COVER SHEET

Title: Diversity of substrate utilization and growth characteristics of sulfate-reducing bacteria isolated from estuarine sediment in Japan

Running head: Sulfate-reducers from estuarine sediment

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Summary

Two different isolation methods, the dilution colony-counting method (colony-isolation) and enrichment culture, were used to isolate sulfate-reducing bacteria (SRBs) from estuarine sediment in Japan. Lactate was used as an electron donor for colony-isolation, and lactate or propionate was used for enrichment culture. All isolates were classified into six different phylogenetic groups according to the 16S rRNA gene-based analysis. The closest relatives of the colony-isolates (12 strains) were species in the genera of *Desulfobacterium, Desulfofrigus, Desulfovibrio* and *Desulfomicrobium*. The closest known relative of the lactate-enrichment isolates was *Desulfovibrio acrylicus* and that of the propionate-enrichment isolates was *Desulfobulbus mediterraneus*. All isolates were incompletely-oxidizing SRBs. Overall patterns of utilization of electron donors and acceptors, as well as fermentative substrates, differed depending on the affiliation of the strain. Furthermore, even if several strains used the same substrate, the growth rates were often significantly different depending on the strain. It was strongly suggested that various species of SRBs should coexist in the sediment by competing for common substrates as well as taking priority in favorable or specific substrates for each species and the community of SRBs should be able to oxidize almost all major intermediates of anaerobic decomposition of organic matter such as lower fatty acids, alcohols and H_2 as well as amino acids. Thus, it was indicated by the phylogenetic and physiological analyses of the isolates that the

SRBs community composed of diverse lineages of bacteria living in anoxic estuarine sediment should be able to virtually play an extensive role in carbon cycle as well as sulfur cycle on the earth.

Key words: *Deltaproteobacteria*; *Desulfopila*; *Desulfobulbus*; *Desulfofrigus*; *Desulfomicrobium*; *Desulfovibrio*; estuarine sediment; 16S rRNA gene; sulfate-reducing bacteria

Introduction

Sulfate-reducing microorganisms, which commonly reduce oxidized sulfur compounds to sulfide as a functional group, include phylogenetically diverse bacterial species as well as some archaeal species (Castro et al., 2000; Rabus et al., 2000). Recently, various novel sulfate-reducing bacteria (SRBs) have been isolated from a wide range of anoxic environments such as marine sediments (Bale et al., 1997; Boyle et al., 1999; Isaksen and Teske, 1996; Jeanthon et al., 2002; Knoblauch et al., 1999a; Rabus et al., 1993; Sass et al., 2002; Sievert and Kuever, 2000; Sun et al., 2000; Sun et al., 2001; Van der Maarel et al., 1996), deep subterranean groundwater (Motamedi and Pedersen 1998), oil fields (Beeder et al., 1995; Lien et al., 1998; Miranda-Tello et al., 2003), hot springs (Mori et al., 2003) and rice field soil (Ouattara et al., 1999).

SRBs are capable of utilizing various compounds such as H_2, fatty acids, alcohols, amino acids and

sugars, as well as aliphatic and aromatic hydrocarbons as electron donors (Hansen, 1993; Rabus et al., 2000). Some SRBs can reduce Fe(III), nitrate and halogenated compounds as the sole electron acceptor (Boyle et al., 1999; Dannenberg et al., 1992; Holmes et al., 2004; Rabus et al., 2000; Sun et al., 2000; Sun et al., 2001). In addition, it is known that many SRBs can oxidize some substrates such as pyruvate and fumarate, even in the absence of available electron acceptors (Hansen, 1993; Rabus et al., 2000). Thus, SRBs play extensive roles in the environment. Since sulfate is present at rather high concentrations in marine environments, it has been reported that SRBs are responsible for up to 50% of the organic carbon mineralization in marine sediments (Jørgensen, 1982; Sørensen et al., 1981) and thus play an important role in carbon and sulfur cycles on the earth.

Many studies on the diversity of SRBs in natural environments have been performed using cultivation-independent molecular techniques based on PCR amplification of the 16S rRNA gene or dissimilatory sulfite reductase gene (Devereux and Mundfrom, 1994; Dhillon et al., 2003; Hines et al., 1999; Joulian et al., 2001; Minz et al., 1999; Purdy et al., 2001; Purdy et al., 2002; Voordouw et al., 1996; Wagner et al., 1998). These studies have revealed that phylogenetically diverse un-cultivated SRBs are present in natural ecosystems. It may be possible to estimate the physiological features of a microbe from its sequence by comparing it with sequences of the closest known relatives. However, if the sequence similarity with the closest relatives is lower than that at the species level, the physiology of the closest known relatives does not necessarily indicate that of un-cultivated microbes recovered

by the molecular techniques.

In this study, we isolated SRBs from estuarine sediment in Japan using different methods in order to isolate SRBs as diverse as possible from the sediment. The bacteria were isolated by (i) the anaerobic roll tube method and picking up colonies of SRBs that appeared after the dilution colony-counting technique with lactate as an electron donor, and (ii) enrichment cultures with lactate or propionate. Phylogenetic affiliations of the isolates were determined by 16S rRNA gene sequences, and their phenotypic features were determined in detail to compare and infer the ecological roles of each group of isolates as well as to understand the functional structure of the SRBs community.

Matherials and Methods

Source of organisms. Sediment cores were collected with a core sampler (5 cm in diameter) from sediment at a water depth of 2 m in the Niida River estuary of Sakata Harbor, which is located on the Sea of Japan side (38^{o} 54.5′ N, 139^{o} 50.6′ E) of Japan. The sediment cores were obtained from sediment depths of 6 cm and 10 cm on the 24th of June 1999 and the 12th of November 2000, respectively.

Media and cultivation. Two basal media (seawater medium and defined medium) were used in this study. The seawater medium contained (per liter of seawater): 0.5 g of KH_2PO_4, 0.3 g of NH_4Cl, 0.1 g of yeast extract, 1 mg of resazurin-Na, 10 ml of the trace element solution (per liter: 10 ml of 25%

(vol/vol) HCl, 1.5 g of $FeCl_2 \cdot 4H_2O$, 0.19 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $MnCl_2 \cdot 4H_2O$, 0.07 g of $ZnCl_2$, 0.062 g of H_3BO_3, 0.036 g of $Na_2MoO_4 \cdot 2H_2O$, 0.024 g of $NiCl_2 \cdot 6H_2O$ and 0.017 g of $CuCl_2 \cdot 2H_2O$) and 0.5 g of L-cysteine·HCl·H_2O, as well as an appropriate electron donor.

The pH was adjusted to 7.2-7.4 with 1 N NaOH. Agar (Difco) (1.5%, wt/vol) was added to the medium and used for the anaerobic roll tube method and maintenance of isolates in slant cultures. For colony counts, 0.1 g of thioglycollate-Na, 0.1 g of ascorbic acid and 0.025 g of $Na_2S_2O_4$ were added (per liter) as reducing agents in place of L-cysteine·HCl·H_2O. To detect sulfide production by colonies of SRBs, the medium was supplemented with $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.28 g per liter) as an Fe compound.

The defined medium contained (per liter): 0.5 g of KH_2PO_4, 1.0 g of NH_4Cl, 1.0 g of Na_2SO_4, 2.0 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.5 g of yeast extract, 1 mg of resazurin-Na, 10 ml of the trace element solution, 15 or 30 g of NaCl (depending on the optimum NaCl concentration for growth of each strain) and 0.5 g of L-cysteine·HCl·H_2O. The pH was adjusted to 7.2-7.4 with 1 N NaOH (Nakamoto et al., 1996; Ueki et al., 1980; Widdel and Bak, 1992). The defined medium was used for the general physiological characterization of isolates. Each electron donor was added at a final concentration of 20 mM. Cultivation and transfer of the isolates were performed under an O_2-free N_2 (100%) atmosphere. All isolates were cultivated at 30^{o}C, unless otherwise stated.

