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predominant in freshwater lake environments**

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2014

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Doctoral thesis

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Graduate School of Environmental Science

Hokkaido University

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Chapter 1

General introduction

1.1 Introduction

Chemolithotrophic sulfur oxidizing prokaryotes are capable of oxidizing reduced sulfur compounds as sources of energy for growth (1). This ability is widely distributed among prokaryotic lineages. At the time of writing, the known sulfur oxidizers are affiliated to three phyla (*Aquificae*, *Firmicutes* and *Proteobacteria*) within the domain *Bacteria* and the phylum *Crenarchaeota* within the domain *Archaea*. The majority of them belong to the phylum *Proteobacteria*, and are classified into the class *Epsilon*-, *Alpha*-, *Gamma*-, and *Betaproteobacteria*.

As can be inferred from the phylogenetic diversity of sulfur chemolithotrophs, they are also diverse in physiology (1). They oxidize a range of inorganic and organic compounds as electron donor, and can use inorganic carbon as their cell carbon. To accept electrons from the oxidation of the electron donor, oxygen is generally used by sulfur chemolithotrophs, but other oxidizing agents are used under anoxic conditions (1). One of them is nitrate or nitrite, which is reduced to gaseous end product, such as nitrogen, nitric oxide, and nitrous oxide. This process is referred to as denitrification (2). These characteristics allow sulfur chemolithotrophs to link sulfur, carbon, and nitrogen cycling in natural environments.

Sulfur chemolithotrophs occur in where reduced sulfur compounds are present (1). In their environmental habitats, reduced inorganic sulfur compounds are supplied from sulfate reduction by sulfate reducers, in addition to geochemical and human activities. In the last decades, genomic analysis of sulfur chemolithotrophs has been taken place focusing on those living in where a sufficient amount of reduced inorganic sulfur compounds are available (3-8). These are providing novel insights into their unique physiological features, and also have revealed the presence of diverse sulfur oxidation pathways. On the other hand, their counterparts living in where the availability of reduced inorganic sulfur compounds is low

have been overlooked. Freshwater lake environments are generally characterized by low sulfide and sulfate concentration (9). In such environments, major sulfur oxidizers are still unknown, and even any genomic information is unavailable.

The aim of this thesis is to gain insights into the genomic features of sulfur chemolithotrophs predominant in freshwater lake environments. Assemblages of sulfur oxidizers were specifically investigated by constructing clone libraries from freshwater lake sediments and anoxic water body of a stratified freshwater lake (Chapter 2, 3). Genomic analysis was carried out on representative sulfur chemolithotrophs predominant in the studied sites focusing on the composition and phylogeny of genes for sulfur oxidation, carbon fixation, and denitrification (Chapter 4, 5). Proteomic analysis was conducted to determine the expression of proteins encoded by these genes. Moreover, comparative proteomics was applied to elucidate the molecular basis of clod adaptation, which is thought to have essential significance for sulfur chemolithotrophs to access growth substrate in natural habitats (Chapter 4).

This thesis provides novel genetic insights into the sulfur oxidation pathway of sulfur chemolithotrophs living in freshwater lake environments.

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Chapter 2

Diversity of sulfur-cycle prokaryotes in freshwater lake sediments investigated using *aprA* as functional marker gene

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2.1 Introduction

Sulfur cycling, one of the active biological processes in aquatic sediments, is catalyzed by sulfate-reducing prokaryotes (SRPs) and sulfur-oxidizing prokaryotes (SOPs). Dissimilatory sulfate reduction is the major anaerobic process in the mineralization of carbon compounds, accounting for over half of the mineralization in marine sediments, along with sulfide production (1). Dissimilatory sulfur oxidation occurs where reduced inorganic sulfur compounds are available as a result of abiotic processes or the activity of SRPs (2). Recently, the significant role of dissimilatory sulfur oxidation in sulfide removal and primary production in marine sediment has been reported (3). In contrast, very few studies have been performed on sulfur-cycle prokaryotes living in general freshwater habitats where the sulfate concentration is much lower than marine water. Sulfate reduction is limited by low availability of sulfate (4), but SRPs still play a role in the degradation of carbon compounds in the low-sulfate sediment (5).

A key question about the sulfur-cycle in freshwater ecosystems is the identity of the major microbial populations involved in this process. Identification of SRPs in freshwater lake sediments has been mainly carried out using 16S rRNA targeted probes specific for members of genera of interest. Compared to marine environments, very little is known about SOPs living in natural freshwater environments. The phylogenetic complexity of sulfur-cycle prokaryotes hampers the concomitant detection of SRPs and SOPs in natural habitats using only a single 16S rRNA gene targeted primer pair or probe, and their relatively low abundance in low-sulfate environments makes their detection even more difficult. In addition, 16S rRNA gene-based analysis cannot provide a link between the genetic identities of uncultured microorganisms and their physiology, and thus such restriction results in failure of the discovery of novel microorganisms involved in sulfur cycling.

An alternative molecular approach to overcome these problems is the use of functional genes. One candidate functional marker gene is *aprA* encoding the adenosine-5'-phosphosulfate (APS) reductase alpha subunit. APS reductase consisting of two subunits (AprB and AprA) converts APS to sulfite and adenosine monophosphate (AMP) in the dissimilatory sulfate reduction process, and is also postulated to operate in the reverse direction in the dissimilatory sulfur oxidation process (6). The *aprA* gene was originally employed to assess SRP diversity (7, 8). Thereafter, a comprehensive *aprBA* database covering most of the SRP genus and *apr*-harboring SOPs was established from the phylogenetic studies of nearly the entire *aprBA* gene (6, 9). On the basis of the high conservation and phylogenetic resolution of *aprA*, Meyer and Kuever (10) developed an *aprA* primer pair for concomitant detection of both SRPs and SOPs. As a result, they achieved simultaneous determination of environmental SRP and SOP diversity with improved species coverage and detection sensitivity (10).

