Desulfopila inferna sp. nov., a sulfate-reducing bacterium isolated from the subsurface of a tidal sand-flat

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A Gram-negative, rod-shaped, sulfate-reducing bacterium (strain JS_SRB250Lac^T) was isolated from a tidal sand-flat in the German Wadden Sea. 16S rRNA gene sequence analysis showed that strain JS_SRB250Lac^T belonged to the *Desulfobulbaceae* (*Deltaproteobacteria*), with *Desulfopila aestuarii* MSL86^T being the closest recognized relative (94.2 % similarity). Higher similarity (96.6 %) was shared with '*Desulfobacterium corrodens*' IS4, but this name has not been validly published. The affiliation of strain JS_SRB250Lac^T to the genus *Desulfopila* was further supported by analysis of *aprBA* gene sequences and shared physiological characteristics, in particular the broad range of organic electron donors used for sulfate reduction. Compared with *Desulfopila aestuarii* MSL86^T, strain JS_SRB250Lac^T additionally utilized butyrate and succinate and grew chemolithoautotrophically with hydrogen as an electron donor. CO dehydrogenase activity was demonstrated, indicating that the reductive acetyl-CoA pathway (Wood–Ljungdahl pathway) was used for CO_2 fixation. Results of cellular fatty acid analysis allowed chemotaxonomic differentiation of strain JS_SRB250Lac^T from *Desulfopila aestuarii* MSL86^T by the presence of $C_{17:0}$ cyclo and the absence of hydroxy and unsaturated branched-chain fatty acids. Based on phylogenetic, physiological and chemotaxonomic characteristics, strain JS_SRB250Lac^T represents a novel species of the genus *Desulfopila*, for which the name *Desulfopila inferna* sp. nov. is proposed. The type strain is JS_SRB250Lac^T (=DSM 19738^T =NBRC 103921^T).

Sandy surface sediments of tidal flats in the Wadden Sea are densely populated by micro-organisms and characterized by high remineralization rates (Ishii *et al.*, 2004; de Beer *et al.*, 2005; Billerbeck *et al.*, 2006b; Musat *et al.*, 2006). Due to their high permeability, seawater drains into the sediment and organic matter is filtered and enriched in the sediment surface, fuelling microbial activity (Huettel & Rusch, 2000; D'Andrea *et al.*, 2002). In addition, pore-water can be transported down several metres to deep sediments by a tide-driven hydraulic pressure gradient and provide microbial communities at these depths with utilizable substrates (Billerbeck *et al.*, 2006a; Røy *et al.*, 2008). This phenomenon was recently supported by the demonstration of large total prokaryotic cell numbers and the presence of a highly abundant and potentially active community of sulfate-reducing bacteria (SRB) at a depth of several metres of a tidal sand-flat ('Janssand', German Wadden Sea; Gittel *et al.*, 2008). In the course of this investigation, strain JS_SRB250Lac^T and four closely related strains were isolated from highly diluted sediment samples from different depths and identified as forming a distinct cluster within the *Desulfobulbaceae* (Gittel *et al.*, 2008). It was furthermore confirmed by *in situ* quantification (CARD-FISH; catalysed reporter deposition–fluorescence *in situ* hybridization) that members of the

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Abbreviation: SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences reported in this study are AM774321, FJ548990 and FJ548989 (16S rRNA, *dsrAB* and *aprBA* gene sequences, respectively, of strain JS_SRB250Lac^T) and FJ548988 (*aprBA* gene sequence of *Desulfopila aestuarii* MSL86^T).

A phase-contrast micrograph of cells of strain JS_SRB250Lac^T and maximum-likelihood trees based on DsrAB and AprBA amino acid sequences are available as supplementary material with the online version of this paper.

Desulfobulbaceae and the *Desulfobacteraceae* dominated the sulfate-reducing community. Therefore, the newly isolated strains appeared to be representative of an *in situ* abundant and active fraction of SRB. The five isolated strains exhibited less than 95 % 16S rRNA gene sequence identity to the closest relative with a validly published name, *Desulfopila aestuarii* MSL86^T (Suzuki *et al.*, 2007), but more than 96 % 16S rRNA gene sequence identity was shared with the marine sulfate reducer '*Desulfobacterium corrodens*' IS4 (Dinh *et al.*, 2004). The latter strain has not yet been described taxonomically. Strain JS_SRB250Lac^T was subjected to a phylogenetic, physiological and chemotaxonomic characterization and comparison with '*Desulfobacterium corrodens*' IS4 and *Desulfopila aestuarii* MSL86^T.

