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Isolation and Characterization of Sulfate-reducing Bacteria from Sediments of Kagoshima Bay

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Key words : sulfate-reducing bacteria, Kagoshima Bay, sediment, 16S rDNA, specific primer

Abstract

The present study was carried out to isolate sulfate-reducing bacteria (SRB) from the sediments of four stations in Kagoshima Bay, Japan and to determine their phylogenetic positions based on 16S rDNA sequences. Sediments from Stations 1, 2 and 3 were similarly characterized as fine silt, higher moisture content (79.1 – 87.4%) and higher ignition loss (IL, 13.8 – 15.9%). On the other hand, Station 4 was quite differrent from the other stations because the sediment was composed of fine sand, with lower moisture content (30.4%) and lower IL (2.5%). However, acid volatile sulfide (AVS) contents (8.6 mg/g) and SRB density (56.0 x 10^3 /g) were remarkably high at Station 3 compared with the other 3 stations. The AVS contents in the cultures of strains SR-St-1-1 and SR-St-3-1 isolated from Stations 1 and 3 increased rapidly after 2 days of incubation, while that of strain SR-St-2-1 isolated from Station 2 started to increase after 4 days of incubation. The pH values of liquid cultures of the three strains increased to alkaline (pH 8.8) during the incubation period. Phylogenetic analysis based on 16S rDNA sequences showed that one strain isolated from Station 1 and five strains from Station 3 were most closely related to *Desulfovibrio dechloracetivorans*, while two strains isolated from Stations 2 and 4 have high homologies to *Desulfosporosinus orientis* and *Desulfovibrio acrylicus*, respectively.

Marine coastal areas are, to a large extent, influenced by human activities that lead to increased concentrations of organic and inorganic pollutants.[1, 2)] Much of the particulate organic matter is accumulated in the bottom sediments, which provide anaerobic conditions as a result of the active consumption of oxygen by heterotrophic organisms.[3)] In anoxic sediments, methanogenic and sulfate-reducing bacteria (SRB) are two main groups responsible for the degradation of organic compounds as carbon and energy sources.[4, 5)] Anaerobic sulfate reduction is particularly important in marine environments due to the high abundance of sulfate. Sulfate-reducing bacteria (SRB) contribute to the anaerobic terminal oxidation process in marine sediments, and in estuarine sediments the sulfate reduction process can be responsible for more than 50% of the organic matter degradation.[6)] The major product of sulfate reduction is hydrogen sulfide, which gives the characteristic smell to sediment muds. SRB are a diverse group of anaerobic bacteria that have the ability to use sulfate as a terminal electron acceptor in the consumption of organic matter with the concomitant

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production of hydrogen sulfide. They utilize a very wide spectrum of different low molecular organic compounds such as lactate, acetate, propionate, succinate, pyruvate, sugars and amino acids as electron donors.[7-9] Physical and chemical factors of sediment layers including sulfate and organic matter concentrations, sedimentation rate, turbulence, temperature, salinity and hydrostatic pressure influence on the numbers and distribution of SRB and the rate of sulfate reduction.[10, 11] SRB, which generate large amount of toxic hydrogen sulfide in aquatic ecosystems, are important from ecological reasons as well as from the viewpoint of economy.[12]

Bottom sediments in Kagoshima Bay contain relatively high content of organic matter as reported that ignition loss (IL) ranged from 1.1% to 13.4% and total organic carbon (TOC) and total organic nitrogen (TON) ranged from 0.2 to 2.3% and from 0.04 to 0.22%, respectively. On the other hand, dissolved oxygen concentration was reported to decrease from 1995 to 2000 particularly in the hypolimnion layers of the inner parts of Kagoshima Bay.[13] These conditions are considered to stimulate the development of SRB in the sediment of Kagoshima Bay.

In this study we isolated sulfate-reducing bacteria (SRB) from the sediment samples obtained from Kagoshima Bay and characterized isolated SRB strains bacteriologically and phylogenetically based on 16S rDNA sequences.

Materials and Methods

Sampling stations

Sediment samples were collected from four stations in Kagoshima Bay, Japan by a research vessel, Nansei-Maru of the Kagoshima University. Sediment samples were collected in August 2005, Station 1 (31°41.6′N, 130°44.6′E, water depth of 102 m), Station 2 (31°23.7′N, 130°38.7′E, water depth of 225 m), Station 3 (31°12.5′N, 130°38.1′E, water depth of 37.5 m) and Station 4 (31°12.3′N, 130°42.5′E, water depth of 108 m). Station 1 is located in the inner area, Stations 2 and 3 are in the central area and Station 4 is in the outer area of Kagoshima Bay (Fig. 1).