Isolation. The sediment sample collected in November 2000 was used to determine viable colony

counts of SRBs by the anaerobic roll tube method with lactate as the sole electron donor (Hungate, 1966). The sediment sample was diluted by consecutive 10-fold dilutions with anoxic seawater. The anoxic seawater was prepared by bubbling seawater collected from the sampling site with O_2-free N_2 gas. The diluted (10^{-3}-10^{-6}) samples (0.2 ml) were inoculated into the seawater agar medium (10 ml) containing 20 mM of sodium lactate.

The sediment sample collected in June 1999 was used for enrichment cultures of SRBs. The sediment sample was diluted as described above, and the diluted (10^{-3}-10^{-6}) samples (0.2 ml) were inoculated into the seawater liquid medium containing 20 mM of sodium lactate or sodium propionate. Cultures showing sulfate-reduction by formation of black precipitate in the medium were transferred to fresh medium containing the same electron donor. After two to four subcultures in the same manner, SRBs were isolated using the anaerobic roll tube method with the respective electron donor.

Purified isolates were finally obtained after several purification procedures through colony isolation by the anaerobic roll tube method. The purity of the isolates was checked by observation of cell morphology by microscopy and appearance of colonies on slant cultures.

Phenotypic characterization of isolates. The Gram-reaction and cellular morphology were confirmed by light microscopy. The motility of the cells was examined by phase-contrast microscopy. Growth of the isolates under the aerobic condition was examined in the presence of the electron donor using the basal defined medium without L-cysteine·HCl·H_2O and resazurin-Na. Oxidase and catalase

activities of cells were tested as described previously (Akasaka et al., 2003).

Effects of NaCl concentration and pH on growth of the isolates were examined using the basal defined medium. Effects of temperature on growth were examined in the seawater medium with lactate as an electron donor except for strain Pro1, which was grown in the basal defined medium with 3.0% (wt/vol) NaCl containing propionate. Growth of the isolates was monitored by measurement of optical density at 660 nm with a spectrophotometer (HITACHI U-1000, Katsuta, Japan).

Utilization of electron donors by the isolates was determined using the defined medium containing each compound at a final concentration of 20 mM. H_2 utilization was determined in the presence of acetate (5 mM) with H_2 in the atmosphere. Utilization of electron acceptors was determined with a sulfate-free medium, which contained the same concentrations of chloride in place of sulfate in the defined medium. Sodium sulfite (3 mM), sodium thiosulfate (15 mM) or sodium fumarate (20 mM) was added to the sulfate-free medium as possible electron acceptors. Fermentative utilization of pyruvate, lactate, fumarate or malate (20 mM) was also determined using the sulfate-free medium. Fatty acids and amino acids were used in the form of a sodium salt and added to the medium from sterilized stock solutions. Utilization of each electron donor or acceptor was determined by comparing the growth in the presence or absence of each compound as well as measurement of the concentration in the medium after cultivation.

Analytical methods. Volatile fatty acids and alcohols were analyzed with a gas chromatograph

(Hitachi G-5000 or 263-30, Katsuta, Japan), as described previously (Ueki et al., 1986). Non-volatile fatty acids and formate were analyzed with a high-performance liquid chromatograph (Shimadzu LC-10AD, Kyoto, Japan), as described previously by (Akasaka et al., 2003). Sulfate, sulfite and thiosulfate were analyzed with an ion chromatograph (Dionex 2000i, Dionex), as described previously (Nakamoto et al., 1996).

16S rRNA gene sequencing and phylogenetic analysis. 16S rRNA genes of the isolates were amplified by polymerase chain reaction (PCR) from DNA extracted from the cells of isolates, and the PCR amplifications were purified and sequenced, as described previously (Akasaka et al., 2003). Multiple alignments of the sequences and references obtained from the GenBank database with the BLAST program (Altschul et al., 1997) were performed, and a phylogenetic tree was constructed with the neighbor-joining method (Saitou and Nei, 1987) and bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topology, using the Clustal W program (Felsenstein, 1985; Thompson et al., 1994). All gaps and unidentified base positions in the alignment were excluded before calculation.

Nucleotide sequence accession numbers. Accession numbers of the sequences determined in this study are AB110538-AB110550 and AB232360-232362.

Results

Isolation of SRBs from estuarine sediment

Culturable SRBs in the estuarine sediment in Sakata Harbor were enumerated by the dilution colony-counting technique using the anaerobic roll tube method with lactate as an electron donor on the 12th November 2000 and SRBs in the sediment sample were enumerated at 2.5-4.9 x 10^5 CFU (colony-forming units) (cm^3 of sediment sample)$^{-1}$. Black colonies showing production of iron sulfide were picked up at random from the roll tubes and twelve pure cultures of isolates (strains MSL53, MSL65, MSL71, MSL79, MSL80, MSL86, MSL92, MSL93, MSL94, MSL95, MSL97 and MSL98) were finally obtained. Strains MSL53 and MSL86 were isolates from roll tubes inoculated with 0.2 ml samples of 10^{-5} and 10^{-4} dilutions, respectively, and others were isolates from 10^{-3} dilutions. These isolates were designated colony-isolates.

Strains MSL10 and MSL15 were isolated using the enrichment culture with lactate as an electron donor. Strains Pro1 and Pro16 were isolated from the enrichment culture using propionate as an electron donor. These isolates were designated lactate- or propionate-enrichment isolates, respectively. We also performed enrichment cultures of SRBs with acetate or butyrate as an electron donor. Although enrichment cultures showing sulfate reduction in the presence of these electron donors were obtained, respectively, we did not succeed in isolating pure cultures from them.

Phylogeny of isolates

Based on the 16S rRNA gene-based phylogenetic analysis, all sixteen isolates were affiliated with the *Deltaproteobacteria* class (Fig. 1). The isolates were classified into the following six distinct phylogenetic groups (four groups of colony-isolates and two of enrichment-isolates).

The closest known species of strains MSL53 and MSL86, based on the 16S rRNA gene sequences, was '*Desulfobacterium catecholicum*' with sequence similarities of 94.5% and 94.4%, respectively. The closest known relative of strain MSL71 was *Desulfofrigus fragile* with a sequence similarity of 93.9%, and that of strains MSL79 and MSL80 was *Desulfovibrio dechloracetivorans* with 95.8% and 94.7% similarities, respectively. Seven isolates (MSL65, MSL92, MSL93, MSL94, MSL95, MSL97 and MSL98) were classified into a group with *Desulfomicrobium norvegicum* as the closest known species with sequence similarities of 98.0-99.8%.

Both lactate-enrichment isolates (strains MSL10 and MSL15) were placed near *Desulfovibrio acrylicus* with 98.4% and 99.1% similarities, respectively. The closest known relative of the propionate-enrichment isolates (strains Pro1 and Pro16) was *Desulfobulbus mediterraneus* with 95.2% and 94.8% similarities, respectively.

Phenotypic characteristics of isolates

All isolates grew and reduced sulfate in both the seawater and defined media in the presence of electron donors. Since all isolates oxidized each electron donor to mainly acetate, as described below,

they were all classified as incomplete-type SRBs.

Six representative isolates (MSL86, MSL71, MSL79, MSL92, MSL10 and Pro1) of each phylogenetic group were selected and characterized phenotypically as follows.

(i) Cell morphology. All strains had Gram-negative and motile cells. Cells of strains MSL86 and Pro1 were rods with slightly different sizes. Cells of strains MSL71, MSL79 and MSL92 were curved rods, and those of strain MSL10 were rods or slightly curved rods (Fig. 2). Cells of all isolates except strain MSL10 were catalase-negative, and all were oxidase-negative (Table 1).

(ii) Growth conditions. All strains grew in the presence of NaCl up to 5.0-7.0% (wt/vol). The optimum NaCl concentration of all the colony-isolates was 1.0% or 2.0% (wt/vol), and three of them (MSL86, MSL79 and MSL92) grew even in the absence of NaCl in the defined medium. Both enrichment-isolates (MSL10 and Pro1) required NaCl in the medium for growth, and their optimum NaCl concentration (3.0%, wt/vol) was higher than those of the colony-isolates.