Strain JS_SRB250Lac^T was isolated from sediment at a depth of 2.5 m from a tidal sand-flat in the German Wadden Sea ('Janssand'; 53° 44.177′ N 007° 41.970′ E). Details of the sampling site have been described elsewhere (Beck *et al.*, 2007; Røy *et al.*, 2008). Enrichment and isolation of strain JS_SRB250Lac^T were described previously (Gittel *et al.*, 2008). *Desulfopila aestuarii* MSL86^T was kindly provided by Katsuji Ueki (Yamagata University, Japan). A culture of '*Desulfobacterium corrodens*' IS4 was provided by Dennis Enning (Max Planck Institute for Marine Microbiology, Bremen, Germany).

Cultivation, growth experiments and strain maintenance were performed in an anoxic, carbonate-buffered, mineral medium as described previously (Gittel *et al.*, 2008). Unless otherwise noted, incubations were carried out with an inoculum volume of 5 % (v/v) and at 20 °C in the dark. Gram-staining of heat-fixed cells was carried out as described by Murray *et al.* (1994). Cells of strain JS_SRB250Lac^T were Gram-negative, non-motile, straight rods with rounded ends, 0.3–0.5 μm wide and 1.0–2.0 μm long (Supplementary Fig. S1, available in IJSEM Online). Longer cells, up to 5 μm, developed at low temperatures (<15 °C). Cells of strain JS_SRB250Lac^T formed light-brown colonies in agar tubes and aggregated during growth in liquid medium. Formation of endospores was not observed.

Growth experiments were performed in triplicate and monitored by phase-contrast microscopy combined with photometric sulfide measurement (Cord-Ruwisch, 1985) and/or by the determination of cellular protein content (Bradford, 1976). Growth rates were calculated from linear regression of cellular protein as a function of time. The effect of NaCl concentration on growth was determined in mineral medium with NaCl concentrations between 1 and 50 g l^{-1}. Highest growth rates were observed at NaCl concentrations between 20 and 30 g l^{-1}. Growth did not occur at NaCl concentrations below 5 g l^{-1}. The temperature range for growth was determined in seawater medium (28 g NaCl l^{-1}) and incubation at 4–50 °C. Growth was observed between 10 and 35 °C, with highest growth rates at 28 °C.

Various organic acids, alcohols, amino acids and aromatic compounds and hydrogen were tested as electron donors for sulfate reduction. Organic substrates were added from sterile stock solutions at final concentrations between 2 and 10 mM. Lactate and acetate were quantified with an HPLC system equipped with an Aminex HPX-87H ion-exclusion column (Bio-Rad) and analysed at 60 °C, with 5 mM H_2SO_4 as the mobile phase and UV detection at 210 nm (UVIS 204; Linear Instruments Corp.). Strain JS_SRB250Lac^T utilized a variety of organic substrates including fatty acids and alcohols (Table 1; detailed information is given in the species description). Besides sharing a broad range of substrates with *Desulfopila aestuarii* MSL86^T, strain JS_SRB250Lac^T also utilized hydrogen, butyrate and succinate as electron donors. Lactate was oxidized incompletely to acetate. This was a common feature of strain JS_SRB250Lac^T and its closest relatives, *Desulfopila aestuarii* and '*Desulfobacterium corrodens*', but the calculation of growth rates at optimum temperatures and NaCl concentrations for each strain highlighted that *Desulfopila aestuarii* MSL86^T grew ten times faster (Suzuki *et al.*, 2007) than strain JS_SRB250Lac^T (maximum growth rate 0.198 day^{-1}) and '*Desulfobacterium corrodens*' IS4, with a doubling time of 3–4 days (Dinh *et al.*, 2004).