Sediment samples were collected by K.K. core samplers (Kimata *et al*, 1960).[14] Zinc acetate was added immediately to sediment samples for acid volatile sulfide (AVS) analysis. The sediment samples for enumeration and isolation of SRB were kept at 4ºC and transported to a laboratory.

Physical and chemical analysis of sediments

Particle size of sediments from 0-1 cm layer of sediments was examined by using a laser diffraction particle size analyzer (SALD 3100). Median diameter of sediment particles was expressed as Mdφ (median

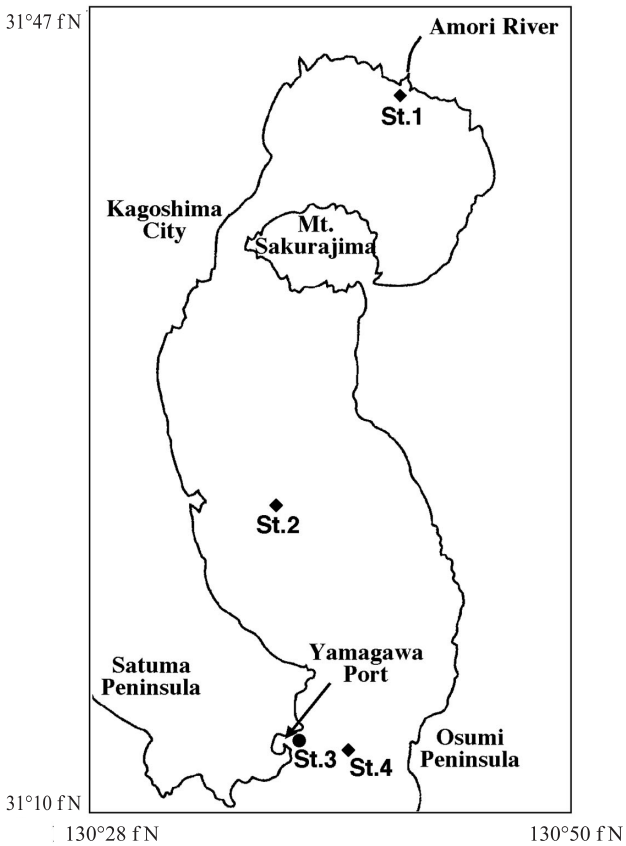

Fig. 1. Location of 4 sampling stations in Kagoshima Bay.

diameter = $1/2^{\varphi}$). Ignition loss (IL) was determined by getting the difference between dry weight (after 24 h at 100°C) of sediment samples and combustion weight of the residue after 2 h at 450°C, expressed in percent. Total acid volatile sulfides (AVS), which include iron monosulfide (FeS) and remaining dissolved hydrogen sulfide, were extracted by steam distillation under acidic condition and trapped in 10% zinc acetate solution. Trapped sulfide content was determined by the spectrophotometric methylene blue method.[15)]

Sulfate-reducing bacteria (SRB) were counted by the MPN method, using medium B described by Postgate (1984).[16)] Growth of SRB was observed by precipitation of FeS (black color).

Isolation and culture of SRB

Five g of sediments were removed from the center part of whole round cores and serially diluted prior to transfer into most-probable-number (MPN) tubes containing modified Postgate's SRB medium B[16)] supplemented with lactate and seawater. The medium contained 0.2 g of KH_2PO_4, 1.0 g of NH_4Cl, 5.8 g of sodium lactate, 1.0 g of yeast extract, 0.1 g of sodium thioglycolate, 0.2 g of $FeSO_4 \cdot 7H_2O$, dissolved into the mixture of 800 ml of natural seawater or artificial seawater (ASW, Herbest's formula composed of NaCl 30 g, KCl 0.7 g, $MgCl_2 \cdot 6H_2O$ 10.8 g, $MgSO_4 \cdot 7H_2O$ 5.4 g and $CaCl_2 \cdot 2H_2O$ 1.0 g per ℓ) and 200 ml of distilled water. The pH was adjusted to 7.6, and the medium was autoclaved at 121°C and then cooled and 1 ml of ascorbic acid was added. Inoculated MPN tubes were incubated at 30°C.

SRB positive cultures were transferred into SRB agar media containing lactate and 0.8% agar. When black colonies developed in agar media at 30°C, they were picked up and inoculated into liquid media. These procedures were repeated until apparent pure cultures were obtained. Bacterial cultures were assessed by Gram staining, aerobic growth, AVS production and 16S rDNA sequence analysis of the cells grown in medium B at 30°C.

