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Dimethyl sulfoxide-respiring bacteria in Suribati Ike, a hypersaline lake, in Antarctica and the marine environment

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Abstract: Dimethyl sulfoxide (DMSO) occurs worldwide, especially in marine environments as well as in lakes and rainwater. DMSO respiration by bacteria is assumed to play an important role in the sulfur cycle in Antarctica and on earth. We first studied whether DMSO-respiring bacteria existed in Antarctica. Eight strains were isolated that grew by DMSO respiration under anaerobic conditions from water of the halocline in a meromictic lake, Suribati Ike, near Syowa Station in Antarctica. All of them were related to known species belonging to the genus *Marinobacter* based on 16S rRNA gene sequences. Using a clone library analysis of 16S rRNA gene sequences, 38 of total 48 clones from water of the halocline were identified as *Marinobacter*. Studies on the various anaerobic respiration capabilities by bacteria in the halocline water found only DMSO respiration. Studies on bacteria with anaerobic respiration abilities in seawater from the Pacific Ocean and Seto Inland Sea, showed that either DMSO-respiring or nitrate-respiring bacteria were present and that all of isolates capable of DMSO respiration were closely related to *Vibrio* species.

key words: Antarctic hypersaline lake, *Marinobacter*, DMSO respiration, marine environment

Introduction

Dimethyl sulfoxide (DMSO) exists at concentrations of up to 220 nmol in seawater. In lakes, rivers and rainwater it ranges from 1 to 70 nmol (Griebler, 1997). DMSO is produced by the oxidation of dimethyl sulfide (DMS) formed by the hydrolysis of dimethyl sulfoniopropionate (DMSP), which exists in marine algae for osmotic balance. Bacterial DMSO reductase converts water soluble DMSO into the insoluble and rather volatile DMS. DMS contributes about 90% of the total sulfur flux from marine environments to the atmosphere (Andreae and Raemdonck, 1983). Atmospheric DMS is eventually transformed into methylsulfonate (MSA) or other oxidized acidic forms by photooxidation, which serves as nucleation sites for the formation of clouds. It is suggested that the sulfur cycle, involving DMS and DMSO as the main components, serves as a biological feedback system to modulate the temperature of the Earth by controlling cloud forma-

tion and hence the albedo of the planet (Stiefel, 1996; Charlson *et al*., 1987). Therefore, DMSO respiration by bacteria appears to play an important role in the global sulfur cycle.

Several bacterial species have the ability of DMSO respiration, including *Escherichia coli* (Weiner *et al*., 1988), *Proteus vulgaris* (Styrvold *et al*., 1984), two phototrophic bacteria *Rhodobacter sphaeroides* (Satoh and Kurihara, 1987) and *R. capsulatus* (McEwan *et al*., 1987), and *Halobacterium* sp. strain NRC-1 (Müller and DasSarma, 2005). As far as the gene of DMSO reductase (*dmsA*), the terminal reductase of DMSO respiration, 16 sequences from the species above and other species such as *Salmonella typhimurium*, *Yersinia pestis*, and *Haemophilus influenzae* are listed in the DNA database (McCrindle *et al*., 2005).

In an ice core of Lake Vostok in Antarctica, the concentration of MSA was found to be higher during the glacial period than that during the interglacial period over the past 160 thousand years (Legrand *et al*., 1967). This may suggest that cloud nucleation is facilitated by increasing the release of DMS by DMSO respiration in the water system and aerosol by volcano eruptions (Petit *et al*., 1999) during low temperature periods. We first investigated the contribution of DMSO respiration by detecting *dmsA* genes from the environmental DNA from a lake, (Suribati Ike in Skarvsnes, Antarctica) by Southern hybridization using probes of *dmsA* from *E. coli* and *R. sphaeroides*. We subsequently tried to study whether DMSO-respiring bacteria actually existed in the lake water.