In this study, I investigated the diversity of sulfur-cycle prokaryotes in freshwater lake sediments using this *aprA* primer pair. In freshwater ecosystems, certain bacterial taxa sometimes dominate the microbial communities in different lakes despite variations in geographic distance and chemical composition (11, 12). For a better understanding of the diversity, I included 6 geographically different lakes in this investigation. Antarctica has been geographically isolated for millions of years, and there are a number of lakes ranging from freshwater to hypersaline that have sustained diverse microorganisms in a polar desert (13). Here, I describe the spatial distribution of *aprA* sequences in sediments from 3 freshwater lakes in Antarctica along with that from 2 freshwater lakes in Japan. In addition, *aprA* sequences were also retrieved from a hypersaline lake for comparison.

2.2 Materials and methods

2.2.1 Sampling of sediments and bottom waters

Samples used in this study were obtained from 2 freshwater lakes in Japan (Lake Biwa, a mesotrophic lake; Lake Okotanpe, an oligotrophic lake), 3 freshwater lakes in Antarctica (Lake Skalle O-Ike, Lake Oyako Ike, Lake Maruwan O-Ike), and a marine-derived, stratified hypersaline lake in Antarctica (Lake Suribachi Ike) (14). The above Antarctic lakes are located along with the Soya Coast, Lützow-Holm Bay, East Antarctica. The additional description about each lake was shown in Table 2-1.

Samples of sediment core and bottom water from Lake Biwa were collected in October 2010 (15). Sampling at Lake Okotanpe took place in June 2007. The sediment core of Lake Okotanpe was obtained by using a core sampler equipped with an acrylic tube (inside diameter 12 cm), and the core sample was subcored (diameter 4.5 cm) after collection. The bottom water sample was sterilized with a 0.20 μm filter (DISMIC-13cp; Advantec, Tokyo, Japan) and then was kept frozen until chemical analysis. Samples of sediments and bottom waters from Antarctic lakes were collected in 47th Japanese Antarctic Research Expedition (December 2005 to February 2006) (16). From 3 Antarctic freshwater lakes, the sediment cores were collected by push-type cores, and the core samples were cut into 2-10 cm intervals (Table 2-2). The sediment sample of Lake Suribachi Ike was collected from sediment surface using Ekman-Birge sediment sampler. These sediment samples were stored at -20°C until DNA extraction. The bottom water samples of Antarctic lakes were filtered by 0.22 μm Sterivex filter (Millipore Co., Bedford, MA) for chemical analysis.

2.2.2 Characterization of bottom water samples

The bottom water parameters (temperature, chloride, sulfate, and dissolved oxygen) of Lake Biwa were cited from a previous study (15). Water temperature and

dissolved oxygen concentration of Lake Okotanpe and Antarctic lakes were measured using multivariable probe (YSI Model 600XLM or YSI Model 6600; YSI Inc., Yellow Spring, OH). Chloride and sulfate concentrations of bottom water samples from Antarctic lakes and Lake Okotanpe were measured with an ion chromatography (DX-120; Dionex, Sunnyvale, CA) equipped with a column for anion analyses (IonPac AS4ASC; Dionex).

2.2.3 Clone library construction

To investigate the diversity of sulfur cycle prokaryotes, *aprA* clone libraries were constructed from the DNA samples obtained from each lake sediment surface (Table 2-2). The clone library of Lake Biwa was constructed using the same DNA sample as in the previous study, which was extracted from the surface layer (0-2 cm) of the sediment core (15). The DNA samples of the other freshwater lakes were extracted from the surface layers of each sediment core using Ultra Clean Soil DNA kit (MoBio Laboratories, Solana Beach, CA). The DNA sample of Lake Suribachi Ike was obtained from the sediment surface using a DNA extraction kit, ISOIL (Nippon Gene, Toyama, Japan). Additionally, the DNA sample extracted from the subsurface layer (2-4 cm) of Lake Maruwan O-Ike was also included in the analysis in order to examine the vertical distribution of sulfur cycle prokaryotes.

From the above DNA samples, *aprA* gene fragments were amplified using a primer pair, AprA-1-FW/AprA-5-RV (10). The reaction mixture (25 μ l) used for PCR contained 0.25 μ M of each primer, 1 $\times$ Ampdirect buffer (Shimadzu, Kyoto, Japan), 0.625 U of Ex Taq (Takara, Shiga, Japan) and 0.5 μ L of DNA solution from each sample. The PCR amplification was performed as follows: Initial denaturation at 94°C for 1 min, then 35 cycles at 94°C for 45 sec, 55°C for 45 sec and 72°C for 45 sec with final elongation at 72°C for 7 min. The PCR products were ligated into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), and the vectors obtained were transformed into TOP10 cells (Invitrogen). Cloned inserts were amplified

using the vector primers M13 forward and reverse (30 cycles of 94°C for 1 min, 55°C for 1min, 72°C for 2 min and an additional extension of 72°C for 7 min), and resulting PCR products of expected size insert were sequenced.