All strains were mesophilic, and the optimum temperature for growth was 35^{o}C except strain MSL71 (30^{o}C). The pH optimum of the strains was in the range of pH 6.3-6.7 except strain MSL86, which had the optimum pH of 7.5-7.6. The growth rate of strain MSL10 at each optimum growth condition (0.287-0.524 h^{-1}) were the highest compared with those of any other isolates (Table 1).

(iii) Utilization of electron donors. Table 2 shows utilization of electron donors and compounds produced by sulfate-reduction as well as the growth rate in the presence of each electron donor.

Three strains (MSL86, MSL71 and MSL10) grew even in the absence of electron donors added to the medium and reduced sulfate with concomitant production of acetate, suggesting that yeast extract or L-cysteine·HCl·H_2O added to the medium was used as an electron donor.

All strains including the propionate-enrichment isolate Pro1 utilized lactate. For all strains, lactate oxidation, sulfate reduction and acetate production proceeded at approximately 2:1:2, which was almost identical to the theoretical ratio. It appeared that lactate was the preferable electron donor to support high growth rates (0.085-0.232 h^{-1}) for all strains except strain MSL92. Pyruvate was also used by all strains and high growth rates were also obtained for strains MSL71, MSL10 and Pro1. The theoretical stoichiometric ratio of pyruvate oxidation, sulfate reduction, and acetate production was 4:1:4, and the ratios were roughly consistent with the value for all isolates except strain MSL86. Formate and ethanol were also used by all strains.

Fumarate was utilized by all strains except strain MSL71, but the amount of sulfate reduced and compounds produced were rather different depending on the strains. Strain MSL79 produced acetate, while other strains produced succinate (MSL86, MSL92 and MSL10) or malate (strain Pro1) in addition to acetate. Malate was utilized by all strains except strain MSL86, and the amounts of sulfate reduced and compounds produced were also different according to the strains in a similar way as that of fumarate. In the presence of malate, strains MSL71, MSL79 and MSL92 formed acetate, while strains MSL10 and Pro1 produced succinate and small amounts of fumarate as well as acetate,

respectively.

Strains MSL79, MSL92, MSL10 and Pro1 used significant amounts of propanol and butanol, while strains MSL86 and MSL71 used these compounds only slightly, if at all. Propanol and butanol were oxidized to their corresponding carboxylic acids according to the approximately theoretical oxidation-reduction balance (2:1:2, respectively) for each strain. Glycerol was utilized by all strains except strain MSL79, and it supported rather high growth rates of strains MSL71 (0.157 h^{-1}) and MSL10 (0.150 h^{-1}). None of colony-isolates used amino acids, while both enrichment-isolates, strains MSL10 and Pro1, used amino acids. For strain MSL10, almost the highest growth rates were obtained with alanine (0.234 h^{-1}) and serine (0.202 h^{-1}) among the electron donors used. All isolates except strain MSL86 used H_2, and based on the growth rates, it was one of the best electron donors for strains MSL79 (0.159 h^{-1}) and MSL92 (0.245 h^{-1}).

Propionate (strain Pro1), butyrate (strain MSL71), succinate (strain MSL79) and serine (strain MSL10) were each utilized by only one strain. None of the strains used acetate, methanol, glycine, aspartate or glutamate.

(iv) Utilization of electron acceptors. Utilization of electron acceptors by the strains was examined in the presence of lactate (all strains except strain Pro1) or propionate (strain Pro1) as an electron donor. All strains utilized thiosulfate in addition to sulfate as an electron acceptor, and all colony-isolates reduced sulfite (Table 3). All strains oxidized the electron donors to acetate with these

electron acceptors. In the presence of fumarate as an electron acceptor, strains MSL92 and MSL10 produced acetate and succinate, while strain MSL86 produced acetate, propionate and succinate (data not shown). Sulfate supported the highest growth rates for most of the strains except for MSL92.

(v) Fermentative utilization of substrates in the absence of electron acceptors. Pyruvate was used by all strains in the absence of electron acceptors. From pyruvate, strains MSL79, MSL92 and MSL10 produced acetate, while strains MSL86 and Pro1 produced propionate and strain MSL71 produced butyrate in addition to acetate (Table 4).

Strains MSL86, MSL79, MSL92 and MSL10 used fumarate in the absence of electron acceptors. Of the four strains, three (MSL86, MSL92 and MSL10) formed acetate and succinate, and strain MSL79 formed acetate, malate and succinate. From malate, strains MSL92 and MSL10 produced acetate and succinate, and strain MSL79 formed small amounts of acetate, fumarate and succinate. Strain Pro1 utilized lactate in the absence of electron acceptors and produced acetate and propionate.

Discussion

We isolated more than twenty strains from the same lactate-enrichment culture as that used for isolation of strains MSL10 and MSL15, and the 16S rRNA gene-based phylogenetic analyses of these strains confirmed that all isolates were closely related to *D. acrylicus* (Van der Maarel et al., 1996) with sequence similarities of higher than 98.0% (data not shown). The result suggested that the

lactate-enrichment culture had enriched exclusively one phylogenetic group of SRBs. The growth rate of strain MSL10, the representative lactate-enrichment isolate, in the defined medium containing 3.0% (wt/vol) NaCl was the highest among the isolates examined, and thus, it was strongly suggested that the high growth rate in the seawater medium used for the enrichment culture resulted in the predominance of the group.

It has been reported that SRBs related to *Desulfovibrio* species were often enriched in cultures using lactate as an electron donor (Postgate, 1984). The result obtained in this study was consistent with this. Although the growth rate of the propionate-enrichment isolate, strain Pro1, in the presence of lactate was rather high, it was still lower than that of strain MSL10. Thus, the propionate-enrichment isolates, as well as other species of lactate-utilizing SRBs present in the sediment, might have been out-competed during the enrichment procedure, and thus, the propionate-utilizing strains were successfully isolated only by using propionate, which was not used by the lactate-enrichment isolates, as an electron donor.

The optimum NaCl concentrations of known marine SRBs are generally in the range of 1.0-3.0% (wt/vol) (Bale et al., 1997; Boyle et al., 1999; Isaksen and Teske, 1996; Jeanthon et al., 2002; Knoblauch et al., 1999a; Kuever et al., 2005; Sass et al., 2002; Sievert and Kuever, 2000; Sun et al., 2000; Sun et al., 2001; Van der Maarel et al., 1996). The optimum NaCl concentrations of all of our isolates were also in this range. Although some colony-isolates grew even in the absence of added

NaCl, both enrichment-isolates, MSL10 and Pro1, essentially required the addition of NaCl to the defined medium. The results suggested that the enrichment procedure selected SRBs adapted to a relatively higher NaCl concentration. Since the NaCl concentration in the sampling site of this study may be affected by flowing freshwater from the river, the environment might provide a variety of niches for microbes in relation to the salt concentration.

Various SRB species have been isolated using positive cultures of the most-probable-number counts from their environments (Castro et al., 2002; Knoblauch et al., 1999b; Muβmann et al., 2005; Sass et al., 2004; Sievert and Kuever, 2000). In our study, the colony-isolation method resulted in isolation of a rather phylogenetically wide range of SRBs even using only one kind of medium. Thus, more diverse SRBs might be isolated by the colony-isolation method using some other media different in composition (electron donors, NaCl concentrations and other components) under various culture conditions including lower temperatures. All our isolates were incompletely-oxidizing SRBs, and we could not isolate any completely-oxidizing SRBs. It has been reported that *Desulfobacter* species, a group of the completely-oxidizing SRBs, are one of the dominant SRBs in estuarine sediments of Japan based on hybridization analysis for the 16S rRNA gene (Purdy et al., 2001; Purdy et al., 2002). Although we could enrich acetate-oxidizing SRBs from the same sediment sample used in this study, we did not succeed in isolating pure cultures of SRBs from the enrichment by the colony isolation using the anaerobic roll tube method. Thus, the isolating procedure from the enrichment cultures

should be improved to obtain pure cultures of completely-oxidizing SRBs.