Suribati Ike is a hypersaline meromictic lake with a maximum depth of 31.2 m and about 1 km across (Hirabayashi and Ossaka, 1977). The salinity of the water deeper than 12 m is 15% which is 5 times higher than that of seawater, and that of water shallower than 10 m ranges from 4 to 12% (Naganuma *et al*., 2005). The upper layer (>10 m) is stirred by the force of the wind, but the lower layer does not mix with the upper layer due to the persistent halocline. Water taken from the lower layer smells of hydrogen sulfide, and no oxygen is observed. Naganuma *et al*. (2005) isolated twenty-three strains of euryhaline halophiles from Suribati Ike, and they were affiliated with the halophilic genera *Halomonas* and *Marinobacter*. The strains from anoxic depths showed no anaerobic growth in LBG medium.

Here, we describe the isolation of DMSO-respiring bacteria from Suribati Ike. We also isolated eight DMSO-respiring bacteria from the Pacific Ocean and Seto Inland Sea water, respectively, to determine taxonomical differences among them.

Materials and methods

Study site, sample collection and enumeration of cells in the Suribati Ike water

Lake Suribati Ike is situated at 69°29'S, 39°41'E in the central Skarvsnes ice-free area along the central Sôya Coast, East Antarctica. It has been suggested that Suribati Ike originated from marine waters due to its surface altitude of −33 m and its salt composition (Hirabayashi and Ossaka, 1977). It shows a typical meromictic stratification of upper (>10 m) and lower (<10 m) layers. The lower layer is anaerobic with a sulfidic odor and blackish sulfide (Naganuma *et al*., 2005).

A water sample at a depth of 10 m from Suribati Ike was collected using autoclaved Heyroth glass bottle as described by Naganuma *et al*. (2005). A coastal surface sea water

sample from the Seto Inland Sea was collected near Miyajima Natural Botanical Garden, Hiroshima University. Pelagic surface samples from the Pacific Ocean were collected from the surface (10°46'N, 150°30'E and 4°19'N, 151°49'E) on a voyage from Japan to Australia.

The total bacterial cell counts in Suribati Ike water were determined by microscopic counting in a Petroff-Hausser chamber (ERMA INC.). The viable bacterial cell counts were determined by counting colony-forming strains on agar plates of solid MTYE medium, which is used as a rich marine medium (Proctor and Gunsalus, 2000). Aerobic cell growth on agar plates in the absence of DMSO was performed at 15°C for 1 month. Anaerobic cell growth on agar plates in the presence of DMSO was performed in a BBL GasPak 100 anaerobic jar with AnaeroPack (Mitsubishi Gas Chemical Co., Inc.) at 15°C for 1 month.

Analysis of 16S rRNA clone libraries from the halocline water

A 1 m*l* water sample from Suribati Ike was centrifuged for 1 min at 10000 ×*g*. The pellet was washed with 100 *μl* of sterilized water, resuspended in 10 *μl* of sterilized water, and used as a template solution for PCR. Partial 16S rRNA sequences (*ca*. 1502 bp) were amplified by PCR with the common primers 27F and 1492R (Hiraishi, 1992; Hiraishi *et al*., 1994) using Ampli Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR conditions were set as 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 1.5 min. The PCR products were gel-purified and then cloned into the pGEM T-Easy vector (Promega, WI, USA). Forty eight randomly selected clones were fully sequenced by the dideoxynucleotide chain termination method using a model 310A DNA Sequencer (Applied Biosystems Co., Foster City, CA, USA).

Isolation of DMSO-respiring bacteria

To isolate the DMSO-respiring bacteria, aliquots (100 *μl*) of water were spread onto the surface of MTYE medium (Proctor and Gunsalus, 2000) containing DMSO at a final concentration of 0.2%, and cultured. Single colonies were repeatedly picked and re-spread on fresh agar plates. Colony-forming strains were maintained on agar plates containing identical ingredients.

Growth experiments

The optimum salt concentration for growth was determined by culturing cells aerobically at 20°C in tubes containing MTYE medium at NaCl concentrations of 0, 1.5, 3, 6, 12 or 24%. The optimum temperature was determined by culturing the cells aerobically in MTYE medium containing 3% NaCl at temperatures of 4, 20, 30, 37 and 40°C. The growth was monitored by measuring optical density at 600 nm.

