Processes (C.L.A.E.N) (contract EVSV-CT92-006), by the Danish Committee on Biotechnology, by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (contract 21/0311221), and by the Max Planck Society.

REFERENCES


