***Desulfomusa hansenii* gen. nov., sp. nov., a novel marine propionate-degrading, sulfate-reducing bacterium isolated from *Zostera marina* roots**

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The physiology and phylogeny of a novel sulfate-reducing bacterium, isolated from surface-sterilized roots of the marine macrophyte *Zostera marina*, are presented. The strain, designated P1^T, was enriched and isolated in defined oxygen-free, bicarbonate-buffered, iron-reduced seawater medium with propionate as sole carbon source and electron donor and sulfate as electron acceptor. Strain P1^T had a rod-shaped, slightly curved cell morphology and was motile by means of a single polar flagellum. Cells generally aggregated in clumps throughout the growth phase. High CaCl₂ (10 mM) and MgCl₂ (50 mM) concentrations were required for optimum growth. In addition to propionate, strain P1^T utilized fumarate, succinate, pyruvate, ethanol, butanol and alanine. Oxidation of propionate was incomplete and acetate was formed in stoichiometric amounts. Strain P1^T thus resembles members of the sulfate-reducing genera *Desulfobulbus* and *Desulforhopalus*, which both oxidize propionate incompletely and form acetate in addition to CO₂. However, sequence analysis of the small-subunit rDNA and the dissimilatory sulfite reductase gene revealed that strain P1^T was unrelated to the incomplete oxidizers *Desulfobulbus* and *Desulforhopalus* and that it constitutes a novel lineage affiliated with the genera *Desulfococcus*, *Desulfosarcina*, *Desulfonema* and '*Desulfobotulus*'. Members of this branch, with the exception of '*Desulfobotulus sapovorans*', oxidize a variety of substrates completely to CO₂. Strain P1^T (= DSM 12642^T = ATCC 700811^T) is therefore proposed as *Desulfomusa hansenii* gen. nov., sp. nov. Strain P1^T thus illustrates the difficulty of extrapolating rRNA similarities to physiology and/or ecological function.

Keywords: propionate, *Desulfobulbus*, incomplete oxidation, *Desulfomusa hansenii* gen. nov., sp. nov.

INTRODUCTION

High sulfate reduction rates have been measured in the rhizosphere of numerous marine macrophytes (Blaabjerg *et al.*, 1998 and references therein) and with isolated roots of the eelgrass *Zostera marina*, *Zostera*

noltii and *Spartina maritima* (Blaabjerg & Finster, 1998; Nielsen *et al.*, 2001). Despite this fact, only a few studies have been carried out describing sulfate-reducer populations in these habitats. For instance, Hines *et al.* (1999), investigating the population composition and biogeochemistry of sulfate-reducing bacteria in the rhizosphere of *Spartina alterniflora*, found that the majority of the rRNA from sulfate-reducing bacteria was from members of the family *Desulfobacteriaceae*. In a previous study, Rooney-Varga *et al.* (1997) showed that within the family *Desulfobacteriaceae*, rRNA clones related to the *Desulfosarcina*/*Desulfococcus*/*Desulfonema* assemblage pre-

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Abbreviations: DSR, dissimilatory sulfite reductase; SSU, small subunit.

The GenBank accession numbers of the 16S rDNA sequence and the dissimilatory sulfite reductase gene sequence of strain P1^T are AF321820 and AF321819, respectively.

dominated. In both papers, the authors used genetic data to speculate upon the ecological role and the physiology of the organisms from which the rRNA was derived. They concluded that members of the *Desulfosarcina*/*Desulfococcus*/*Desulfonema* assemblage that have a versatile substrate spectrum are particularly well adapted to the rhizosphere environment. The number of isolated sulfate reducers from rhizosphere environments is even more limited. Recently, Nielsen *et al.* (1999) reported on the isolation and characterization of a fructose-degrading sulfate reducer from the cortex of *Zostera marina* roots, *Desulfovibrio zosterae* sp. nov. *Desulfovibrio zosterae* is, to our knowledge, the only sulfate reducer that has been isolated from root tissue of a marine macrophyte. From the same batch of surface-sterilized roots from which *Desulfovibrio zosterae* was isolated, a propionate-degrading sulfate reducer, designated strain P1^T, was also enriched and isolated. The following communication reports on the characterization of this micro-organism. Interestingly, strain P1^T shared many phenotypic traits with the incomplete oxidizing genera *Desulfobulbus* and *Desulforhopalus*, but was affiliated phylogenetically with the complete oxidizing members of the *Desulfosarcina*/*Desulfococcus*/*Desulfonema* assemblage.