DNA extraction from SRB cells

For extraction of whole DNA from SRB cells, 1 ml of liquid cultures of isolated SRB strains grown in medium B was centrifuged at 5,000 rpm for 3 min to remove black precipitates in the cultures. After 5 min of centrifugation at 15,000 rpm for 5 min under 4°C, cell pellets were re-suspended in 40 µl of TE buffer. After 4 µl of SDS solution (10% sodium dodecylsulfate) and 4 µl of Proteinase K solution (10 mg per ml) were added to the cell suspensions, the mixtures were incubated at 60°C for 30 min. Their genomic DNAs of SRB strains were extracted by a method described by Tsai and Olsen.[17)] Total DNAs were examined visually on 1% (w/v) agarose gel electrophoresis.

Polymerase chain reaction (PCR)

The 16S rDNA of SRB strains was amplified by PCR according to the standard method. The universal primers, PrSSU.1F (5'-AGAGTTTGATCATGGCTCAG-3') and PrSSU.1R (5'-GGTTGGATCACCTCCTT-3') as forward and reverse primers, respectively, were used. The PCR mixtures were subjected to thermal cycling as follows: an initial denaturation (1 min at 95°C), followed by 30 cycles of denaturation (15 sec at 95°C), annealing (30 sec at 48°C, 51°C, 54°C, 57°C or 60°C), and extension (1.5 min at 72°C), and an additional extension process (7 min at 72°C).

Sequence and phylogenetic analysis of 16S rDNA

To determine 16S rDNA sequences, the PCR products were re-amplified by using the following primers with the same PCR program as described above.

PrSSU.1F (5'-AGAGTTTGATCATGGCTCAG-3'),
PrSSU.2F(5'-TCCTACGGGAGGCAGCA-3'),
PrSSU.2R (5'-TTAAGTCCCGCAACGAGCG-3')
PrSSU.1R (5'-GGTTGGATCACCTCCTT-3')

Nucleotide sequences of 16S rDNA were determined with ABI PRISM 310 Genetic Analyzer (Applied systems, USA) and aligned by using the CLUSTAL X multiple sequence alignment program version 1.8 (Thompson *et al*., 1997)[18] with closely related 16S rDNA sequences found in the GenBank database. The multiple alignments obtained were used for bootstrap re-samplings of 1,000 replications (Felsenstein, 1985).[19] 16S rDNA-based phylogenetic trees were constructed from a distance matrix calculations by the neighbor-joining method using CLUSTAL X. (Felsenstein, 1989).[20]

PCR with primers specific to *Desulfovibrio* spp.

Several specific primers for the genus *Desulfovibrio* have been reported by some investigators. In this study, 2 specific reverse primers were designed based on the references as follows: DSVIB679r (TTACACTCCTAC ACCTGG), Stubner (2004)[21] and DSV-I-695 (CCTCC AGATATCTACGGA), Manz *et al*.(1998).[22]

PCR amplification with the two primer sets such as PrSSU.1F and DSV-I-695r, and PrSSU.2F and DSVIB679r, was carried out using genomic DNAs extracted from isolated SRB strains, *Vibrio harveyi* and *Escherichia coli* as templates. Thermal cycling reactions were carried out as follows: an initial denaturation for 1 min at 95°C, 30 cycles of denaturation (15 sec at 95°C), annealing (30 sec at 60°C and 64°C) and extension (1.5 min at 72°C), and final extension (7 min at 72°C). PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. DNA bands were visualized by UV illumination.

Results

Sediment characteristics and SRB cell counts

Physical and chemical analysis of sediments from four stations are shown in Table 1. The sediments of Stations 1, 2 and 3 are similarly characterized as fine silt containing considerably high moisture contents (79.1 – 87.4%) and high IL (13.8 – 15.9%). On the other hand, the sediment of Station 4 is very differrent from others because of fine sand with 30.4% of moisture and 2.5% of IL. Acid volatile sulfide (AVS) contents (8.6 mg/g) and SRB density (56.0 x 10^3 /g) are remarkably high at Station 3 compared with those of other 3 stations.

Isolation of sulfate-reducing bacteria

Eight colonies were picked up from 10^{-2} to 10^{-3} dillutions of MPN tubes and transferred to flasks containing liquid medium B. The appearance of black colored precipitates in the liquid cultures is an indicator

Table 1. Characterization of sediments obtained from four stations in Kagoshima Bay

Parameters*	Station 1	Station 2	Station 3	Station 4
Mdφ	5.07	5.83	5.21	3.11
σI	1.81	1.74	1.81	3.16
SkI	0.15	0.04	0.13	0.3
Moisture (%)	79.12 ± 0.01	83.45 ± 0.11	87.42 ± 0.03	30.39 ± 2.38
IL (%)	15.41 ± 0.18	13.81 ± 0.61	15.89 ± 0.12	2.52 ± 0.44
AVS (mg/g)	0.11 ± 0.002	0.07 ± 0.002	8.64 ± 1.83	0.01 ± 0.0004
No. of SRB (MPN/g)	15.5 x 10^3	7.8 x 10^3	56.0 x 10^3	0.72 x 10^3