Lithoautotrophic growth was tested by repeated transfers into a medium that was free of organic electron donors and overlaid with a headspace of H_2/CO_2 (90 : 10, v/v). Measurement of enzyme activities (CO dehydrogenase, 2-oxoglutarate : electron acceptor oxidoreductase) was done

Table 1. Selected characteristics for differentiation of strain JS_SRB250Lac^T from related strains

Strains: 1, JS_SRB250Lac^T; 2, '*Desulfobacterium corrodens*' IS4 (data from Dinh, 2003; Dinh *et al.*, 2004); 3, *Desulfopila aestuarii* MSL86^T (Suzuki *et al.*, 2007). All strains formed rod-shaped cells and were positive for utilization of lactate and pyruvate as electron donors for sulfate reduction and fermentation of pyruvate in the absence of sulfate. All strains were negative for utilization of propionate, malate and alanine as electron donors for sulfate reduction and fermentation of lactate and malate.

Characteristic	1	2	3
DNA G+C content (mol%)	50.3	51.9	54.5
Optimum salinity (% NaCl, w/v)	2–3	1–1.5	1
Optimum temperature (°C)	28	28–30	35
Utilization of electron donors (final concentration, mM)			
H_2	+	+	−
H_2 plus acetate (2)	+	+	−
n-Butyrate (5)	+	−	−
Fumarate (10)	+	−	+
Succinate (10)	+	−	−
Utilization of electron acceptors (final concentration, mM)			
Thiosulfate (10)	−	−	+

as described previously (Galushko & Schink, 2000; Kuever *et al.*, 2001). Strain JS_SRB250Lac^T utilized hydrogen as an electron donor and carbon dioxide/bicarbonate as sole carbon sources. In a cell-free extract of strain JS_SRB250Lac^T, active CO dehydrogenase was found [205 nmol (mg protein^{-1}) min^{-1}], whereas 2-oxoglutarate : electron acceptor oxidoreductase (a key enzyme in the citric acid cycle) was not detected. This indicated the operation of the reductive acetyl-CoA pathway (Wood–Ljungdahl pathway) for CO_2 fixation (Thauer, 1988). Growth in sulfate-free medium with lactate (20 mM) as electron donor was observed with sulfite (2 mM) as an electron acceptor, but not with nitrate (5 mM) or thiosulfate (10 mM). Fermentative growth occurred with pyruvate and fumarate, but not with lactate or malate (10 mM each).

'*Desulfobacterium corrodens*' IS4 and JS_SRB250Lac^T were phylogenetically more closely related than were *Desulfopila aestuarii* MSL86^T and JS_SRB250Lac^T, but they clearly differed with respect to their physiological properties. The strains originated from either surface or subsurface sediments and the lower salinity optimum of '*Desulfobacterium corrodens*' IS4 (1.0–1.5 ‰) might be inferred from its exposure to fresh water in surface sediments. Beside its striking ability to oxidize elemental iron anaerobically, '*Desulfobacterium corrodens*' IS4 was shown to utilize only a few other electron donors, e.g. lactate and pyruvate. Cultivation with these alternative electron donors yielded only poor growth compared with the utilization of elemental iron (Dinh *et al.*, 2004). In contrast, strain JS_SRB250Lac^T was nutritionally versatile, utilizing a variety of short-chain organic acids and alcohols as well as hydrogen as electron donors, but did not utilize elemental iron as an electron donor (Dennis Enning, personal communication).

For cellular fatty acid analysis, cells of strain JS_SRB250Lac^T and '*Desulfobacterium corrodens*' IS4 were grown with lactate (20 mM) and sulfate (28 mM) and harvested from the late exponential phase by centrifugation. Fatty acid methyl esters were obtained by saponification, methylation and extraction (Sasser, 1997). Positions of double bonds in fatty acid methyl esters were determined by analysis of their dimethyl disulfide (DMDS) adducts according to the method of Dunkelblum *et al.* (1985). Fatty acid methyl esters were quantified via GC-FID (Hewlett Packard HP 5890 Series II gas chromatograph). The major cellular fatty acids of strain JS_SRB250Lac^T were $C_{16:0}$, $C_{18:0}$, $C_{16:1}\omega7$, $C_{16:1}\omega5$ and $C_{17:0}$ cyclo. Significant differences in the cellular fatty acid profiles of strains JS_SRB250Lac^T and '*Desulfobacterium corrodens*' IS4 compared with the profile of *Desulfopila aestuarii* MSL86^T were the presence and large relative amounts of the cyclopropane fatty acid $C_{17:0}$ cyclo (15.3 and 22.5 %, respectively) and the absence of hydroxy and unsaturated branched-chain fatty acids in the former strains (Table 2).