2.2.4 Phylogenetic and statistical analyses

The nucleic acid sequences were translated using EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/), and the deduced *aprA* amino acid sequences were aligned using ClustalW implemented in eBioX software package version 1.5.1 developed by Erik Lagercrantz (<http://www.ebioinformatics.org/ebiox/>). From the alignment, only unique AprA sequences were selected, and then similarity search was performed using BLASTP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the closest cultured relatives. Pairwise distance matrix was calculated based on Poisson model using MEGA software package version 5.05 (17). The calculated distance matrices were input into Mothur (18) and grouped into operational taxonomic units (OTUs) at a 91% cutoff level. By using MEGA, phylogenetic tree was constructed by neighbor-joining method with aligned AprA sequences of representative clones from each OTU and their cultured relatives. Confidence of the tree topology was evaluated by bootstrap analysis with 1,000 replications.

Estimation of sampling efficiencies was performed on each clone library. Rarefaction curves were generated using Mothur. Good's coverage values were calculated by following formula: $C = (1 - n/N) \times 100$, where n is the number of OTUs with only one clone and N is the total number of clones in the library (19).

2.2.5 Nucleotide sequence accession numbers

The nucleotide sequences determined here have been deposited into the DDBJ/EMBL/GenBank under accession numbers AB749926-AB750351.

2.3 Results

2.3.1 Lake characteristics

The characteristics of bottom waters are shown in Table 2-3. At the sampling dates, the surfaces of Lake Maruwan O-Ike and Lake Skallen O-Ike were covered with the ice of 150 cm and 130 cm thickness, respectively. The temperatures of all bottom waters were not higher than 10°C. The chloride concentrations of the bottom water samples from 5 freshwater lakes ranged from 0.05 mM (Lake Maruwan O-Ike) to 4.78 mM (Lake Oyako Ike), and the sulfate concentrations of them were less than 0.5 mM, which is the typical concentration range in freshwater lakes (4). The highest concentrations of chloride (3177 mM) and sulfate (67.45 mM) were detected in Lake Suribachi Ike.

2.3.2 Clone library characteristics and statistical analyses

In this study, 7 *aprA* clone libraries were constructed from lake sediment samples, and each of them was named as in Table 2-2 (Biwa, Oko, Ska, Oya, Mw0-2, Mw2-4, and Suri). In total, 426 clones contained 194 unique AprA sequences. They were grouped into 43 distinct OTUs based on >91% sequence identity (Table 2-4). Although the rarefaction curves of the clone libraries did not reach saturation (Fig. 2-1), the Good's coverage values ranged from 82.1% to 98.0% (Table 2-2).

2.3.3 Distribution of SRPs and SOPs

To obtain an accurate description of the phylogenetic relationship of each OTU, a phylogenetic tree was constructed (Fig. 2-2). On the basis of the closest cultured relatives determined by BLASTP search and the phylogenetic tree, 166 clones in 26 OTUs, and 180

clones in 15 OTUs were provisionally assigned to SRP and SOP lineages, respectively. The other 80 clones in 2 OTUs were not clearly affiliated with either SRP or SOP lineage (Table 2-4). Four clone libraries (Biwa, Oko, Ska, and Suri) were dominated by SRP-related clones rather than SOP-related clones, and 2 libraries, Mw0-2 and Mw2-4, were dominated by SOP-related clones. Oya consisted of approximately 50% SRP- and 50% SOP-related clones (Table 2-2). Therefore, analysis of SRPs mainly focused on Biwa, Oko, Ska, Oya, and Suri, and analysis of SOPs mainly focused on Mw0-2, Mw2-4, and Oya.

2.3.4 Phylogenetic composition of sulfur cycle prokaryotes

SRP-related OTUs were affiliated with 10 different phylogenetic groups (Table 2-4). To identify the major SRP groups, the relative abundance of these groups was calculated for the 5 clone libraries (Fig. 2-3A), and I focused on the groups that accounted for more than 5% of all SRP-related clones. The majority of SRP-related clones (62% of total SRP-related clones) were affiliated with the family *Desulfobacteraceae* (Fig. 2-3A). This group was divided into 3 clusters: *Desulfofaba*, *Desulfosarcina*, and *Desulfobacterium* (Fig. 2-2). In the cluster containing *Desulfofaba gelida*, 51 of the 52 clones were grouped into OTU10, which accounted for 74% of Suri (Table 2-4). The other clones of *Desulfobacteraceae* were exclusively detected in freshwater sediments. In particular, the clones of OTU12 and OTU14–16 were frequently detected in both Antarctica and Japan. The closest relatives of them were marine SRPs, *Desulfosarcina variabilis* (90–92% sequence identity with OTU12) and *Desulfobacterium indolicum* (90–94% sequence identity with OTU14–16). A total of 33 clones (corresponding to 19.9% of total SRP-related clones) were affiliated with *Desulfobulbaceae* (Fig. 2-3A), and the OTUs of this group were detected in freshwater lakes in Antarctica and Japan, except for OTU22 (Table 2-4). Nearly half of the clones of *Desulfobulbaceae* were grouped into OTU17, which formed a distinct cluster with