*Mdφ, median diameter of sediments (φ scale); σI, sorting coefficient; SkI, skewness; IL, ignition loss; AVS, acid volatile sulfide; SRB, sulfate-reducing bacteria.

of SRB growth as shown in Fig. 2. The back colored precipitates and hydrogen sulfide occurred during four days in the cultures of the strains isolated from Stations 1 and 3. On the other hand, the strains isolated from Stations 2 produced black colored precipitates and the smell of hydrogen sulfide in 10 days. Generally, SRB strains were known to grow faster in the liquid medium with natural sea water than with artificial seawater. In order to ensure the culture purity, one culture from each station was chosen and repeatedly inoculated to fresh media. Culture purity was confirmed by observation of a uniform cell form of Gram staining cells grown under an anaerobic condition and analysis of 16S rDNA sequences.

Micrographs of Gram-staining cells of the isolated strains from Stations 1, 2 and 3, which were tentatively named as SR-St-1-1, SR-St-2-1, SR-St-2-2 and SR-St-3-1, are shown in Fig. 3. Strains SR-St-1-1 and SR-St-3-1 isolated from Stations 1 and 3 were Gram-negative, curved or straight rods (0.3-0.7 by 1.0-3.0 μm), and

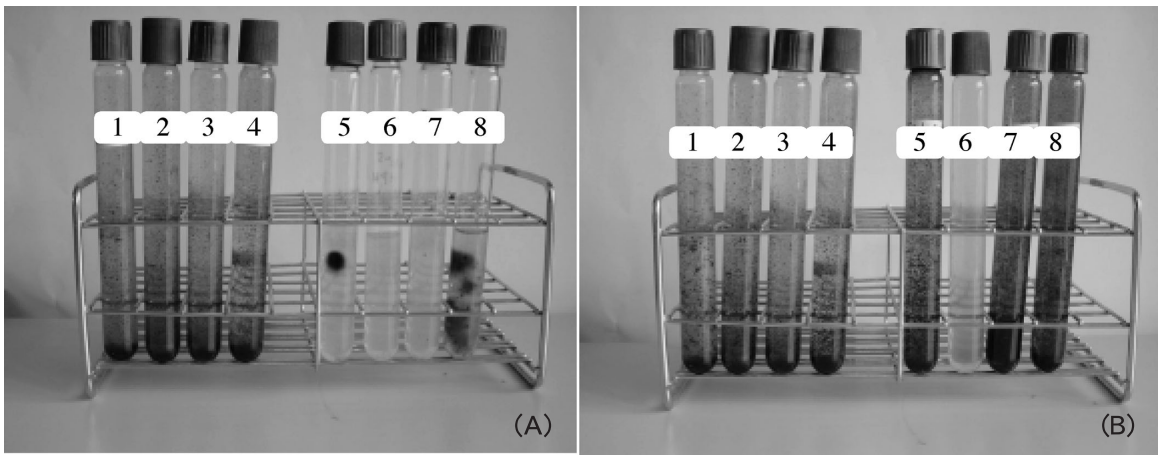

Fig. 2. Growth of SRB strains in liquid and soft agar media.
(A): Test tubes 1-4 are liquid cultures and test tubes 5-8 are soft agar cultures. The test tubes 1 and 5, SR-St-1-1; 2 and 6, SR-St-2-1; 3 and 7, SR-St-2-2; 4 and 8, SR-St-3-1.
(B): Test tubes 1-4 are liquid cultures with natural seawater and test tubes 5-8 are liquid cultures with artificial sea water. The test tubes 1 and 5, SR-St-1-1; 2 and 6, SR-St-2-1; 3 and 7, SR-St-2-2; 4 and 8, SR-St-3-1.