METHODS

Source of organisms. Strain P1^T was isolated from a mixed culture obtained from surface-sterilized roots of the eelgrass *Zostera marina*. Root surface sterility was achieved by washing sediment-free roots in a saline 1.05% (w/v) hypochlorite solution for 30 s (Blaabjerg & Finster, 1998). Isolation of strain P1^T was achieved by repeated application of the agar-shake dilution method in an iron-rich APW medium previously described by Coates *et al.* (1995). Propionate (10 mM) and sulfate (10 mM) served as electron donor/carbon source and electron acceptor, respectively. Colonies of sulfate reducers were identified by their black colour due to ferrous sulfide precipitation. Six morphologically identical strains were isolated. One strain, designated strain P1^T, was studied in detail. Culture purity was examined in sulfate-free APW medium, which was supplemented with fumarate (5 mM), pyruvate (5 mM), glucose (5 mM) and 0.1% (w/w) yeast extract. In addition, cultures were regularly checked for purity by phase-contrast microscopy.

Desulfovibrio desulfuricans subsp. *desulfuricans* DSM 1926 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. It was used as a positive control in the desulfoviridin test. *Rhodococcus* sp. was obtained from our own culture collection. It served as a positive control in the Gram-staining test.

Culture medium and substrates. Pure cultures of strain P1^T were routinely cultivated in defined APW medium described by Coates *et al.* (1995), with the modification that iron was only added to the enrichment medium in trace amounts. The modification, which did not affect growth of strain P1^T allowed measurement of the population density by OD determination. Iron as reducing agent was replaced by Na₂S · 9H₂O. The medium was prepared according to

Widdel & Bak (1992). The following sterile solutions were added aseptically to 1 l autoclaved salt medium: NaHCO₃ solution (1 M), 30 ml; Na₂S solution (0.2 M), 7.5 ml; trace metal solution, 1 ml (SL 10a); vitamin solutions; and selenite-tungstate solution (0.02 mM), 1 ml. The medium was distributed into 50 ml bottles. The bottles were closed with rubber-sealed aluminium screw caps. Electron donors, carbon sources and acceptors were added from sterile stock solutions to give the concentrations desired.

Electron donor and acceptor tests. Electron donor/acceptor tests were carried out in 20 ml screw-capped test tubes in duplicate. Hydrogen consumption was studied in 50 ml glass bottles containing 20 ml medium and a headspace filled with a mixture of hydrogen gas and CO₂ (90:10 v/v). Acetate (1 mM) was added as carbon source. Substrate fermentation was tested in sulfate-free medium. Controls contained basal medium and inoculum but no additional electron donor or acceptor. Diazotrophic growth was tested for in NH₄Cl-free medium.

Analytical procedures. Propionate and acetate were analysed by ion-exclusion chromatography using an Aminex HPX-87H column (Bio-Rad) for compound separation. H₂SO₄ (0.05 mM) was used as eluent. The flow rate was 0.9 ml min⁻¹, oven temperature was 65 °C and the injected sample volume was 100 µl. The compounds were measured with a UV detector at 210 nm. The samples were centrifuged and filter-sterilized (0.2 µm) prior to analyses. Sulfate was determined by suppressed ion chromatography as described by Isaksen & Finster (1996). The pH, salt and temperature tolerances of strain P1^T were studied by growth tests in which the change in OD₆₀₀ was monitored. Experiments were carried out in duplicate. Generation times were calculated from the increase in culture OD over time. Polyglucose was determined as glucose equivalents. Exponentially grown cells were harvested by centrifugation, washed in fresh medium and treated with 1 M H₂SO₄ for 6 h. Glucose was measured by a UV method according to the manufacturer's instructions (Boehringer Mannheim). The presence of poly-β-hydroxybutyrate was examined by staining cells with Sudan black B made up in ethylene glycol and microscopic inspection of the stained specimen. The appearance of red fluorescence in light of 366 nm after cells were treated with 2 M NaOH was used to determine the presence of desulfoviridin (Widdel & Pfennig, 1984). Catalase was assayed by treating a dense cell suspension with few drops of a 3% H₂O₂ solution and examination of bubble formation.

The G + C content was determined at the DSMZ according to a standard protocol, which included methods developed by Mesbah *et al.* (1989), Tamaoka & Komagata (1984) and Visuvanathan *et al.* (1989).