Desulfocapsa thiozymogenes. Seven clones in OTU18 and OTU19 shared 92–94% sequence identity with *Desulfobulbus elongatus*. The remaining clones in OTU20 and OTU21 were related to *Desulfobacterium catecholicum* with 92–95% sequence identity. Twelve clones grouped into OTU9 were affiliated with *Peptococcaceae* (Table 2-4). OTU9 was detected only in Suri, and belonged to the gram-positive SRP genus *Desulfotomaculum* (Fig. 2-2). The other 18 SRP-related clones were affiliated with 7 different SRP groups. The phylogenetic affiliations for 5 of these groups (Group 1, 2, 3, 4, and 5) were not determined because of proposed lateral gene transfer (LGT) events and low bootstrap values of the respective nodes (Fig. 2-2). Group 1, 2, 3, and 4 branched with the Gram-positive AprA lineage. These groups included *Desulfobacterium anilini*, *Desulfarculus baarsii*, *Desulfomonile tiedjei*, and the members of *Syntrophobacteraceae*. LGT of *aprA* genes from putative donor strains of Gram-positive sulfate-reducing bacteria (SRB) to these SRB has been proposed on the basis of the presence of shared insertion and deletion sequences among respective AprBA sequences (9). The occurrence of the LGT event and the need for taxonomic reclassification of *Desulfobacca acetoxidans* was also postulated based on the AprA sequence composition analysis (9).

All the 180 SOP-related clones in 15 OTUs diverged into 2 distantly related lineages defined by Meyer and Kuever as *aprA* lineage 1 and lineage 2 (Fig. 2-2) (6). Although most SOP-related clones were not assigned to certain genera, the SOP community mainly consisted of 4 major OTUs (>5% of total SOP-related clones) (Fig. 2-3B). OTU38 encompassed 57.8% of total SOP-related clones, but was not detected in Suri (Table 2-4). Phylogenetic analyses revealed that OTU38 is affiliated with *aprA* lineage 1, even though it was not assigned to a single genus (Table 2-4). The 31 clones of OTU26 (corresponding to 17.2% of total SOP-related clones) were detected in Oya and Mw2-4 (Fig. 2-3B), and 11 clones of OTU25 were detected in Biwa, Oko, Ska, and Oya. The closest cultured relative of

OTU26 and OTU25 was *Sulfuricella denitrificans* with 92–98% and 88–92% sequence identity, respectively (Table 2-4). OTU40, which accounted for 5% of total SOP-related clones, was phylogenetically distant from any AprA sequence of known SOPs (Fig. 2-2). The remaining 25 clones in 11 OTUs constituted a minor part of the SOP community. OTU29 formed a cluster with the AprA sequence of *Thiodictyon* sp. strain f4 (93–97% identity). Two clones grouped into OTU30 were related to *Sulfuritalea hydrogenivorans* (96% and 97% identity). The other remaining SOP-related OTUs had low sequence identity with cultured relatives, 7 of which contained only 1 clone (Table 2-4).

The remaining 80 clones, which were not clearly affiliated with either SRP or SOP lineage, were grouped into OTU36 and OTU37. The clones of OTU36 comprised 75 clones and dominated Biwa, Oko, Ska, and Oya, but were not found in Mw0-2, Mw2-4, or Suri (Table 2-4). The closest cultured relative of OTU37 was *Thermodesulfobacterium islandicus* with low sequence identity (68–72%).

2.4 Discussion

The published bacterial 16S rRNA clone library of Lake Biwa, derived from the same DNA sample used in this study, contained only 1 clone (out of 83 clones) belonging to the genera of known sulfur cycle prokaryotes (15) indicating that the known sulfur cycle prokaryotes constitute a small fraction of the whole bacterial community in Lake Biwa. Although the limited number of *aprA* clones obtained in this study was probably insufficient to fully elucidate the microbial diversity, their sequences provide several insights into the identity of microorganisms potentially involved in the sulfur-cycle.

The microbial community composition of hypersaline lake sediment clearly differed from that of freshwater lake sediments. In Lake Suribachi Ike, a low complexity community

represented by AprA sequences related to *Desulfofaba* and *Desulfotomaculum* was observed. The SRPs related to *Desulfofaba* appear to be derived from marine water, because all cultured members of this genus were isolated from marine environments (20, 21). Most *Desulfotomaculum* spp. grow better at lower salt concentrations, but a halophilic species has been also isolated (22). In Lake Suribachi Ike, the occurrence of sulfate reduction is suggested by the presence of sulfide in lower-layer water (23), and thus the putative members of *Desulfofaba* and hypersaline-adapted *Desulfotomaculum* species might be key sulfate reducers in Lake Suribachi Ike.

In 4 freshwater lake sediments, the most frequently detected OTU was OTU36. Although the sequences of OTU36 were phylogenetically distant from any known prokaryotes, the closest cultured relative was the freshwater sulfate-reducer *Thermodesulfovibrio islandicus* (24). Both AprA sequences have 12 amino acid residues between positions 172 and 185 (Fig. 2-4). From the alignment of 122 AprA sequences of SRPs and SOPs available from the public database (GenBank) (Fig. 2-5), same signature was found only in 8 AprA sequences of *Thermodesulfovibrio* spp., *Desulfonauticus* spp., and the several members of the *Chlorobiaceae* (distribution of *aprA* gene among green sulfur bacteria is restricted). The sequences of *Desulfonauticus* spp., which are thermophilic SRPs, completely differ from OTU36, but the several members of the *Chlorobiaceae* belong to a phylogenetic cluster, which consist of OTU36 and *Thermodesulfovibrio* spp. (Fig. 2-2). It has been proposed that *aprBA* genes of *Thermodesulfovibrio* donor lineage were horizontally transferred to *Chlorobiaceae* after its diversification into marine and freshwater strains (9). Considering the fact that OTU36 was not detected in hypersaline lake, Lake Suribachi Ike, the microorganisms represented by the AprA sequences of OTU36 are likely to play an important role in sulfur cycle of freshwater environments. However, whether they are involved in sulfate reduction or sulfur oxidation cannot be evaluated from the presence of these insertion

sequences.