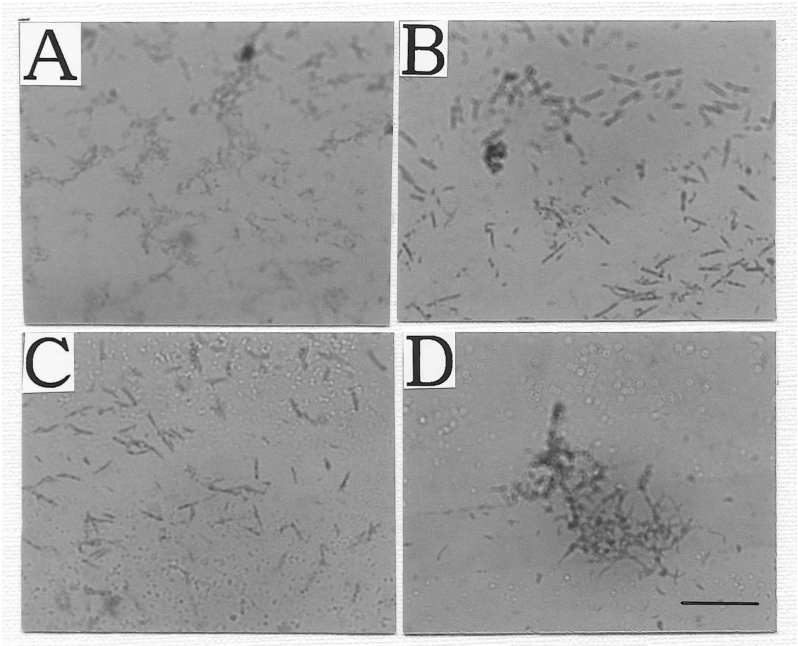

Fig. 3. Gram-stained cells of SRB strains from different sediment samples.
A, SR-St-1-1; B, SR-St-2-1; C, SR-St-2-2; D, SR-St-3-1. Bar indicates 10 μm.

both strains of SR-St-2-1 and SR-St-2-2 were Gram-negative rods (0.5-1.0 by 3-5 μm) with densely stained particle-like structures.

AVS production and pH change in liquid cultures

The growth of SRB strains were estimated by the increase of AVS production during incubation in liquid medium B. As shown in Fig. 4, AVS contents in strains SR-St-1-1 and SR-St-3-1 increased rapidly after 2 days of incubation. On the other hand, AVS content in the liquid culture of SR-St-2-1 started increasing from 6 days of incubation. AVS content in SR-St-1-1 culture continued increasing until 10 days, while that in SR-St-3-1 and SR-St-2-1 cultures decreased from 6 or 8 days of incubation, respectively.

The pH values of the liquid cultures of three strains increased from 7.6 to 8.8 during 10 days incubation and showed that SR-St-2-1 had relatively longer lag-time for pH increasing (Fig. 5).

PCR products of 16S rDNA

The 16S rDNAs were amplified by PCR with the SRB genomic DNAs as templates. The optimal annealing temperatures for PCR of each SRB culture were examined and the results of agarose gel electrophoresis of PCR products from strains SR-St-1-1, SR-St-2-1, SR-St-3-1 and SR-St-4-1, which was isolated from Station 4,

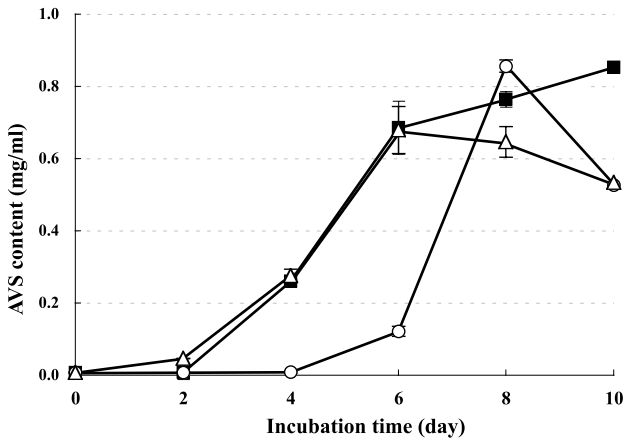

Fig. 4. AVS production during 10 days incubation of SRB strains in liquid medium B.
Data indicates an average of duplicate cultures for each strain.
The standard deviations are shown as vertical lines.

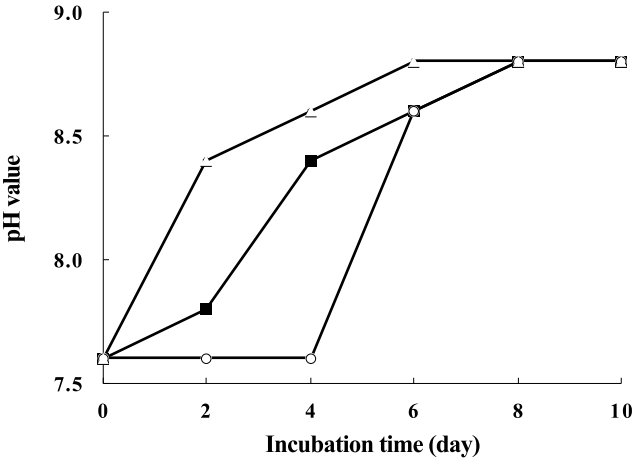

Fig. 5. pH change during 10 days incubation of SRB strains in liquid medium B.
Data indicates an average of duplicate cultures for each strain.