Phylogenetic sequence analysis

The sequences were aligned using the CLUSTAL W multiple-alignment program (Thompson *et al*., 1997). Positions with gaps were ignored by using the TOSSGAPS function of CLUSTAL W. The 16S rRNA sequences were examined for similarity using FASTA (Pearson and Lipman, 1988) and BLAST (Karlin and Altschul, 1990; Tatusova

and Madden, 1999) at the DNA Data Bank of Japan (DDBJ). The phylogenetic tree was constructed using TreeView (Page, 1996) by the neighbor-joining method after 1000 bootstrap replications and drawn by using TreeView (Page, 1996). The nucleotide sequences of 16S rRNA gene from eight isolates have been submitted to GenBank-EMBL-DDBJ under the accession no. AB252056-AB252063.

Results and discussion

Total and viable bacterial cell counts in halocline water

The total bacterial cell count in water from the halocline layer at 10 m depth counted by microscopic counting was 1.7×10^7 cells/m*l*. The number of viable bacterial cells grown on MTYE medium under aerobic conditions is 8.3×10^3 cells/m*l*, indicating that the viable count was less than 0.1% of the total bacteria. The number of cells grown under anaerobic conditions on MTYE medium in the presence of DMSO as the sole terminal electron acceptor was 7.4×10^3 cells/m*l*, indicating that 90% of cells grown aerobically also have DMSO respiration activity. This confirms that all of DMSO-respiring bacteria reported so far are able to grow aerobically (McCrindle *et al*., 2005).

Clone sequences of 16S rRNA gene of halocline water

A clone library of 16S rRNA genes was constructed from a cell suspension of halocline water. Forty eight clones were isolated randomly that had an insert of the correct size corresponding to positions 27–1492 of the *E. coli* 16S rRNA gene, and their sequences of 16S rRNA genes were determined (Table 1). Using a clone library analysis, 38 out of a total of 48 clones were identified as the genus belonging to *Marinobacter*, 4% to *Halomonas*, 2% to *Pseudomonas* and 2% to *Halocella*, and 10% were not identified. Bowman *et al*. (2000) have detected the members of *Marinobacter*, *Halomonas* and *Pseudomonas* in a clone library from three limnologically disparate hypersaline Antarctic lakes. Recently, Naganuma *et al*. (2005) report the vertical distribution of *Marinobacter*,

Table 1. Phylogenetic affiliations and frequencies of 16S rRNA gene sequences retrieved from the meromictic lake, Suribati Ike, Antarctica.

Closest identified relative	Similarity (%)	Number of clones
Marinobacter sp. SKA 35 (AF261056)	97.3–99.5	17
Marinobacter hydrocarbonoclasticus (AB021372)	96.9–99.3	14
Marinobacter sp. HB7 (AB089804)	97.1–98.5	2
Marinobacter sp. SBS (AF482686)	98.0–98.1	2
Marinobacter daepoensis strain SW-156 (AY517633)	98.3–99.1	2
Marinobacter sp. ANT8277 (AY167267)	98.4	1
Halomonas variabilis strain HTG7 (AY204638)	97.1–98.3	2
Halomonas sp. SYO J42 (AB085653)	99.2	1
Pseudomonas halophila (AB021383)	97.2	1
Halocella cellulolsilytica (X89072)	97.1	1
unidentified		5
		Total 48

Halomonas and *Idiomarina* in Suribati Ike. Our results are consistent with those by Bowman *et al*. and Naganuma *et al*. in that *Marinobacter* and *Halomonas* are included in the hypersaline Antarctic lakes.

Characterization of strains grown anaerobically with DMSO

Suribati Ike lake water (10 μl) was placed onto solid MTYE plates containing DMSO and cultured under anaerobic conditions at 15°C for two weeks, and 96 colonies were isolated. Subsequently, eight colonies were selected by their differing shapes. Their sequences of 16S rRNA gene showed that they all were affiliated to limited phylogenetic clusters, *Marinobacter*, with a similarity between 99.6 and 100% (Fig. 1). All eight isolates also grew under aerobic conditions. Although several bacterial species are known to have the ability of DMSO respiration, *Marinobacter* isolates from marine environments have not been tested for this ability. This is the first report of *Marinobacter* being able to