In freshwater lake sediments, I observed a common distribution of 2 major SRP groups, *Desulfobacteraceae* and *Desulfobulbaceae* (Fig. 2-3A). Although the abundance of these groups differed from lake to lake, any of them were found in all freshwater lake sediments. The frequent detection of *Desulfobacteraceae* clones in freshwater sediments is unexpected, because most of the described species of this class have been isolated from marine environments. The clones of *Desulfobacteraceae* found only in freshwater sediments were divided into 2 clusters corresponding to *Desulfosarcina* and *Desulfobacterium*. *Desulfosarcina* spp. are frequently found in marine and brackish habitats (25, 26). On the other hand, environmental AprA sequences very similar to OTU12 (97–98% sequence identity) were previously detected in a stratified freshwater lake, Lake Pavin (27), suggesting the *Desulfosarcina*-like SRPs are present also in freshwater lakes. *Desulfobacterium* spp. are widespread in brackish and marine environments, but the 16S rRNA sequences of them have been frequently reported in various freshwater sediments (28-30). Cultured members of this genus completely oxidize a wide range of organic compounds, including acetate and other short chain fatty acids, and are capable of chemolithoautotrophic growth with hydrogen. This genus also includes a species that grows by fermentation and iron-reduction (31, 32). The high abundance and the wide distribution of putative members of the genus *Desulfobacterium* appears to be maintained by metabolic versatility (33).

The clones affiliated with the *Desulfobulbaceae* were related to 3 genera that are commonly found in freshwater habitats. *Desulfocapsa thiozymogenes*, the closest relative of OTU17, grows either heterotrophically by incomplete oxidation of alcohols or chemoautotrophically by disproportionation of thiosulfate, sulfite, or elemental sulfur (34). The members of *Desulfobulbus* are common SRPs in freshwater lake sediments, and cultured members of this genus can grow by fermentation and reduction of nitrate and Fe (III) (32, 35).

Desulfobacterium catecholicum (closest relative of OTU20 and 21) can completely oxidize organic acids with dissimilatory nitrate reduction to ammonia (36). The cultured members affiliated with *Desulfobulbaceae* can use alternatives to sulfate as electron acceptors. These observations support the idea that some SRPs with the ability to use various electron acceptors other than sulfate are advantageous for survival in low-sulfate environments (5, 37).

As to phylogenetic analysis of SOP community, it was not possible to clearly assign a majority of OTUs to certain genera, partially because of the limited number of reference *aprA* sequences. Moreover, difficulty in such assignment is illustrated by low bootstrap values and close phylogenetic distances between the several beta- and gammaproteobacterial species, as apparent in the phylogenetic tree (Fig. 2-2). In such a situation, the assignment to families, or even to classes, was also impossible for many OTUs including OTU38, which contained the largest number of clones. OTU38 fell into the *aprA* lineage 1 cluster, which included a chemolithoautotrophic betaproteobacterium *Thiobacillus thioparus* and a phototrophic gammaproteobacterium *Thiocapsa rosea* (Fig. 2-2). The phenotypic characteristics of microorganisms corresponding to OTU38 cannot be inferred from their phylogeny because of the different physiological traits exhibited by the cultured relatives within this cluster. On the other hand, the second most frequently detected SOP-related OTU, found in 2 Antarctic freshwater lake sediments, formed a distinct cluster with a betaproteobacterial sulfur oxidizer, *Sulfuricella denitrificans*. This OTU was detected in Mw2-4 with high frequency but was not detected in Mw0-2. These results indicate the effect of sediment depth on SOP community composition. *S. denitrificans* was isolated from a meromictic freshwater lake in Japan, and is one of the few known cold-adapted SOPs (38, 39). Sulfur-oxidizers related to *S. denitrificans* might thrive in a certain depth of sediment in cold freshwater habitats.

In this study, I concomitantly detected SRPs and SOPs in sediments from several

lakes. As a result, SRPs which are considered metabolically flexible were widely distributed in freshwater lake sediments across geographical distances. In addition, this study indicated the high abundance of SRPs related to the genus *Desulfosarcina*, which was not previously regarded as important in this kind of environment. Although the SOP-related AprA sequences provided little phylogenetic information, one of the major groups was clearly affiliated with the genus *Sulfuricella*. It should be noted that chemolithoautotrophic sulfur-oxidizing epsilonproteobacteria, such as *Sulfurimonas denitrificans* and *Sulfuricurvum kujiense*, lack an *aprA* gene (40, 41). This functional gene-based approach suggested the importance of the microorganisms represented by *aprA* sequences distantly related to *Thermodesulfovibrio islandicus*. Isolation and characterization of these microorganisms will lead to a better understanding of the sulfur-cycle in freshwater lake ecosystems.

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Table 2-1. Geographical descriptions of 6 lakes and sampling sites.