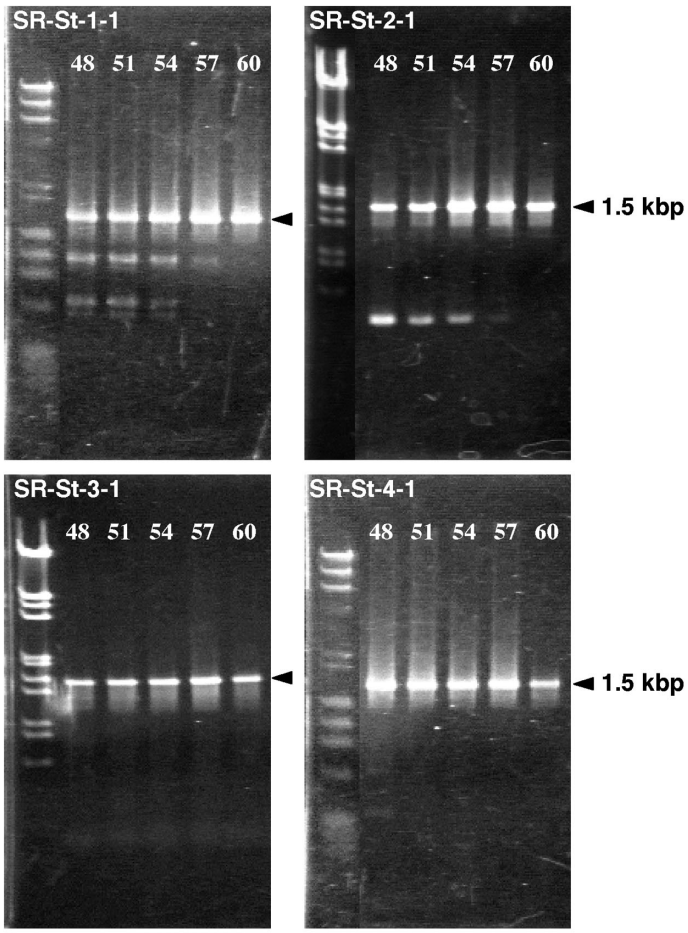

Fig. 6. Agarose gel electrophoresis of 16S rDNA amplified by PCR at various annealing temperatures using genomic DNA of the SRB strains as templates.
Numbers denote the annealing temperature. The 100 bp ladder markers are also shown.

were shown in Fig. 6. Three strains of SR-St-1-1, SR-St-3-1, and SR-St-4-1 generated single bands corresponding to 1,500 bp at annealing temperature of 60°C. In the case of SR-St-2-1, an additional band with a small size was obtained.

Phylogenetic analysis of isolated strains

The PCR products from 4 strains were purified and subjected to sequencing by an automated DNA sequencer. Phylogenetic trees inferred from the 16S rDNA of the isolated SRB strains constructed by the neighbor-joining method indicated that the strain SR-St-1-1 isolated from Station 1, SR-St-3-1, SR-St-3-2, SR-St-3-3, SR-St-3-4, and SR-St-3-5 from Station 3, and SR-St-4-1 from Station 4 are members of *Desulfovibrio* group as shown in Fig. 7. Among them, the strains SR-St-1-1, SR-St-3-1, SR-St-3-2, SR-St-3-3, SR-St-3-4 and SR-St-3-5 are most closely related to *Desulfovibrio dechloracetivorans* with similarity of 98% and the strain SR-St-4-1 is close to *Desulfovibrio acrylicus* with similarity of 98%. On the other hand, the strain SR-St-2-1 is a member of *Desulfosporosinus* group and closely related to *Desulfosporosinus orientis* with similarity of 98%.

PCR by using primers specific to *Desulfovivrio*

Two reverse primers specific to *Desulfovibrio* spp. were used to identify the *Desulfovibrio* isolates among the SRB strains. As shown in Fig. 8, the primer set of PrSSU.1F and DSV-I-695r produced a single band at