Electron microscopy. Whole-cell photographs were obtained with cells that were fixed on a carbon/celloidin-impregnated copper grid (Electron Microscopy Sciences) and stained with uranyl acetate. TEM pictures were taken with a Philips CM 20 transmission electron microscope operating at 120 keV. Thin sections were prepared according to standard methodologies. Pictures were taken with a Zeiss 10 b transmission electron microscope.

Nucleic acid extraction, PCR amplification and sequencing. Nucleic acids were extracted using the FastDNA spin kit for soil (Bio 101) according to the manufacturer's instructions. The small-subunit (SSU) rDNA was PCR-amplified and sequenced as described by Lane (1991). A sequence of 1519 nt was obtained after sequencing both strands with

multiple primers. The dissimilatory sulfite reductase (DSR) gene was amplified using the selective primers designed by Wagner *et al.* (1998). Direct sequencing of the DSR gene only produced short sequences. Therefore, DSR amplicates were ligated into a pCR-XL-TOPO vector and transformed into ONE SHOT *Escherichia coli* cells according to the manufacturer's directions (TOPO XL PCR Cloning; Invitrogen). Randomly selected clones containing inserts of the right length were sequenced with a thermosequenase fluorescent cycle sequencing kit (Pharmacia Biotech) and an ALFexpress DNA sequencer (Pharmacia Biotech). A partial sequence of 1263 nt was obtained.

Phylogenetic analysis. rDNA sequences were aligned to the RDP alignment version 7.1 using the on-line services of RDP (<http://www.cme.msu.edu/rdp/>). Sequences from close relatives and phylogenetically representative SSU were also obtained from RDP. The alignment was corrected manually with SEQPUP version 0.6. Only sequence positions that were unambiguously aligned were included in the phylogenetic analysis. DSR nucleotide sequences were translated to amino acids and aligned manually with the Genetic Data Environment version 2.2 sequence editor implemented in the ARB software environment (January 1996 version; Strunk & Ludwig, 1998). Trees of both SSU rDNA sequences and DSR amino acid sequences were constructed using the program PAUP* version 4.0 (Swofford, 2000). The nucleotide data matrix was analysed by distance matrix and maximum-likelihood approaches, whereas the amino acid matrix was evaluated by parsimony and distance matrix analysis. All analyses used the default settings in PAUP* 4.0. Bootstrap analyses based on 100 replicates were performed with the distance matrix datasets. Branching patterns that were not supported by more than 50% of the bootstrap runs were eliminated by making multifurcations at the appropriate basal node. This was done to avoid showing phylogenetic affiliations that were not well supported by the data.

RESULTS

Enrichment and isolation

A sulfate-reducing enrichment culture from surface-sterilized roots of the eelgrass *Zostera marina* was obtained within 2 weeks of incubation with propionate as electron donor and sulfate as electron acceptor. The culture was dominated by motile, straight to slightly curved, rod-shaped cells. Cells generally aggregated in clumps throughout the growth phase.

Cell morphology

Cells were straight to slightly curved, 3–6 µm long and 2–3 µm wide. They were motile by means of a single polar flagellum (Fig. 1). Cells of old cultures lost their motility. Cells of young cultures were characterized by a spore-like shiny appearance under phase-contrast microscopy. The shiny appearance vanished in old

Fig. 1. Electron micrograph of negatively stained cells of strain P1^T. Bar, 5 µm.

Fig. 2. Electron micrograph of a thin section of strain P1^T with bright spherical inclusions. Bar, 1 µm.

Fig. 3. Phylogenetic tree based on SSU rRNA sequences showing the relationship between strain P1^T and its closest relatives as well as other reference organisms of the δ -Proteobacteria. The SSU rRNA sequence of *E. coli* was used as outgroup. The tree was constructed using a heuristic search algorithm with default settings for the distance matrix analysis in PAUP* 4.0. Only nodes supported by bootstrap numbers greater than 50% are shown. Other nodes are represented as multifurcations. Numbers on nodes indicate bootstrap values and 1404 unambiguous aligned nucleic acids of the SSU rDNA were included in the analysis. Bar, mean number of substitutions per site.

cultures where cells appeared grey. In these cultures, cell stages were observed which contained shiny inclusions. Growing cells contained large numbers of spherical inclusions that were 0.1–0.2 μ m in diameter (Fig. 2). The nature of the inclusion could not be elucidated. However, glucose formation from either polyglucose or poly- β -hydroxybutyrate was not detected. Cells stained Gram-negative.