Lake	Location (area)	Area (m ²)	Maximum depth (m)	Sampling date	Water depth at sampling site (m)	GPS coordinate of sampling site	
						Latitude	Longitude
Biwa	Japan (Shiga)	674,000,000	104	28.Oct.10	89.0	N 35°39'	E 136°12'
Okotanpe	Japan (Hokkaido)	413,000	21	27.Jun.07	20.0	N 42°48' 06.05"	E 141°15' 36.03"
Skallen O-Ike	Antarctica (Skallen)	209,000	9.3<	25.Dec.05	9.3	S 69°40' 17.08"	E 39°24' 44.00"
Oyako Ike	Antarctica (Skarvsnes)	54,000	8.1<	10.Jan.06	8.1	S 69°28' 31.05"	E 39°36' 08.23"
Maruwan O-Ike	Antarctica (Rundvagshetta)	252,000	37	20.Dec.05	20.2	S 69°54' 27.08"	E 39°02' 24.05"
Suribachi Ike	Antarctica (Skarvsnes)	406,000	31.2	6.Jan.06	16.0	S 69°29' 11.06"	E 39°40' 04.03"

Table 2-2. Clone number and library coverage for *aprA* clone libraries constructed from each sediment sample.

Clone library		Clone no.				Good's coverage (%)
name	Sediment sample (Lake)	SRP lineage	SOP lineage	Uncertain	Total	
Biwa	0-2 cm core (Biwa)	21	11	25	57	86.0
Oko	0-1 cm core (Okotanpe)	25	6	8	39	82.1
Ska	0-10 cm core (Skallen O-Ike)	21	10	23	54	94.4
Oya	0-5 cm core (Oyako Ike)	37	44	19	100	98.0
Mw0-2	0-2 cm core (Maruwan O-Ike)	4	50	0	54	90.7
Mw2-4	2-4 cm core (Maruwan O-Ike)	1	58	5	64	95.3
Suri	Sediment surface (Suribachi Ike)	57	1	0	58	94.8

Table 2-3. Characteristics of bottom water.

Lake	Water depth (m)	Temperature (°C)	Cl ⁻ (mM)	SO ₄ ²⁻ (mM)	^a DO (mg/l)
^b Biwa	89	8.1	0.29	0.097	3.26
Okotanpe	15	7.4	0.06	0.416	9.68
Skallen O-Ike	9	6.0	3.33	0.147	13.52
Oyako Ike	7	7.4	4.78	0.232	4.41
Maruwan O-Ike	19	4.0	0.05	0.011	^c -
Suribachi Ike	16	^d 10.0	3177	67.45	^e LTD

^a Dissolved oxygen concentration.

^b The bottom water parameters of Lake Biwa were cited from a previous study (Kojima et al., 2012).

^c No data.

^d The temperature was measured at a depth of 15.3m.

^e Less than detection limit.

Kojima H, Tsutsumi M, Ishikawa K, Iwata T, Mußmann M, Fukui M. 2012. Distribution of putative denitrifying methane oxidizing bacteria in sediment of a freshwater lake, Lake Biwa. *Syst. Appl. Microbiol.* **35**: 233-238.

Table 2-4. Distribution of clone number per OTU among 7 *aprA* clone libraries and phylogenetic affiliation based on the closest cultured relatives determined according to the results of BLASTP search.