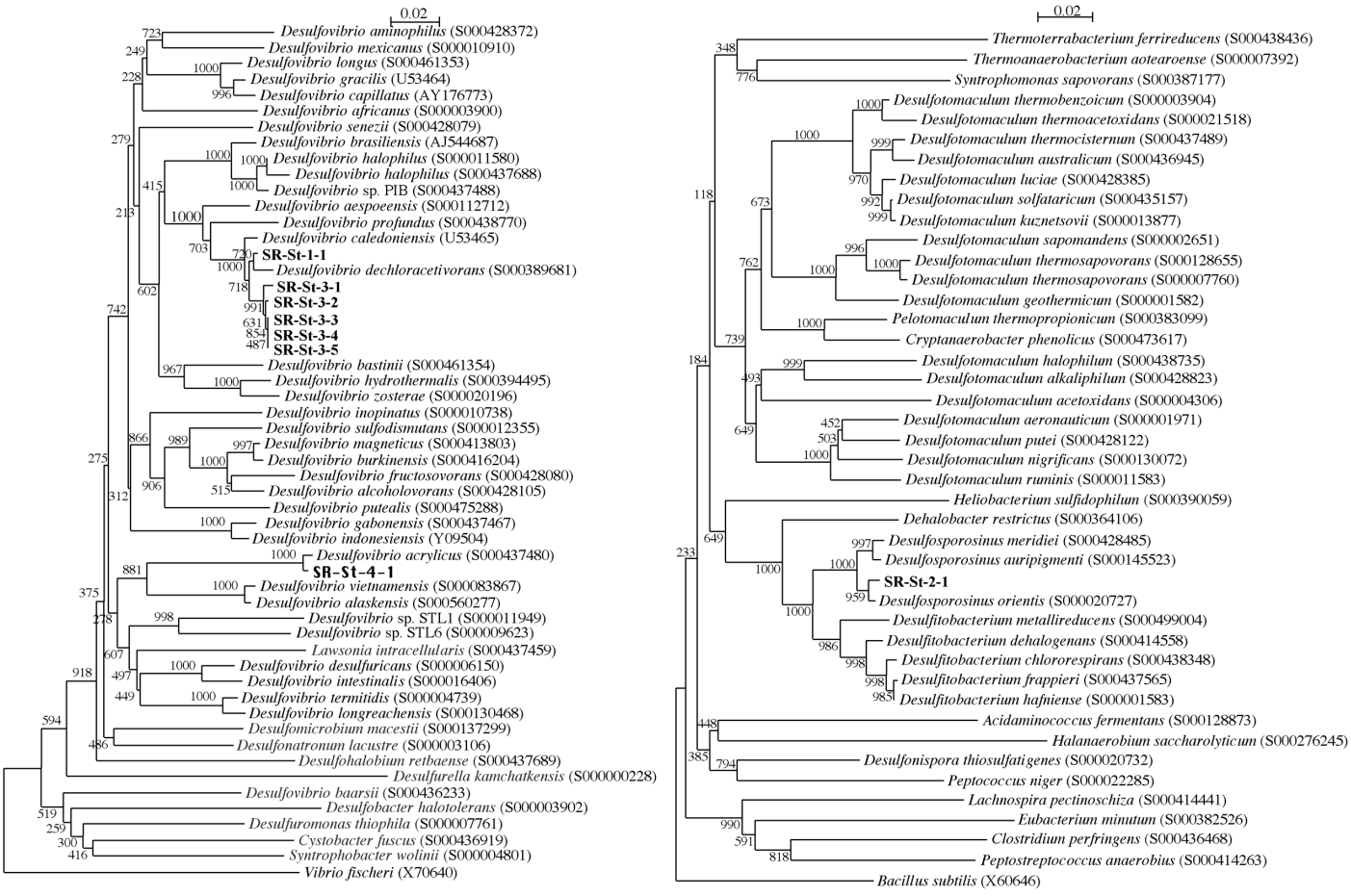

Fig. 7. Phylogenetic trees inferred from 16S rDNA sequences of the isolated SRB strains and related bacteria from a database. The tree was constructed by the neighbor-joining method. A scale bar indicates genetic distance. Numbers at the branching points indicate bootstrap values of 1,000 replicates.