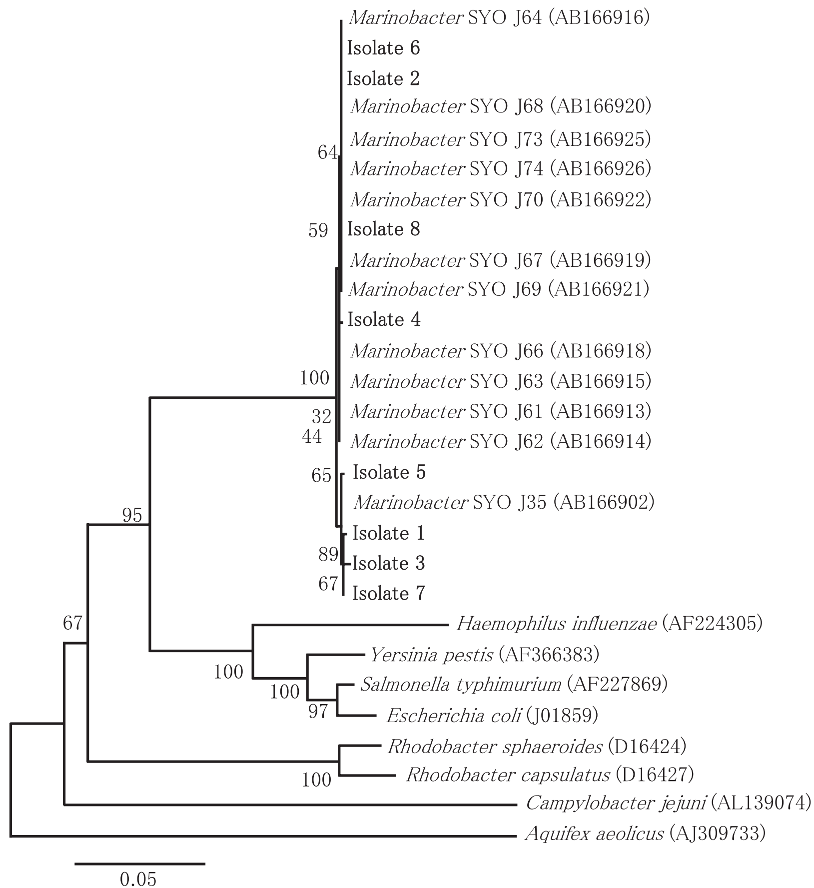

Fig. 1. Phylogenetic tree based on 16S rRNA sequences of isolates from the meromictic Antarctic lake, Suribati Ike. The scale bar indicates 0.05 fixed substitution per nucleotide position.

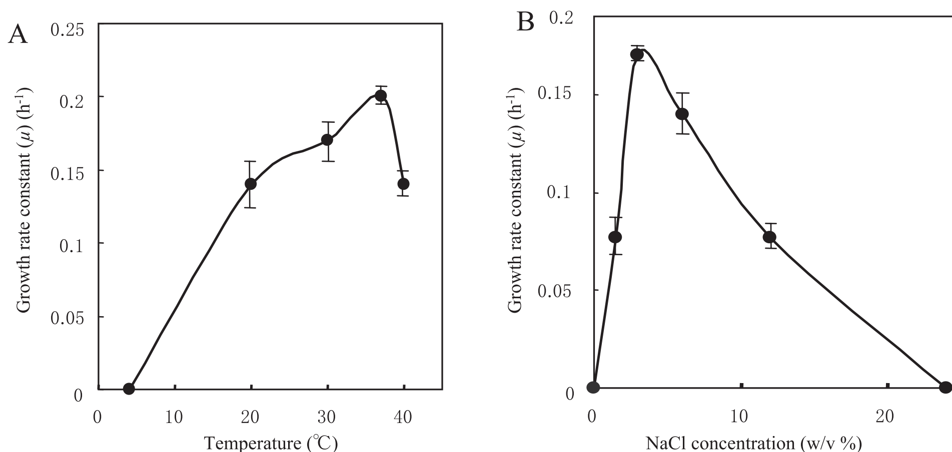

Fig. 2. Effects of salinity at 20°C (A) and temperature (B) at a NaCl concentration of 30 g/m*l* on the growth constant rate of the isolate. Growth rate constant (μ) was calculated as $0.693/g$. g is a generation time (h). Each value corresponds to the mean of triplicate values (error bars, standard errors of the means).

grow anaerobically utilizing DMSO respiration.