Putative function	Phylogenetic affiliation	OTUs	Number of clones								Closest cultured relatives	identity %
			Japan		Antarctica				Sum.			
			Biwa	Oko	Ska	Oya	Mw0-2	Mw2-4		Suri		
Sulfate reduction	Group 1	OTU1	0	0	0	2	0	0	0	2	<i>Desulfobacterium anilini</i>	82
		OTU2	1	0	0	0	0	0	0	1	<i>Desulfobacterium anilini</i>	84
	Group 2	OTU3	1	0	0	0	0	0	0	1	<i>Desulfomonile tiedjei</i> DSM 6799	91
		OTU4	0	1	0	0	0	0	0	1	<i>Desulfomonile tiedjei</i> DSM 6799	91
	Group 3	OTU5	0	2	0	0	0	0	0	2	<i>Desulfotomaculum kuznetsovii</i> DSM 6115	84
		OTU6	0	1	0	0	0	0	0	1	<i>Desulfotomaculum kuznetsovii</i> DSM 6115	84
		OTU7	1	0	0	0	0	0	0	1	<i>Desulfotomaculum kuznetsovii</i> DSM 6115	87
	Group 4	OTU8	0	0	0	2	0	0	0	2	<i>Syntrophobacter pfennigii</i>	83
	<i>Peptococcaceae</i>	OTU9	0	0	0	0	0	0	12	12	<i>Desulfotomaculum gibsoniae</i> DSM 7214	91-95
	<i>Desulfobacteraceae</i>	OTU10	0	4	0	4	0	0	43	51	<i>Desulfofaba gelida</i>	88-96
		OTU11	1	0	0	0	0	0	0	1	<i>Desulfofaba gelida</i>	88
		OTU12	9	6	0	9	1	0	0	25	<i>Desulfosarcina variabilis</i> str. Montpellier	90-92
		OTU13	0	2	0	0	0	0	0	2	<i>Desulfococcus biacutus</i>	92
		OTU14	2	0	0	0	0	0	0	2	<i>Desulfobacterium indolicum</i>	94
		OTU15	1	0	7	11	0	0	0	19	<i>Desulfobacterium indolicum</i>	90-92
		OTU16	1	2	0	0	0	0	0	3	<i>Desulfobacterium indolicum</i>	94
	<i>Desulfobulbaceae</i>	OTU17	3	3	9	0	0	0	0	15	<i>Desulfocapsa thiozymogenes</i>	92-98
		OTU18	0	1	0	0	2	0	0	3	<i>Desulfobulbus elongatus</i>	92
		OTU19	0	0	1	3	0	0	0	4	<i>Desulfobulbus elongatus</i>	92-94
		OTU20	0	2	0	0	0	0	0	2	<i>Desulfobacterium catecholicum</i>	94
		OTU21	0	0	0	6	1	0	1	8	<i>Desulfobacterium catecholicum</i>	92-95
		OTU22	0	0	0	0	0	0	1	1	<i>Desulfobacterium corrodens</i>	97
	<i>Desulfovibrionales</i>	OTU23	0	1	0	0	0	0	0	1	<i>Desulfococcus biacutus</i>	81
		OTU24	0	0	1	0	0	0	0	1	<i>Desulfovibrio fructosovorans</i> JJ	90
	Group 5	OTU35	1	0	3	0	0	0	0	4	<i>Desulfobacca acetoxidans</i> DSM 11109	97-100
	Archaea	OTU43	0	0	0	0	0	1	0	1	<i>Archaeoglobus veneficus</i>	74
Sulfur oxidation	<i>aprA</i> lineage 2	OTU25	3	1	3	4	0	0	0	11	<i>Sulfuricella denitrificans</i>	88-92
		OTU26	0	0	0	12	0	19	0	31	<i>Sulfuricella denitrificans</i>	92-98
		OTU27	0	0	0	3	1	0	0	4	<i>Sulfuricella denitrificans</i>	88-92
		OTU28	0	0	1	5	0	0	0	6	<i>Sulfuricella denitrificans</i>	82-86
		OTU29	2	0	3	1	0	0	0	6	<i>Thiodictyon</i> sp. f4	93-97
		OTU30	0	1	0	0	0	1	0	2	<i>Sulfuritalea hydrogenivorans</i>	96-97
		OTU31	0	0	0	0	0	0	1	1	<i>Sulfuritalea hydrogenivorans</i>	88
		OTU32	0	1	0	0	0	0	0	1	<i>Sulfuricella denitrificans</i>	85
		OTU33	0	0	0	0	0	1	0	1	<i>Thiocapsa pendens</i>	86
		OTU34	1	0	0	0	0	0	0	1	<i>Sulfuricella denitrificans</i>	76
	<i>aprA</i> lineage 1	OTU38	5	3	3	18	38	37	0	104	^a <i>Thiocapsa rosea</i>	87-94
		OTU39	0	0	0	1	0	0	0	1	^a <i>Thiobacillus thioparus</i>	91-97
		OTU40	0	0	0	0	9	0	0	9	<i>Thiobacillus plumbophilus</i>	91
		OTU41	0	0	0	0	1	0	0	1	<i>Thiobacillus plumbophilus</i>	79-81
		OTU42	0	0	0	0	1	0	0	1	<i>Thiobacillus plumbophilus</i>	76
									1	<i>Thiobacillus plumbophilus</i>	84	
Uncertain	Unclassified	OTU36	25	8	23	19	0	0	0	75	<i>Thermodesulfovibrio islandicus</i>	68-72
		OTU37	0	0	0	0	0	5	0	5	<i>Thermodesulfovibrio islandicus</i>	71-72

^a The closest cultured relative of 62 out of 104 clones in OTU39 was *Thiocapsa rosea*, and that of the other 42 clones was *Thiobacillus thioparus*.