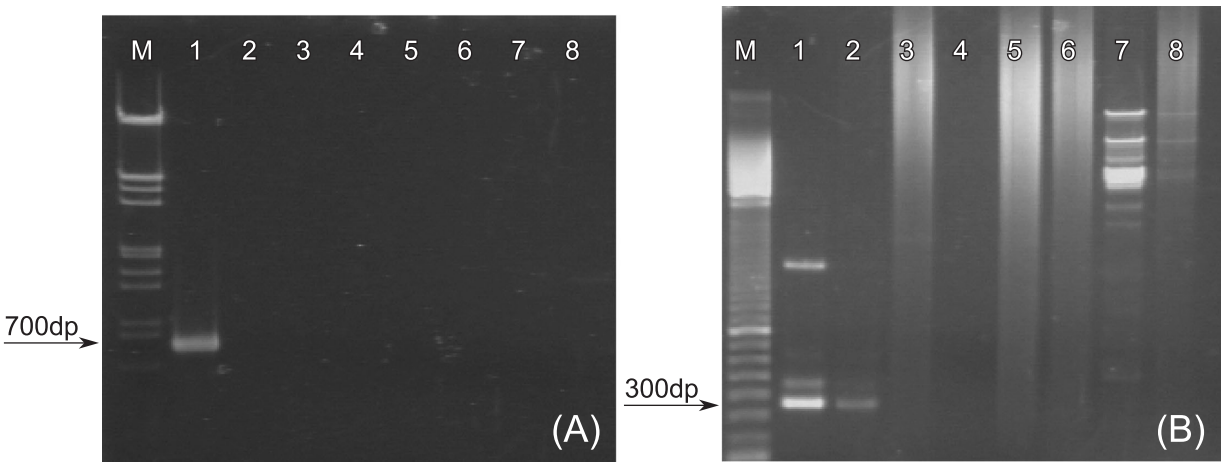

Fig. 8. Agarose gel electrophoresis of 16S rDNA of the isolated SRB strains amplified by PCR using *Desulfovibrio* spp.-specific primer sets. (A): PrSSU-1F and DSV-I-695r were used as primers. (B): PrSSU-2F and DSVIB679r were used as primers. M1, λ/*Eco*RI+*Hin*dIII marker; M2, 100 bp ladder marker. Lanes l and 2, SR-St-3-4; lanes 3 and 4, SR-St-2-1; lanes 5 and 6, *Vibrio harveyi*; lanes 7 and 8, *Escherichia coli*. The annealing temperatures of the thermal cycling are 61°C (lanes 1, 3, 5 and 7) or 64°C (lanes 2, 4, 6 and 8). The sizes predicted from the primer design are shown as arrows.

annealing temperature of 61°C. This single band has the size of about 700 bp predicted from the primer design. The application of the primer set of PrSSU.2F and DSVIB679r yielded the band of the expected size of 300 bp as well as different size bands.

Discussion

Kagoshima Bay is located between 30°59'N to 31°43'N latitude and 130°31'E to 130°48'E longitude. It has a length of about 75 km from north to south and a width of about 25 km. The bay is divided into three areas including the inner, central and bay mouth. Mt. Sakurajima (volcano) is located between the inner and central areas of the bay. The channel between Sakurajima and Kagoshima City is narrow (1.9 km), with a depth of about 40 m. The bay mouth connected with an open sea is also narrow (8.7 km) with a depth of about 80 m (Fig. 1). The bottom topography of Kagoshima Bay presents a shape similar to two conical bowls connected together. Due to these conditions, exchange of seawater in the bay is very inefficient, especially in the inner area. The central area is the widest among the three areas and presents a basin-like topography with the maximum depth of about 230 m.

Station 1 located in the inner area is known to be characterized by low oxygen concentration in the bottom water probably owing to a stagnant condition of water and high content of organic matter. Station 2 in the central area had relatively high concentrations of organic matter and dissolved oxygen. Sediments collected from Station 3 in Yamagawa Harbor have undergone a heavy impact by organic pollutant loads as compared to other stations in Kagoshima Bay. Sediment muds from Station 3 are characterized by high concentrations of both moisture and organic matter (high IL) and had uniformly black color with very strong smell of volatile hydrogen sulfide. The AVS content in this station was much higher than other stations. The sediments from Station 4 in the outer area are characterized by sandy-clay sediments with very low moisture level as well as low organic matter content.

In this study, we isolated SRB strains from sediments of the above four stations in Kagoshima Bay. Among them three representative strains from Stations 1, 2 and 3 were used to determine AVS production during 10 days of incubation. The AVS contents in the cultures of

SR-St-1-1 and SR-St-3-1 isolated from Stations 1 and 3 increased rapidly after 2 days of incubation. While, the AVS content in the liquid culture of strain SR-St-2-1 isolated from Station 2 started to increase after 4 days of incubation. This result indicates that the strains SR-St-1-1 and SR-St-3-1 grew faster than SR-St-2-1 in the culture conditions. The pH values of liquid cultures of the three strains increased to alkaline (pH 8.8) during the incubation period. Postage (1984)[16] described that the increase of sulfide production in an incubation medium yielded the pH value increasing to alkaline pH and the high content of sulfide caused inhibition for the growth of SRB.

Comparison of the 16S rDNA sequences of the SRB strains isolated from the sediments of 4 stations indicated that the SRB isolated from Staions 1, 3 and 4 are a member of the delta subdivision of *Proteobacteria*.[23] On the other hand, 5 strains of SRB isolated from Station 3 and one strain from Station 1 were closely related to *Desulfovibrio dechloracetivorans*, and a strain isolated from Station 4 had higher homology to *Desulfovibrio acrylicus*, a strain isolated from Station 2 was highly close to the Firmicute *Desulfosporosinus oriensis*. The application of specific primers to identify *Desulfovibrio* spp. demonstrated that the primers of DSV-I-695r and DSVIB679r are suitable to detect 16S rDNA of this bacterial group. So and Young (1999)[24] reported that an alkane-degrading sulfate-reducing bacterial strain isolated from estuarine sediment was most closely related to the genera *Desulfosarcina, Desulfonema* and *Desulfococcus* in the delta subdivision of *Proteobacteria*.

In sulfate- and organic matter-rich environments such as estuary and coastal regions, sulfate reducing bacteria (SRB) play an important role in the degradation of organic pollutants. Further work is needed to estimate the more precise distribution of SRB and to define their ecological role in marine environments.

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