Various products such as fatty acids (e.g., formate, acetate, propionate, butyrate, lactate, pyruvate, succinate, malate and fumarate), alcohols, amino acids and H_2 are formed by diverse fermentative bacteria in anoxic environments as intermediates of anaerobic decomposition of organic matter. In this study, we examined utilization of these compounds of the isolates to know their ecological roles and to understand the functional structure of the SRBs community in the estuarine sediment. The results indicated that the ranges of electron donors, as well as electron acceptors, used were different depending on the affiliation of the isolate, and growth rates under a certain condition were also significantly different. All strains tested used lactate, pyruvate and ethanol, which have been reported probably usable by most SRBs (Hansen, 1993; Holt et al., 1994; Kuever et al., 2005; Rabus et al., 2000). Out of these common electron donors, lactate and pyruvate generally supported rapid growth of most of the isolates, indicating that these compounds are suitable electron donors for many of the SRBs in the sediment. Formate was also used by all strains. H_2 was also used by all strains except MSL86; however, the growth rate of each strain was usually lower than that with lactate, except for strain MSL92, which had a significantly high growth rate with H_2. Furthermore, some substrates such as propionate, glycerol, alanine and serine supported high growth rates of some strains. Although propionate and butyrate are the most important intermediates of anaerobic decomposition of organic matter and SRBs are known to take significant roles in oxidation of these compounds in various

environments (Sørensen et al., 1981; Ueki et al., 1986), a relatively few species of SRBs have been described to oxidize these fatty acids (Kuever et al., 2005). In fact, only one strain out of our six representatives isolates utilized propionate (strain Pro1) and butyrate (strain MSL71), respectively. Succinate, also an important product of some fermentative bacteria, was also utilized by only one strain (MSL79). By the isolation of these strains, however, it is distinctly shown that SRBs as one of microbial communities composed of various species relating to our isolates are able to oxidize all the major intermediates of anaerobic decomposition of organic matter including some amino acids and glycerol. Although acetate-oxidizing pure cultures are not included in our collection, it is certain that acetate is also oxidized by some species of SRBs in the estuarine sediment, since acetate was oxidized depending on sulfate reduction in the enrichment culture inoculated with the same sediment sample as described above.

Since growth rates of the isolates were determined by using batch cultures in the presence of excess amounts of substrates in this study, they might not necessarily reflect growth properties of the isolates in their natural habitats. However, the results obtained strongly indicated that various species of SRBs as a physiological group of microbes should coexist in the environment by competing for common substrates as well as taking priority in preferred or specific substrates for each species. This should allow a wide range of functions to the overall community of SRBs in the estuarine sediment.

It is known that fumarate serves as both an electron donor and acceptor as well as a fermentable

substrate for SRBs. Malate performs similarly. When fumarate or malate was used as an electron donor for the isolates, the main product was acetate, although some isolates (strains MSL86, MSL92 and MSL10) produced succinate in addition to acetate. Almost all strains using fumarate or malate as an electron donor could also grow with these substrates even in the absence of electron acceptors. Strain MSL92 produced acetate and succinate from fumarate or malate in the absence of electron acceptors, while strain MSL86 produced propionate in addition to acetate and succinate using fumarate as an electron acceptor. Thus, the roles of fumarate and malate in relation to sulfate reduction are rather complex, and the physiology of fumarate and malate utilization by SRBs should be examined more definitively.

Determining the physiological characteristics of representative isolates demonstrated that most of the isolates had distinct features from those of known related species. Based on the additional physiological and chemotaxonomic characterization of strain MSL86, we proposed a novel genus and species *Desulfopila aestuarii* gen. nov., sp. nov., to accommodate the strain (the type strain = MSL86^T = JCM 14042^T = DSM 18488^T) (Suzuki et al., in press a). Furthermore, strain Pro1 was affiliated with the genus *Desulfobulbus* depending on the comprehensive characterizations and we proposed a novel species *Desulfobulbus japonicus* for the strain (the type strain = Pro1^T = JCM 14043^T = DSM 18378^T) (Suzuki et al., in press b). For other strains having distinct features from the closest relatives (such as strains MSL71 and MSL79), further physiological and chemotaxonomic characterizations for the

description of these strains are now in progress. The results obtained in this study, however, indicated that even if the similarity of the 16S rRNA gene sequence to the closest relative is rather high (e.g., MSL92 and MSL10), some physiological features of the isolates are often significantly different from those of the closest relatives. For instance, strain MSL92 had several different properties of those of the closest relative, *D. norvegicum* (Sharak Genthner et al., 1994; Sharak Genthner et al., 1997), especially in relation to fumarate utilization. In addition, strain MSL10 has catalase activity, while the closest relative, *D. acrylicus*, does not, and the former does not use succinate as an electron donor, while the latter use (Van der Maarel et al., 1996). Presence of these physiological diversities among closely related microbial groups should be taken into consideration to understand the ecological function of each microbial group.

It has been shown that diverse SRBs are present in natural environments including marine sediments by using cultivation-independent molecular techniques (Devereux and Mundfrom, 1994; Dhillon et al., 2003; Joulian et al., 2001; Minz et al., 1999; Purdy et al., 2001; Purdy et al., 2002; Voordouw et al., 1996; Wagner et al., 1998). In this study, we could really isolate phylogenetically diverse SRBs including novel lineages from an estuarine sediment using different isolation methods. Furthermore, we showed a physiological diversity of SRBs present in the sediment by determining various growth characteristics of the isolates. It was shown from the investigations that each lineage of the SRBs in the estuarine sediment occupies a specific niche in the community according to the

differences in the range of substrate utilization as well as in the priority to each substrate and the SRBs as an essential physiological group in the anaerobic microbial community in the sediment have extensive functions in the decomposition of organic matter as well as in the sulfur cycle.

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References

Akasaka, H., Izawa, T., Ueki, K., and Ueki. A. (2003) Phylogeny of numerically abundant culturable anaerobic bacteria associated with degradation of rice plant residue in Japanese paddy field soil. *FEMS Microbiol*. *Ecol*., **43**, 149-161.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSIBLAST: a new generation of protein database search programs. *Nucleic Acids Res*., **25**, 3389-3402.

Bale, S. J., Goodman, K., Rochelle, P. A., Marchesi, J. R., Fry, J. C., Weightman, A. J., and Parkes, R. J. (1997) *Desulfovibrio profundus* sp. nov., a novel barophilic sulfate-reducing bacterium from deep sediment layers in the Japan Sea. *Int*. *J*. *Syst*. *Bacteriol*., **47**, 515-521.

Beeder, J., Torsvik, T., and Lien, T. (1995) *Thermodesulforhabdus norvegicus* gen. nov., sp. nov., a novel thermophilic sulfate-reducing bacterium from oil field water. *Arch*. *Microbiol*., **164**, 331-336.

Boyle, A. W., Phelps, C. D., and Young, L. Y. (1999) Isolation from estuarine sediments of a *Desulfovibrio* strain which can grow on lactate coupled to the reductive dehalogenation of 2,4,6-tribromophenol. *Appl*. *Environ*. *Microbiol*., **65,** 1133-1140.

Castro, H., Reddy, K. R., and Ogram, A. (2002) Composition and function of sulfate-reducing

prokaryotes in eutrophic and pristine areas of the Florida Everglades. *Appl. Environ. Microbiol*., **68**, 6129-6137.

Castro, H. F., Williams, N. H., and Ogram, A. (2000) Phylogeny of sulfate-reducing bacteria. *FEMS Microbiol. Ecol*., **31**, 1-9.

Dannenberg, S., Kroder, M., Dilling, W., and Cypionka, H. (1992) Oxidation of H_2, organic compounds and inorganic sulfur compounds coupled to reduction of O_2 or nitrate by sulfate-reducing bacteria. *Arch. Microbiol*., **158**, 93-99.

Devereux, R., and Mundfrom, G. W. (1994) A phylogenetic tree of 16S rRNA sequences from sulfate-reducing bacteria in a sandy marine sediment. *Appl. Environ. Microbiol*., **60**, 3437-3439.