The G+C content of the genomic DNA was determined by means of HPLC at the Identification Service of the DSMZ (Braunschweig, Germany). Nucleic acid extraction, amplification, cloning and sequencing of the 16S rRNA gene of JS_SRB250Lac^T were performed as described previously (Gittel *et al.*, 2008). Phylogenetic trees based on 16S rRNA gene sequence datasets (>1400 nt) were constructed using the neighbour-joining and maximum-likelihood algorithms implemented in the ARB program package (Ludwig *et al.*, 2004). In addition, a fragment containing approx. 1.9 kb of the *dsrAB* gene (encoding the α- and β-subunits of the dissimilatory sulfite reductase) was amplified, cloned and sequenced according to Kjeldsen *et al.* (2007). A custom-designed internal primer (5′-GTGCCTTTGATC-TGCAG-3′) was used to complete the sequence. *aprBA* genes (encoding the adenosine-5′-phosphosulfate reductase α- and β-subunits) of strain JS_SRB250Lac^T and *Desulfopila aestuarii* MSL86^T were determined using two sets of degenerate primers (AprB-1-FW/AprA-5-RV and AprA-1-FW/Apr-10-RV) as described previously (Meyer & Kuever, 2007). DsrAB and AprBA amino acid sequence-based phylogenetic trees were inferred using the PhyML program (maximum-likelihood method; http://www.atgc-montpellier.fr/phyml/). Datasets (deduced from *dsrAB* and *aprBA* gene sequences) including all available unambiguously aligned DsrAB and AprBA amino acid sequence positions of members of the family *Desulfobulbaceae* were analysed. Maximum-likelihood trees were constructed using the WAG amino acid substitution model matrices. The robustness of inferred trees was tested by bootstrap analysis with 1000 (16S rRNA gene) and 100 (DsrAB, AprBA) resamplings.

Table 2. Cellular fatty acid compositions of strain JS_SRB250Lac^T and related strains

Strains: 1, JS_SRB250Lac^T; 2, '*Desulfobacterium corrodens*' IS4 (data from this study); 3, *Desulfopila aestuarii* MSL86^T (data from Suzuki *et al.*, 2007). Values are percentages by weight of total fatty acids; −, not detected/not reported.

Fatty acid	1	2	3
Saturated straight-chain			
$C_{14:0}$	1.1	1.2	1.4
$C_{15:0}$	1.6	1.3	−
$C_{16:0}$	23.3	15.6	33.6
$C_{17:0}$	8.3	5.9	3.4
$C_{18:0}$	11.5	3.9	2.5
Unsaturated straight-chain			
$C_{15:1}\omega6$	−	1.2	1.1
$C_{16:1}\omega9$	0.7	−	−
$C_{16:1}\omega7$	18.3	11.1	6.0
$C_{16:1}\omega5$	11.4	21.9	17.1
$C_{17:1}\omega6$	−	1.6	13.7
$C_{18:1}\omega7$	8.6	7.6	1.7
$C_{18:1}\omega5$	−	3.9	2.7
Cyclic fatty acids			
$C_{17:0}$ cyclo	15.3	22.5	−
Hydroxy fatty acids	−	−	4.5
Unsaturated branched-chain fatty acids	−	−	4.6

Phylogenetic analyses of 16S rRNA gene sequence datasets showed that strain JS_SRB250Lac^T is grouped within the deltaproteobacterial family *Desulfobulbaceae* (Fig. 1). '*Desulfobacterium corrodens*' IS4, isolated from marine sediment, was identified as the closest cultured relative of strain JS_SRB250Lac^T, sharing 96.6 % 16S rRNA gene sequence similarity (Dinh *et al.*, 2004), but this name has not been validly published. The closest relative of strain JS_SRB250Lac^T with a validly published name was *Desulfopila aestuarii* MSL86^T (94.2 % similarity), which was isolated from an estuarine sediment in Japan (Suzuki *et al.*, 2007). The affiliation of strain JS_SRB250Lac^T to the *Desulfobulbaceae* was confirmed by DsrAB and AprBA amino acid sequence analyses (Supplementary Fig. S2). AprBA amino acid sequence analyses additionally supported its close relationship to '*Desulfobacterium corrodens*' DSM 15630 (=IS4) (94 % identity) and *Desulfopila aestuarii* MSL86^T (90 % identity). *dsrAB* gene sequences for *Desulfopila aestuarii* MSL86^T (Katsuji Ueki, personal communication) and '*Desulfobacterium corrodens*' IS4 (this study) could not be determined with the standard procedure and primer variations as described by Loy *et al.* (2002).