Optimal growth conditions

The optimal temperature and NaCl concentration for growth were studied using one of the isolates (Fig. 2). Growth was observed at temperatures from 20 to 40°C with an optimum at 37°C in media containing 3% NaCl, and no growth was observed at 4°C (Fig. 2A), suggesting that the isolate is psychrotolerant to mesophilic rather than psychrophilic conditions, as observed by Naganuma *et al.* (2005). As for the optimal NaCl concentration (Fig. 2B), growth was observed within a wide range of NaCl concentrations from 1.5 to 12%. The optimal concentration was 3–6% NaCl, and no growth was observed at either 0 or 24% NaCl, indicating that the strain is a moderate halophile. Although the NaCl concentration of the halocline water is 15% (Naganuma *et al.*, 2005), the growth rate of the isolate at 15% was half that at 3%. It appears that this isolate can grow in an extended range of salinities (3–15% NaCl), which is advantageous for survival in environments with differing salinities such as Suribati Ike.

Growth by anaerobic respiration of bacteria from Suribati Ike, Pacific Ocean and Seto Island Sea

Anaerobic respiration, such as that utilizing DMSO, nitrate or fumarate, in the ocean plays an important role in the circulation of sulfur, nitrogen and carbon at a global level (Atlas and Bartha, 1998). Therefore, it was investigated whether water samples from Suribati Ike contained bacteria capable of anaerobic growth in the presence of nitrate and fumarate as terminal electron acceptors rather than DMSO (Table 2). Water samples from the Pacific Ocean and Seto Inland Sea were also investigated to compare the anaerobic growth abilities. A 1 m*l* water sample was put into an 18-m*l* screw-capped tube and the

Table 2. Anaerobic growth with various electron acceptors of bacteria from Suribati Ike, the Pacific Ocean and Seto Inland Sea.

Sample	Electoron acceptor		
	DMSO	NO_3^-	Fumarate
Antarctic (Suribati Ike)	±	–	–
Pacific Ocean	+	+	±
Seto Inland Sea	±	+	±

Glyserol was used as the carbon source.
+ : good growth with the optical density at 660 nm more than 1.0,
± : weaker growth with the optical density less than 1.0,
– : no growth.

tube was filled with MTYE medium containing 0.2% DMSO, nitrate, or fumarate and cultured at 20°C for one month. The cultures with DMSO of Suribati Ike became slightly turbid, although no growth of bacteria was observed in the cultures with nitrate or fumarate (Table 2). On the other hand, cultures of the Pacific Ocean and Seto Inland Sea became turbid in the presence of either DMSO or nitrate, and in the presence of fumarate also became slightly turbid. From the water samples of both the Pacific Ocean and Seto Inland Sea, 8 colonies were isolated from the solid plate of the MTYE medium containing DMSO cultured anaerobically. The 16S rRNA sequences of isolates indicated that they all belonged to species of the genus *Vibrio*. These results indicate that the principal DMSO-respiring bacteria in Suribati Ike may be different from that in the marine environment.

It is interesting that the majority of DMSO-respiring bacteria in Suribati Ike are different from those in the marine environment. The bacteria belonging to *Vibrio* sp. are one of the main marine ones (Tranzo *et al*., 2005) and found as free-living or associated to the intestinal microbiota of marine fish (Pujalte *et al*., 2003). Most Antarctic lakes and ponds including Suribati Ike host no fish. This might reflect the difference of the genus of main DMSO-respiring bacteria living between Suribati Ike and marine environment. Since the halocline is markedly abundant in flagellated algae in Antarctic meromictic lakes such as Suribati Ike (Ban *et al*., 2001), DMSP in flagellated algae may be converted to DMSO by oxidation. *Marinobacter* species are ubiquitous in marine environments (Kaye and Baross, 2000), and several *Marinobacter* strains have broad catabolic capacities to be enriched from coastal sediments (Hedlund *et al*., 2001) and the deep-sea floor (Kaye and Baross 2000). Therefore, it could be considered that *Marinobacter* species are the dominant DMSO-respiring bacteria in Suribati Ike.

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