Dhillon, A., Teske, A., Dillon, J., Stahl, D. A., and Sogin, M. L. (2003) Molecular characterization of sulfate-reducing bacteria in the Guaymas Basin. *Appl. Environ. Microbiol*., **69**, 2765-2772.

Felsenstein, L. (1985) Confidence limits on phylogenies: an approach using bootstrap. Evolution, **39**, 783-791.

Hansen, T. A. (1993) Carbon metabolism of sulfate-reducing bacteria. *In* The Sulfate-Reducing Bacteria: Contemporary Perspectives, ed. By Odom, J. M., and Singleton, R., Jr, Springer Verlag, New York, pp. 21−40.

Hines, M. E., Evans, R. S., Sharak Genthner, B. R., Willis, S. G., Friedman, S., Rooney-Varga, J. N., and Devereux, R. (1999) Molecular phylogenetic and biogeochemical studies of

sulfate-reducing bacteria in the rhizosphere of *Spartina alterniflora*. *Appl*. *Environ*. *Microbiol*., **65**, 2209-2216.

Holmes, D. E., Bond, D. R., and Lovley, D. R. (2004) Electron transfer by *Desulfobulbus propionicus* to Fe(III) and graphite electrodes. *Appl*. *Environ*. *Microbiol*., **70**, 1234-1237.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (1994) Group 7: Dissimilatory sulfate- or sulfur- reducing bacteria. *In* Bergey's Manual of Determinative Bacteriology, 9th ed., Williams and Willkins, Baltimore, Maryland, pp. 335-339.

Hungate, R. E. (1966) The Rumen and Its Microbes. Academic Press, New York.

Isaksen, M. F., and Teske, A. (1996) *Desulforhopalus vacuolatus* gen. nov., sp. nov., a new moderately psychrophilic sulfate-reducing bacterium with gas vacuoles isolated from a temperate estuary. *Arch*. *Microbiol*., **166**, 160-168.

Jeanthon, C., L'Haridon, S., Cueff, V., Banta, A., Reysenbach, A.-L., and Prieur, D. (2002) *Thermodesulfobacterium hydrogeniphilum* sp. nov., a thermophilic, chemolithoautotrophic, sulfate-reducing bacterium isolated from a deep-sea hydrothermal vent at Guaymas Basin, and emendation of the genus *Thermodesulfobacterium*. *Int*. *J*. *Syst*. *Evol*. *Microbiol*., **52**, 765-772.

Jørgensen, B. B. (1982) Mineralization of organic matter in the sea bed-the role of sulphate reduction. *Nature*, **296**, 643-645.

Joulian, C., Ramsing, N. B., and Ingvorsen, K. (2001) Congruent phylogenies of most common

small-subunit rRNA and dissimilatory sulfite reductase gene sequences retrieved from estuarine sediments. *Appl. Environ. Microbiol.*, **67**, 3314-3318.

Knoblauch, C., Sahm, K., and Jørgensen, B. B. (1999a). Psychrophilic sulfate-reducing bacteria isolated from permanently cold Arctic marine sediments: description of *Desulfofrigus oceanense* gen. nov., sp. nov., *Desulfofrigus fragile* sp. nov., *Desulfofaba gelida* gen. nov., sp. nov., *Desulfotalea psychrophila* gen. nov., sp. nov. and *Desulfotalea arctica* sp. nov. *Int. J. Syst. Bacteriol.*, **49**, 1631-1643.

Knoblauch, C., Jørgensen, B. B., and Harder, J. (1999b) Community size and metabolic rates of psychrophilic sulfate-reducing bacteria in Arctic marine sediments. *Appl. Environ. Microbiol.*, **65**, 4230-4233.

Kuever, J., Rainey, F. A., and Widdel, F. (2005) Class IV. *Deltaproteobacteria* class nov. *In* Bergey's Manual of Systematic Bacteriology, Vol. 2, Part C, 2nd ed., ed. Brenner, D. J., Krieg, N. R., Staley, J. T. and Garrity, G. M., Springer, New York, pp. 922-1144.

Lien, T., Madsen, M., Steen, I. H., and Gjerdevik, K. (1998) *Desulfobulbus rhabdoformis* sp. nov., a sulfate reducer from a water-oil separation system. *Int. J. Syst. Bacteriol.*, **48**, 469-474.

Minz, D., Flax, J. L., Green, S. J., Muyzer, G., Cohen, Y., Wagner, M., Rittmann, B. E., and Stahl, D. A. (1999) Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial

mat characterized by comparative analysis of dissimilatory sulfite reductase genes. *Appl. Environ. Microbiol*., **65**, 4666-4671.

Miranda-Tello, E., Fardeau, M.-L., Fernández, L., Ramírez, F., Cayol, J.-L., Thomas, P., Garcia, J.-L., and Ollivier, B. (2003) *Desulfovibrio capillatus* sp. nov., a novel sulfate-reducing bacterium isolated from an oil field separator located in the Gulf of Mexico. *Anaerobe*, **9**, 97-103.

Mori, K., Kim, H., Kakegawa, T., and Hanada, S. (2003) A novel lineage of sulfate-reducing microorganisms: *Thermodesulfobiaceae* fam. nov., *Thermodesulfobium narugense*, gen. nov., sp. nov., a new thermophilic isolate from a hot spring. *Extremophiles*, **7**, 283-290.

Motamedi, M., and Pedersen K. (1998) *Desulfovibrio aespoeensis* sp. nov., a mesophilic sulfate-reducing bacterium from deep groundwater at Äspö hard rock laboratory, Sweden. *Int. J. Syst. Bacteriol*., **48**, 311-315.

Muβmann, M., Ishii, K., Rabus, R., and Amann, R. (2005) Diversity and vertical distribution of cultured and uncultured *Deltaproteobacteria* in an intertidal mud flat of the Wadden Sea. *Environ. Microbiol*., **7**, 405-418.

Nakamoto, M., Ueki, A., and Ueki, K. (1996) Physiological properties of a sulfate-reducing bacterium isolated from municipal sewage sludge and its possible role as a syntrophic acidogen in the ecosystem. *J. Gen. Appl. Microbiol*., **42**, 109-120.

Ouattara, A. S., Patel, B. K. C., Cayol, J.-L., Cuzin, N., Traore, A. S., and Garcia, J.-L. (1999) Isolation and characterization *Desulfovibrio burkinensis* sp. nov. from an African ricefield, and phylogeny of *Desulfovibrio alcoholivorans*. *Int. J. Syst. Bacteriol*., **49**, 639-643.

Postgate, J. R. (1984) The Sulphate-reducing Bacteria. Cambridge: Cambridge University Press.

Purdy, K. J., Nedwell, D. B., Embley, T. M., and Takii, S. (2001) Use of 16S rRNA-targeted oligonucleotide probes to investigate the distribution of sulphate-reducing bacteria in estuarine sediments. *FEMS Microbiol*. *Ecol*., **36**, 165-168.

Purdy, K. J., Embley, T. M., and Nedwell, D. B. (2002). The distribution and activity of sulphate reducing bacteria in estuarine and coastal marine sediments. *Antonie van Leewenhoek*, **81**, 181-187.

Rabus, R., Nordhaus, R., Ludwig, W., and Widdel, F. (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. *Appl*. *Environ*. *Microbiol*., **59**, 1444-1451.

Rabus, R., Hansen, T., and Widdel, F. (2000) Dissimilatory sulfate- and sulfur-reducing prokaryotes. *In* The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community, 3rd ed., ed. by Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. and Stackebrandt, E., Springer-Verlag, New York.

Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing

phylogenetic trees. *Mol. Biol. Evol.* **4**, 406-425.

Sass, H., Overmann, J., Rütters, H., Babenzien, H.-D., and Cypionka, H. (2004) *Desulfosporomusa polytropa* gen. nov. sp. nov., a novel sulfate-reducing bacterium from sediments of an oligotrophic lake. *Arch. Microbiol.*, **182**, 204-211.