Based on the phylogenetic, physiological and chemotaxonomic characteristics described above, strain JS_SRB250Lac^T should be classified as a member of the genus *Desulfopila* and we propose that it represents a novel species, *Desulfopila inferna* sp. nov.

Description of *Desulfopila inferna* sp. nov.

Desulfopila inferna (in.fer′na. L. fem. adj. *inferna* that which is, or comes from, below, referring to the isolation of the type strain from a subsurface sediment).

Cells are straight rods with rounded ends, 0.3–0.5 μm wide and 1.0–2.0 μm long. Non-motile. The NaCl range for growth is 0.5–5 % (w/v) with an optimum between 2 and 3 % (w/v). The temperature range for growth is 10–35 °C with an optimum at 28 °C. Utilizes formate, n-butyrate, lactate, fumarate, pyruvate, succinate, valerate, caproate, caprate, ethanol, 1-propanol, 1-butanol, glycerol and proline as electron donors for sulfate reduction. Chemolithoautotrophic growth with H_2 plus CO_2/bicarbonate. Does not utilize acetate, propionate, malate, glycine, alanine, serine, betaine, choline, benzoate, sorbitol or mannitol. Sulfate and sulfite serve as electron acceptors. Thiosulfate and nitrate are not utilized. Pyruvate and fumarate are fermented in the absence of electron acceptors. Malate and lactate are not fermented. The genomic DNA G+C content of the type strain is 50.3 mol%. The major cellular fatty acids are $C_{16:0}$, $C_{18:0}$, $C_{16:1}\omega7$, $C_{16:1}\omega5$ and $C_{17:0}$ cyclo.

The type strain is JS_SRB250Lac^T (=DSM 19738^T =NBRC 103921^T), isolated from subsurface sediment from a tidal sand-flat of the German Wadden Sea.

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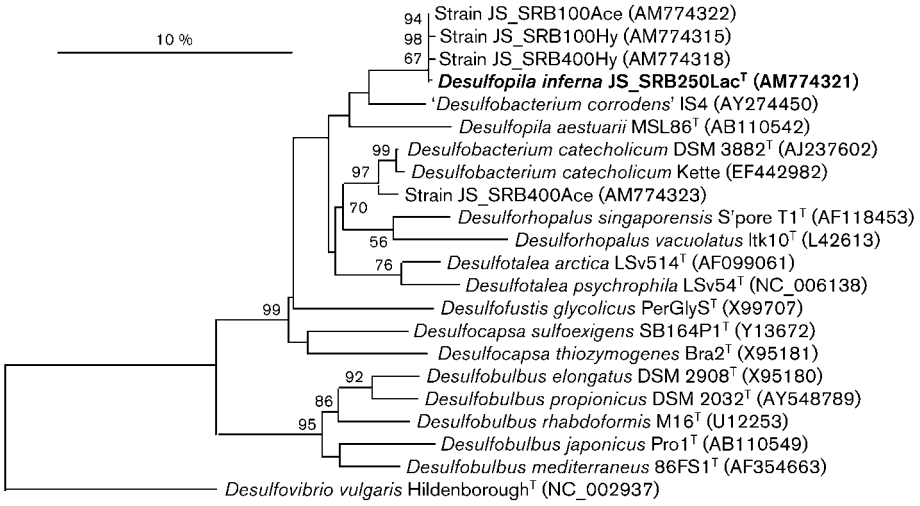

Fig. 1. Neighbour-joining tree showing the affiliation of the 16S rRNA gene sequence of strain JS_SRB250Lac^T to selected reference sequences from members of the *Deltaproteobacteria*. Bootstrap values are percentages based on analysis of 1000 replicates; only values >50 % are indicated near nodes. The tree topology inferred from maximum-likelihood analysis was similar to that obtained from the neighbour-joining method (not shown). Bar, 10 % sequence divergence.

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