Physiological and biochemical characteristics

Strain P1^T used sulfate (20 mM), sulfite (5 mM) and elemental sulfur as electron acceptors with propionate (15 mM) as electron donor. Nitrate (2 mM) and thiosulfate (20 mM) were not used. Propionate (15 mM), alanine (15 mM), ethanol (15 mM), butanol

(10 mM), pyruvate (5 mM), succinate (10 mM) and fumarate (10 mM) served as carbon sources and electron donors when sulfate was present as electron acceptor. Hydrogen was also used as an electron donor; growth with H₂ was only observed in the presence of acetate as carbon source. The following compounds were not utilized as electron donors: acetate (15 mM), butyrate (5 mM), long-chain fatty acids (C₅–C₁₆; 2 mM), 1-propanol (5 mM), 2-propanol (5 mM), lactate (15 mM), glucose (5 mM), fructose (5 mM), sucrose (5 mM), benzoate (1 mM), nicotinate (1 mM), choline (5 mM), α -ketoglutarate (5 mM) and betaine (5 mM). A fermentative type of metabolism was not observed.

Strain P1^T required brackish-marine medium for growth (DSMZ signature 198 for *Desulfosarcina*

Fig. 4. Phylogenetic tree based on DSR gene amino acid sequences showing the relationship between strain P1^T and its closest relatives as well as other sulfate reducing bacteria. The DSR gene sequence of *Archaeoglobus* sp. was used as outgroup. The tree was constructed using a heuristic search algorithm with default settings for the distance matrix analysis in PAUP* 4.0. Only nodes supported by bootstrap numbers greater than 50% are shown. Other nodes are represented as multifurcations. Numbers on nodes indicate bootstrap values and 252 unambiguous aligned amino acids of the DSR gene were included in the analysis. Bar, mean number of substitutions per site.

variabilis). However, CaCl₂ and MgCl₂ at concentrations of 10 and 50 mM, respectively, were required for optimal growth. Growth was inhibited at CaCl₂ and MgCl₂ concentrations below 4 and 20 mM, respectively. Optimum growth rate of strain P1^T was observed at pH 7.2–7.4 and at 20 °C. Growth occurred between 30 and 8 °C. At 30 °C, cells were generally bigger than those grown at 25 °C or lower. Cells were catalase-positive. Strain P1^T was desulfovibridin-negative.

Propionate metabolism and G + C content

Propionate was incompletely oxidized to acetate in the presence of sulfate; 1 mol acetate was formed from

1 mol propionate. The following equation for propionate oxidation is proposed:

The amount of propionate that was consumed for cell material synthesis was calculated by the following equation:

thus, 0.0118 mmol propionate are required for 1.0 mg (dry weight) cells and 3.25 g biomass was produced per

mole propionate. Consequently, 4.3 % consumed propionate was incorporated into cell biomass.

The G + C content of DNA of strain P1^T was 53.4 ± 0.3 mol % (mean value of three determinations).

Phylogeny based on comparison of SSU rDNA

Strain P1^T is a member of the δ -*Proteobacteria*. It belongs to a coherent group of micro-organisms, which consists of the genera *Desulfosarcina*, *Desulfococcus* and *Desulfonema* (Fig. 3). All organisms of that group oxidize long-chain fatty acids and, with the exception of '*Desulfobotulus sapovorans*', the oxidation of organic electron donors to CO₂ is complete. Within this phylogenetic cluster, strain P1^T was more closely related to the genus *Desulfosarcina* and to the species *Desulfosarcina variabilis* (91.1 %) than to members of the other genera.

Phylogeny based on comparison of DSR genes

Phylogeny derived from DSR sequences was largely congruent with SSU-based phylogeny (Fig. 4). Since the number of DSR sequences that are available for comparison is lower than the number of SSU rRNA sequences, it is not surprising that minor differences in the branching pattern were observed for the two trees. Nonetheless, the basic association of strain P1^T with the genera *Desulfosarcina*, *Desulfococcus* and *Desulfonema* and not with *Desulfobulbus* was supported by DSR sequence analysis.