Sass, A., Rütters, H., Cypionka, H., and Sass, H. (2002) *Desulfobulbus mediterraneus* sp. nov., a sulfate-reducing bacterium growing on mono- and disaccharides. *Arch. Microbiol.*, **177**, 468-474.

Sharak Genthner, B. R., Mundfrom, G., and Devereux, R. (1994) Characterization of *Desulfomicrobium escambium* sp. nov. and proposal to assign *Desulfovibrio desulfuricans* Norway 4 to the genus *Desulfomicrobium*. *Arch. Microbiol.*, **161**, 215-219.

Sharak Genthner, B. R., Friedman, S. D., and Devereux, R. (1997) Reclassification of *Desulfovibrio desulfuricans* Norway 4 as *Desulfomicrobium norvegicum* comb. nov. and confirmation of *Desulfomicrobium escambiense* (corrig., formerly "*escambium*") as a new species in the genus *Desulfomicrobium*. *Int. J. Syst. Bacteriol.*, **47**, 889-892.

Sievert, S. M., and Kuever, J. (2000) *Desulfacinum hydrothermale* sp. nov., a thermophilic, sulfate-reducing bacterium from geothermally heated sediments near Milos Island (Greece). *Int. J. Syst. Evol. Microbiol.*, **50**, 1239-1246.

Sørensen, J., Christensen, D., and Jørgensen, B. B. (1981) Volatile fatty acids and hydrogen as

substrates for sulfate-reducing bacteria in anaerobic marine sediment. *Appl. Environ. Microbiol*., **42**, 5-11.

Sun, B., Cole J. R., Sanford, R. A., and Tiedje J. M. (2000) Isolation and characterization of *Desulfovibrio dechloracetivorans* sp. nov., a marine dechlorinating bacterium growing by coupling the oxidation of acetate to the reductive dechlorination of 2-chlorophenol. *Appl. Environ. Microbiol*., **66**, 2408-2413.

Sun, B., Cole, J. R., and Tiedje, M. (2001) *Desulfomonile limimaris* sp. nov., an anaerobic dehalogenating bacterium from marine sediments. *Int. J. Syst. Evol. Microbiol*., **51**, 365-371.

Suzuki, D., Ueki, A., Amaishi, A., and Ueki, K. (2007) *Desulfopila aestuarii* gen. nov., sp. nov., a novel, Gram-negative, rod-like sulfate-reducing bacterium isolated from an estuarine sediment in Japan. *Int. J. Syst. Evol. Microbiol*., **57**, 520-526..

Suzuki, D., Ueki, A., Amaishi, A., and Ueki, K. (2007) *Desulfobulbus japonicus* sp. nov., a novel, Gram-negative, propionate-oxidizing, sulfate-reducing bacterium isolated from an estuarine sediment in Japan. *Int. J. Syst. Evol. Microbiol*., **57**, 849-855.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res*., **22**, 4673-4680.

Ueki, A., Minato, H., Azuma, R., and Suto, T. (1980) Enumeration and isolation of anaerobic

bacteria in sewage digester fluids: Enumeration of sulfate-reducers by the anaerobic roll tube method. *J. Gen. Appl. Microbiol*., **26**, 25-35.

Ueki, A., Matsuda, K., and Ohtsuki, C. (1986) Sulfate reduction in the anaerobic digestion of animal waste. *J. Gen. Appl. Microbiol*., **32,** 111-123.

Van der Maarel, M. J. E. C., van Bergeijk, S., van Werkhoven, A. F., Laverman, A. M., Meijer, W. G., Stam, W. T., and Hansen, T. A. (1996) Cleavage of dimethylsulfoniopropionate and reduction of acrylate by *Desulfovibrio acrylicus* sp. nov. *Arch. Microbiol*., **166**, 109-115.

Voordouw, G., Armstrong, S. M., Reimer, M. F., Fouts, B., Telang, A. J., Shen, Y., and Gevertz, D. (1996) Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. *Appl. Environ. Microbiol*., **62**, 1623-1629.

Wagner, M., Roger, A. J., Flax, J. L., Brusseau, G. A., and Stahl, D. A. (1998) Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *J. Bacteriol*., **180**, 2975-2982.

Widdel, F., and Bak, F. (1992) Gram-negative mesophilic sulfate-reducing bacteria. *In* The Prokaryotes, 2nd ed., ed. by Balows, A., Trüper, H. G., Dworkin, M., Harder, W. and Schleifer, K.-H., Springer-Verlag, New York, pp. 3352-3378.

Legends for figures

Fig. 1.
Phylogenetic relationships between isolates and closely related organisms based on 16S rRNA gene sequences. Bootstrap values shown are based on analysis of 1000 replicates. The scale bar represents an estimated difference of 2% in nucleotide sequence positions. As the outgroup, *Escherichia coli* was used.

Fig. 2.
Phase-contrast photomicrographs of isolates.
A, MSL86; B, MSL71; C, MSL79; D, MSL92; E, MSL10; F, Pro1

Fig. 1.

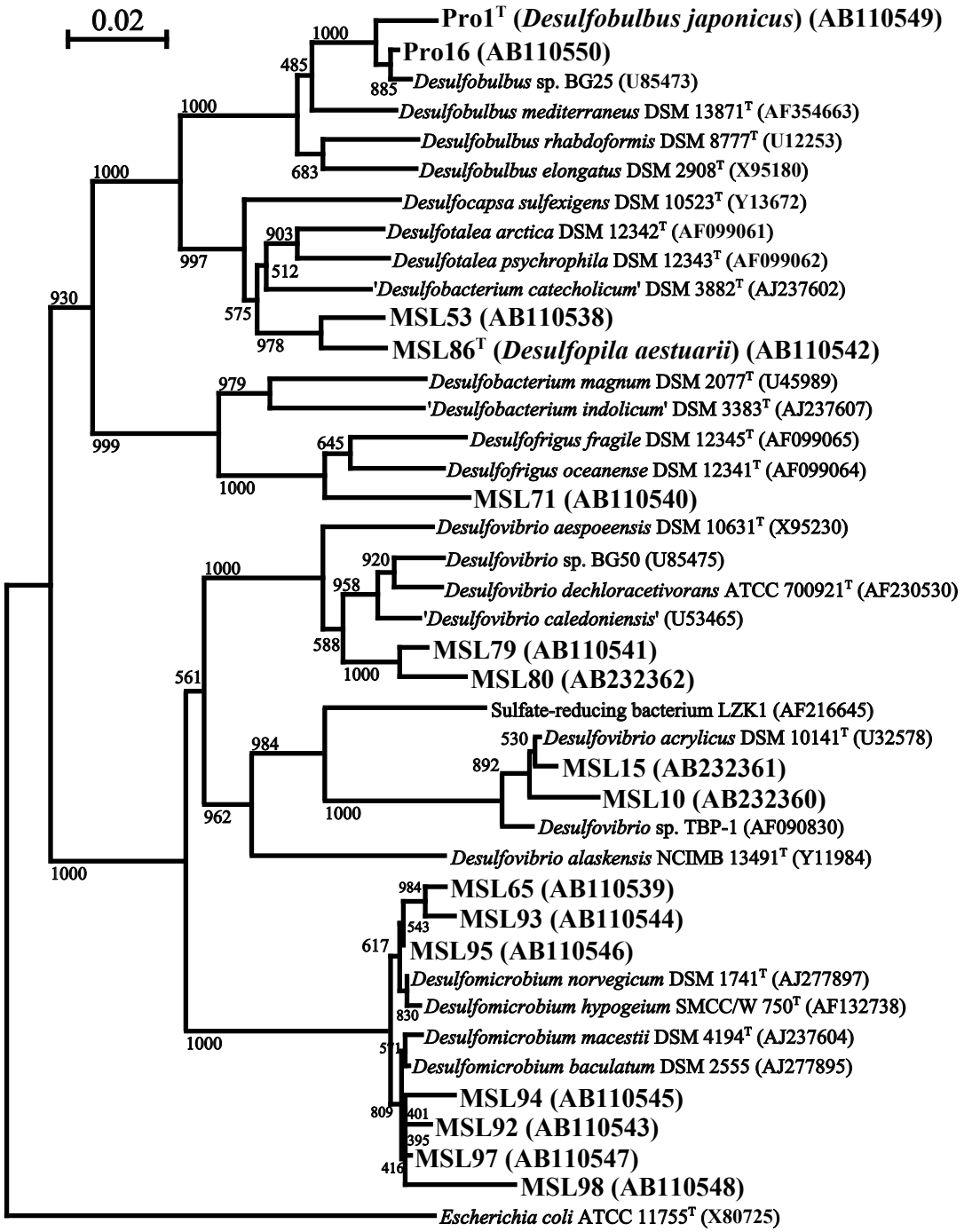
0.02
Pro1T (Desulfobulbus japonicus) (AB110549)
Pro16 (AB110550)
Desulfobulbus sp. BG25 (U85473)
Desulfobulbus mediterraneus DSM 13871T (AF354663)
Desulfobulbus rhabdoformis DSM 8777T (U12253)
Desulfobulbus elongatus DSM 2908T (X95180)
Desulfocapsa sulfexigens DSM 10523T (Y13672)
Desulfotalea arctica DSM 12342T (AF099061)
Desulfotalea psychrophila DSM 12343T (AF099062)
'Desulfobacterium catecholicum' DSM 3882T (AJ237602)
MSL53 (AB110538)
MSL86T (Desulfopila aestuarii) (AB110542)
Desulfobacterium magnum DSM 2077T (U45989)
'Desulfobacterium indolicum' DSM 3383T (AJ237607)
Desulfofrigus fragile DSM 12345T (AF099065)
Desulfofrigus oceanense DSM 12341T (AF099064)
MSL71 (AB110540)
Desulfovibrio aespoeensis DSM 10631T (X95230)
Desulfovibrio sp. BG50 (U85475)
Desulfovibrio dechloracetivorans ATCC 700921T (AF230530)
'Desulfovibrio caledoniensis' (U53465)
MSL79 (AB110541)
MSL80 (AB232362)
Sulfate-reducing bacterium LZK1 (AF216645)
Desulfovibrio acrylicus DSM 10141T (U32578)
MSL15 (AB232361)
MSL10 (AB232360)
Desulfovibrio sp. TBP-1 (AF090830)
Desulfovibrio alaskensis NCIMB 13491T (Y11984)
MSL65 (AB110539)
MSL93 (AB110544)
MSL95 (AB110546)
Desulfomicrobium norvegicum DSM 1741T (AJ277897)
Desulfomicrobium hypogeium SMCC/W 750T (AF132738)
Desulfomicrobium macestii DSM 4194T (AJ237604)
Desulfomicrobium baculatum DSM 2555 (AJ277895)
MSL94 (AB110545)
MSL92 (AB110543)
MSL97 (AB110547)
MSL98 (AB110548)
Escherichia coli ATCC 11755T (X80725)

Fig. 2.

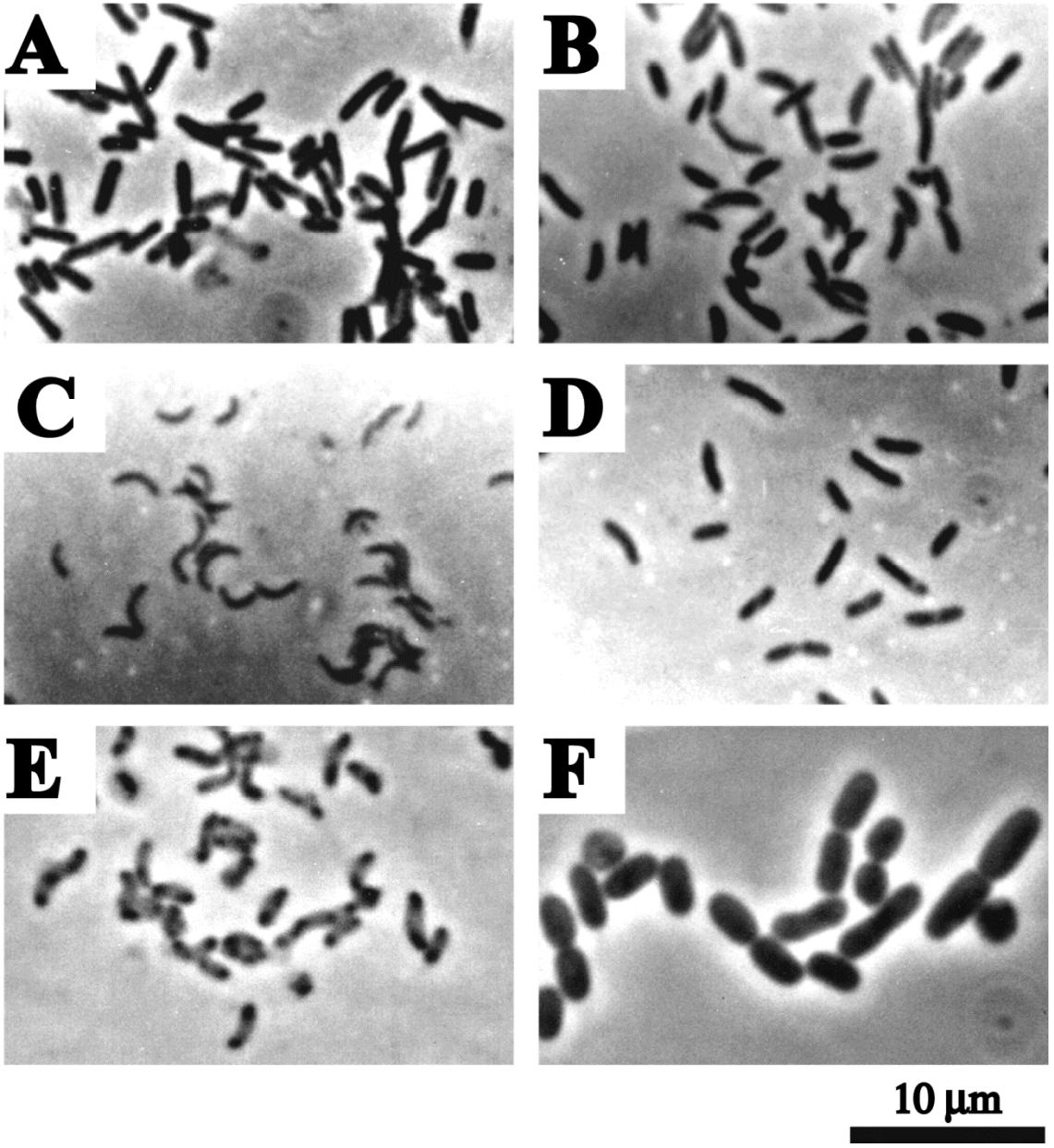

Table 1. Phenotypic properties of isolates of sulfate-reducing bacteria.

	MSL86	MSL71	MSL79	MSL92	MSL10	Pro1
Morphology	rods	curved rods	curved rods	curved rods	curved rods	rods
Cell size (μm)						
Length	1.9-3.8	1.6-3.4	1.8-2.3	1.8-2.8	1.7-2.5	1.4-2.9
Width	0.7-1.2	0.8-0.9	0.7-1.0	0.7-1.0	0.4-0.7	0.8-1.6
Motility	+	+	+	+	+	+
Gram-staining	-	-	-	-	-	-
Catalase	-	-	-	-	+	-
Oxidase	-	-	-	-	-	-
NaCl (%, wt/vol)						
optimum/range	1.0/0-5.0	2.0/1.0-6.5	2.0/0-6.5	1.0/0-5.0	3.0/1.0-6.5	3.0/1.0-7.0
growth rate (h^{-1}) at optimum[a]						
	0.137	0.206	0.207	0.178	0.306	0.149
Temperature(℃)						
optimum/range	35/10-40	30/10-35	35/10-40	35/15-40	35/10-45	35/15-35
growth rate (h^{-1}) at optimum[b]						
	0.189	0.196	0.243	0.138	0.524	0.080
pH						
optimum/range	7.5/6.3-8.5	6.3/5.6-8.5	6.5/5.6-8.5	6.5/5.6-8.5	6.5/5.7-8.4	6.7/6.1-7.5
growth rate (h^{-1}) at optimum[a, c]						
	0.175	0.167	0.186	0.149	0.287	0.073

[a] Determined at 30°C.