DISCUSSION

In this communication, a newly isolated marine sulfate-reducing bacterium, designated strain P1^T, is reported. Like members of the related genera *Desulfobulbus* and *Desulforhopalus*, strain P1^T used propionate as electron donor and carbon source and oxidized it incompletely to acetate and CO₂ with sulfate as electron acceptor (Widdel & Pfennig, 1982; Lien *et al.*, 1998; Isaksen & Teske, 1996). As with members of *Desulfobulbus* and *Desulforhopalus*, strain P1^T also shared the ability to utilize lactate, ethanol, pyruvate, fumarate and hydrogen (Lien *et al.*, 1998; Isaksen & Teske, 1996). However, in contrast to members of these genera, strain P1^T was not able to grow by a fermentative type of metabolism (Laanbroek *et al.*, 1982; Isaksen & Teske, 1996) or to use thiosulfate as electron acceptor. The large inclusions that were observed in cells of strain P1^T were not reported from any of the known representatives of the genera *Desulfobulbus* and *Desulforhopalus*. Smaller inclusions containing polyglucose were, however, found in cells of *Desulfobulbus propionicus* (Stams *et al.*, 1983). Identification of the compound(s) that constituted the inclusions of strain P1^T awaits further investigations. Despite the fact that strain P1^T shared many phenotypic traits with members of the genera *Desulfobulbus* and *Desulforhopalus*, it was only distantly related to

them. A comparison of the SSU rDNA revealed that strain P1^T phylogenetically fell into the *Desulfosarcina/Desulfococcus/Desulfosarcina* assemblage, where it was most closely related to *Desulfosarcina variabilis* with a sequence similarity of 91.1 %. This affiliation was also supported by DSR sequence similarities. Physiologically, members of the *Desulfosarcina/Desulfococcus/Desulfonema* assemblage are generally characterized by their capacity to oxidize their energy sources completely to CO₂. However, like strain P1^T, '*Desulfobotulus sapovorans*' represents an exception to the rule (Widdel & Bak 1992). '*Desulfobotulus sapovorans*' is also an incomplete oxidizer but, in contrast to strain P1^T, '*Desulfobotulus sapovorans*' degrades long-chain fatty acids, a metabolic trait that is characteristic for genera within this assemblage. In addition to long-chain fatty acids, members of the assemblage utilize a wide range of substrates, including aromatic compounds and alkanes (So & Young, 1999). In contrast, the range of substrates used by strain P1^T was relatively restricted and in good agreement with the substrate pattern of the genera *Desulfobulbus* and *Desulforhopalus*.

On the basis of a combined phenotypic and genotypic characterization, strain P1^T was placed in a new genus for which the name *Desulfomusa* gen. nov. is proposed. Strain P1^T is proposed as the type strain of a novel species within this genus with the species name *Desulfomusa hansenii* sp. nov.

Description of *Desulfomusa* gen. nov.

Desulfomusa (De.sul.fo.mu'sa. L. *de* from; L. n. *sulfur* sulfur; M.L. n. *musa* banana; N.L. fem. n. *Desulfomusa* banana-shaped bacterium that reduces sulfate).

Strain P1^T uses sulfate as electron acceptor and reduces it to sulfide. Propionate is incompletely oxidized to acetate and CO₂. Long-chain fatty acids, acetate and aromatic compounds are not oxidized. Sulfate, sulfite and elemental sulfur serve as electron acceptors. A fermentative type of metabolism is not observed. *Desulfomusa* belongs to the δ -*Proteobacteria*; to date the closest relative is *Desulfosarcina variabilis*. According to Rule 20c of the Bacteriological Code (Lapage *et al.*, 1992), *Desulfomusa hansenii*, the only species, is the type species of the genus.

Description of *Desulfomusa hansenii* sp. nov.

Desulfomusa hansenii (han.sen'i.i. N.L. gen. n. *hansenii* of Hansen, named to honour Theo Hansen of The Netherlands, who made important contributions to our understanding of the pathways of organic matter oxidation in sulfate-reducing bacteria).

Cells are straight to slightly curved 2–3 × 3–6 µm. Motile by means of a single polar flagellum. Gram-negative. Cells contain spherical inclusions, which are 0.1–0.2 µm in diameter. CaCl₂ and MgCl₂ are required for growth (optimum concentrations 10 and 50 mM,

respectively). Propionate, alanine, ethanol, butanol, pyruvate, succinate and fumarate serve as carbon sources and electron donors. Optimal pH and temperature for growth are 7.2–7.4 and 20 °C, respectively. Grows diazotrophically. Catalase is present. The DNA G + C content is 53.4 mol % (determined by HPLC). Habitat: roots of the marine macrophyte *Zostera marina*. Type strain is P1^T (= DSM 12642^T = ATCC 700811^T).

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