[b] The seawater medium was used for all strains except for strain Pro1. For strain Pro1, the basal defined medium containing 3.0% (wt/vol) NaCl was used.

[c] The basal defined medium containg 1.5% (wt/vol) NaCl was used for all strains execpt for strains MSL10 and Pro1. For strains MSL10 and Pro1, the basal defined medium containing 3.0% (wt/vol) NaCl was used.

Table 2. Utilization of substrates as electron donors and compounds produced by isolates through sulfate-reduction.

Strain	Substrates[a]	Substrates consumed (mmol/l)	Sulfate reduced (mmol/l)	Compounds produced (mmol/l)		Growth rate (h^{-1})
				Acetate	Others	
MSL86	No addition	n.d.	2.34	2.09	–	0.097
	Formate	18.8	2.31	–	–	0.094
	Pyruvate	22.0	4.52	5.89	—	0.108
	Lactate	15.1	5.80	15.4	—	0.085
	Fumarate	18.6	6.28	8.28	Succinate (2.92)	0.094
	Ethanol	13.2	5.06	15.7	—	0.087
	Glycerol	n.d.	7.26	11.9	—	0.091
MSL71	No addition	n.d.	0.76	2.75	—	0.091
	Formate	15.0	1.71	1.82	—	0.058
	Butyrate	18.8	10.8	34.6	—	0.081
	Pyruvate	22.0	4.65	17.7	—	0.197
	Lactate	18.5	10.7	21.3	—	0.175
	Malate	17.4	6.09	15.3	—	0.098
	Ethanol	4.04	3.17	6.39	—	0.104
	Glycerol	n.d.	8.69	17.7	—	0.157
	H_2	n.d.	1.56	1.08	—	0.111
MSL79	No addition	n.d.	—	1.02	—	n.d.
	Formate	21.1	4.18	—	—	0.100
	Pyruvate	22.0	4.06	19.8	—	0.082
	Lactate	18.5	10.6	21.7	—	0.171
	Fumarate	19.7	9.82	16.3	—	0.120
	Malate	17.4	6.74	15.7	—	0.121
	Succinate	21.1	12.6	20.7	—	0.052
	Ethanol	19.6	9.80	19.7	—	0.096
	Propanol	16.0	9.38	—	Propionate (19.4)	0.101
	Butanol	10.3	6.98	—	Butyrate (12.9)	0.075
	H_2	n.d.	8.63	—	—	0.159
MSL92	No addition	n.d.	—	0.74	—	n.d.
	Formate	21.1	5.46	—	—	0.063
	Pyruvate	22.0	4.86	17.9	—	0.115
	Lactate	18.5	10.6	20.9	—	0.130
	Fumarate	17.0	3.00	9.69	Succinate (4.80)	0.023
	Malate	17.4	7.09	15.6	—	0.053
	Ethanol	22.4	10.0	18.7	—	0.076
	Propanol	16.0	9.20	—	Propionate (17.9)	0.074
	Butanol	8.63	4.40	—	Butyrate (6.79)	0.078
	Glycerol	n.d.	2.55	1.82	—	0.018
	H_2	n.d.	9.30	—	—	0.245
MSL10	No addition	n.d.	1.80	4.60	—	0.150
	Formate	21.9	5.40	1.40	—	0.078
	Pyruvate	13.7	2.83	10.8	—	0.166
	Lactate	19.0	10.4	22.0	—	0.232
	Fumarate	21.3	2.70	9.10	Succinate (11.1)	0.077
	Malate	19.3	4.60	11.2	Succinate (11.6)	0.101
	Ethanol	20.1	10.7	19.8	—	0.081
	Propanol	5.70	3.50	—	Propionate (7.70)	0.087
	Butanol	8.40	4.10	—	Butyrate (8.70)	0.081
	Glycerol	n.d.	2.33	3.40	—	0.150
	Alanine	n.d.	5.49	7.28	—	0.234
	Serine	n.d.	6.95	16.1	—	0.202
	H_2	5.50	2.80	—	—	0.091
Pro1	No addition	n.d.	0.92	0.82	—	n.d.
	Formate	16.3	7.11	—	—	0.120
	Propionate	18.9	12.9	16.6	—	0.147
	Pyruvate	19.3	5.30	18.5	—	0.168
	Lactate	22.3	12.9	21.5	—	0.173
	Fumarate	12.7	4.00	6.30	Malate (6.19)	0.031
	Malate	5.20	2.45	5.83	Fumarate (1.28)	0.018
	Ethanol	18.2	13.4	25.1	—	0.106
	Propanol	21.7	8.80	—	Propionate (19.2)	0.088
	Butanol	15.5	6.63	—	Butyrate (13.2)	0.044
	Glycerol	n.d.	15.0	13.0	—	0.071
	Alanine	n.d.	13.2	14.1	Propionate (1.10)	0.063
	H_2	n.d.	8.04	—	—	0.074

[a] Substrates tested as electron donors were formate, acetate, propionate, butyrate, pyruvate, lactate, fumarate, malate, succinate, methanol, ethanol, propanol, butanol, glycerol, glycine, alanine, serine, aspartate, glutamate and H_2. Electron donors tested but not utilized were not shown. All strains were negative for utilization of acetate, methaol, glycine, aspartate, and glutamate as an electron donor.

n.d., Not determined.

—, Not detected.

Table 3. Utilization of electron acceptors by isolates of sulfate-reducing bacteria.

Strain	Sulfate	Sulfite	Thiosulfate	Fumarate
MSL86	+ (0.085)[a]	+ (0.052)	+ (0.038)	+ (0.096)
MSL71	+ (0.175)	+ (0.160)	+ (0.080)	−
MSL79	+ (0.171)	+ (0.145)	+ (0.136)	−
MSL92	+ (0.130)	+ (0.130)	+ (0.132)	+ (0.119)
MSL10	+ (0.232)	−	+ (0.160)	+ (0.197)
Pro1	+ (0.147)	−	+ (0.068)	−

[a] Lactate (20 mM) was used for all strains except for strain Pro1 as an electron donor.

Propionate (20 mM) was used for strain Pro1.Values in parentheses indicate growth rate (h^{-1}).
+, Utilized; −, not utilized.

Table 4. Utilization of substrates in the absence of electron acceptors by isolates of sulfate-reducing bacteria.

Strain	Substrates[a]	Substrates consumed (mmol/l)	Compounds produced (mmol/l)		Growth rate (h^{-1})
			Acetate	Others	
MSL86	Pyruvate	10.4	7.79	Propionate (2.30)	0.018
	Fumarate	13.0	9.52	Succinate (6.98)	0.075
MSL71	Pyruvate	18.8	18.2	Butyrate(1.79)	0.107
	Malate	5.14	4.00	—	0.033
MSL79	Pyruvate	6.95	7.50	—	0.057
	Fumarate	18.2	4.64	Malate (4.24), Succinate (6.80)	0.057
	Malate	7.57	1.47	Fumarate (1.78), Succinate (2.00)	0.034
MSL92	Pyruvate	11.1	9.44	—	0.098
	Fumarate	18.6	6.03	Succinate (10.0)	0.015
	Malate	19.8	5.92	Succinate (18.7)	0.013
MSL10	Pyruvate	10.8	8.11	—	0.221
	Fumarate	17.8	6.76	Succinate (11.1)	0.112
	Malate	19.3	8.29	Succinate (12.5)	0.199
Pro1	Pyruvate	5.62	3.28	Propionate (1.08)	0.048
	Lactate	5.69	2.73	Propionate (3.57)	0.040

[a] Substrates tested were pyruvate, lactate, fumarate and malate. Substrates tested but not utilized were not shown.

—, Not detected.