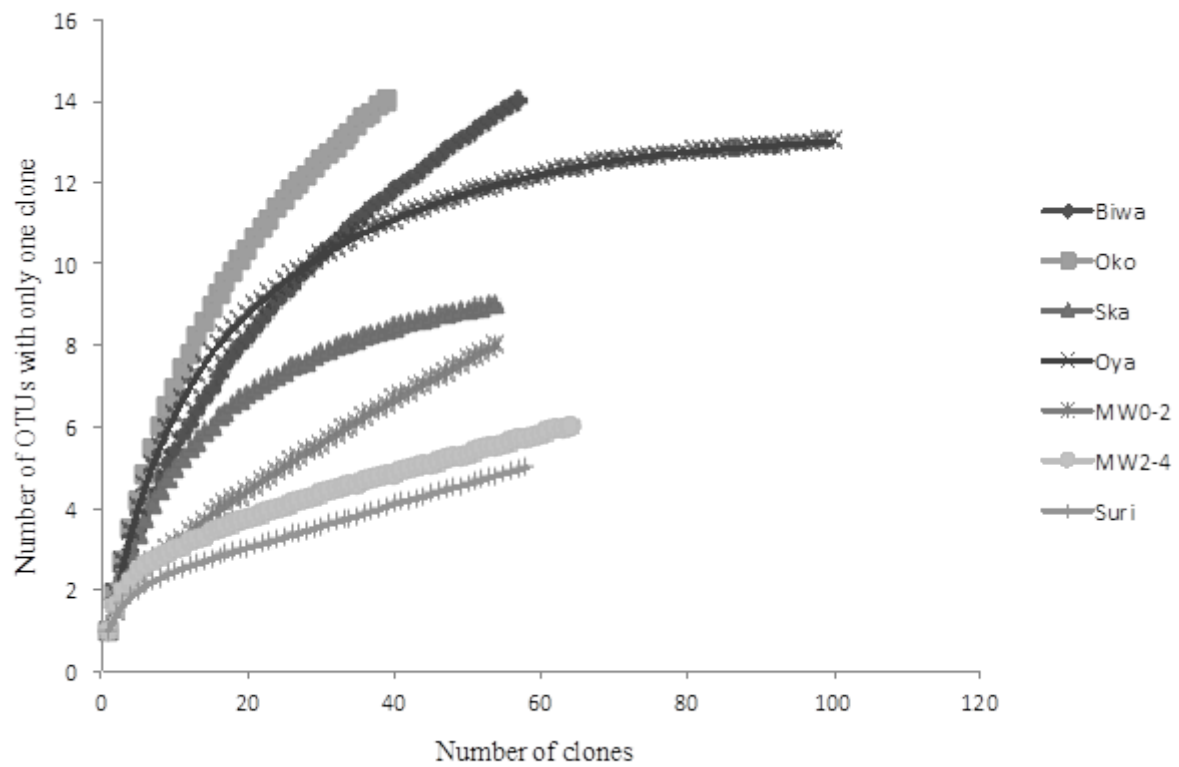


Fig. 2-1. Rarefaction curves generated from *aprA* clones of each clone library.

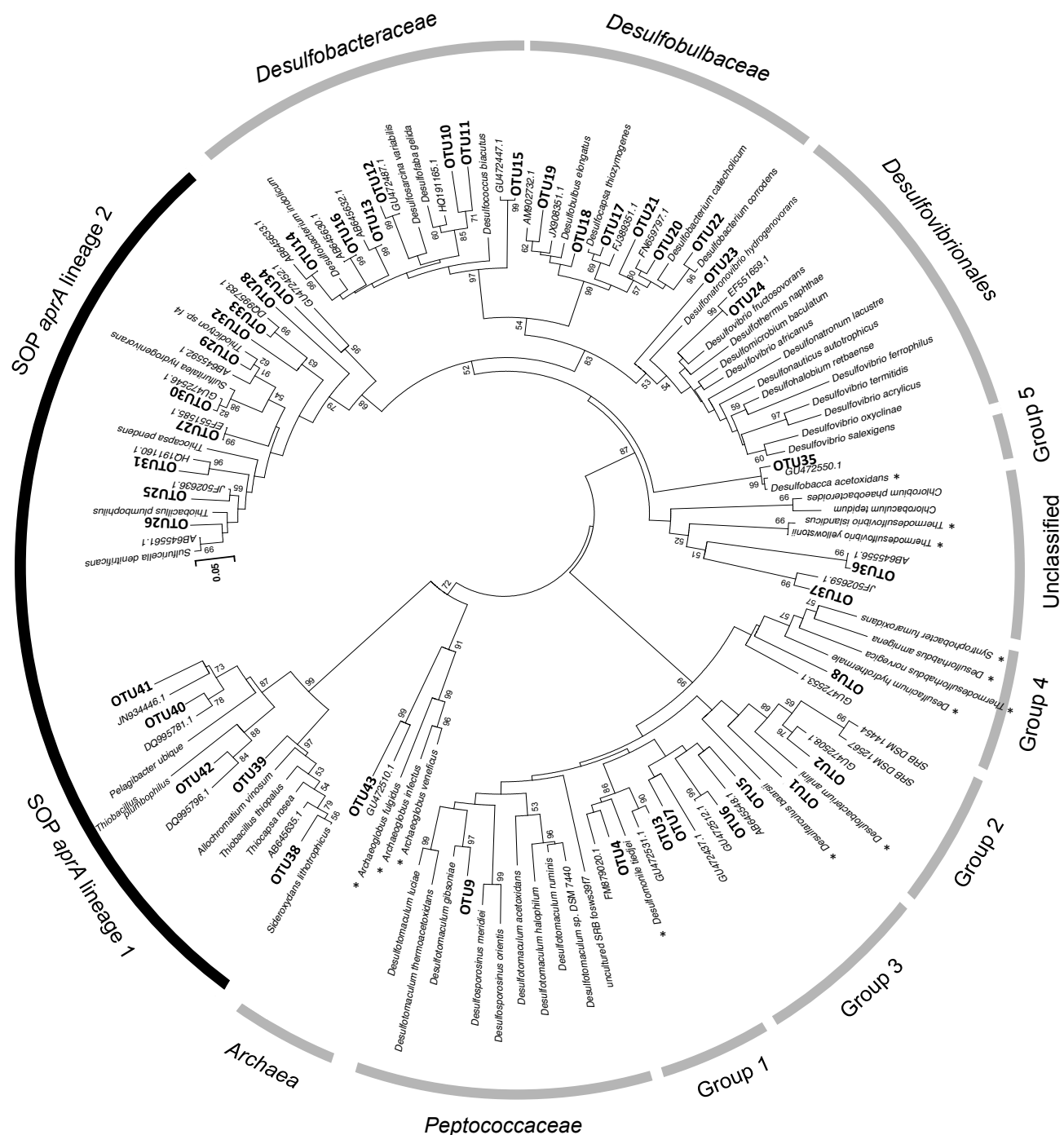


Fig. 2-2. Phylogenetic tree based on AprA sequences including uncultured and cultured close relatives. The tree was constructed by neighbor-joining method. The sequences obtained in this study are highlighted in bold type. The environmental AprA sequences are represented by accession numbers. SRPs with laterally transferred *aprA* genes are marked with an asterisk. Numbers at nodes indicate confident values from 1,000 bootstrap resamplings (values > 50% are shown).

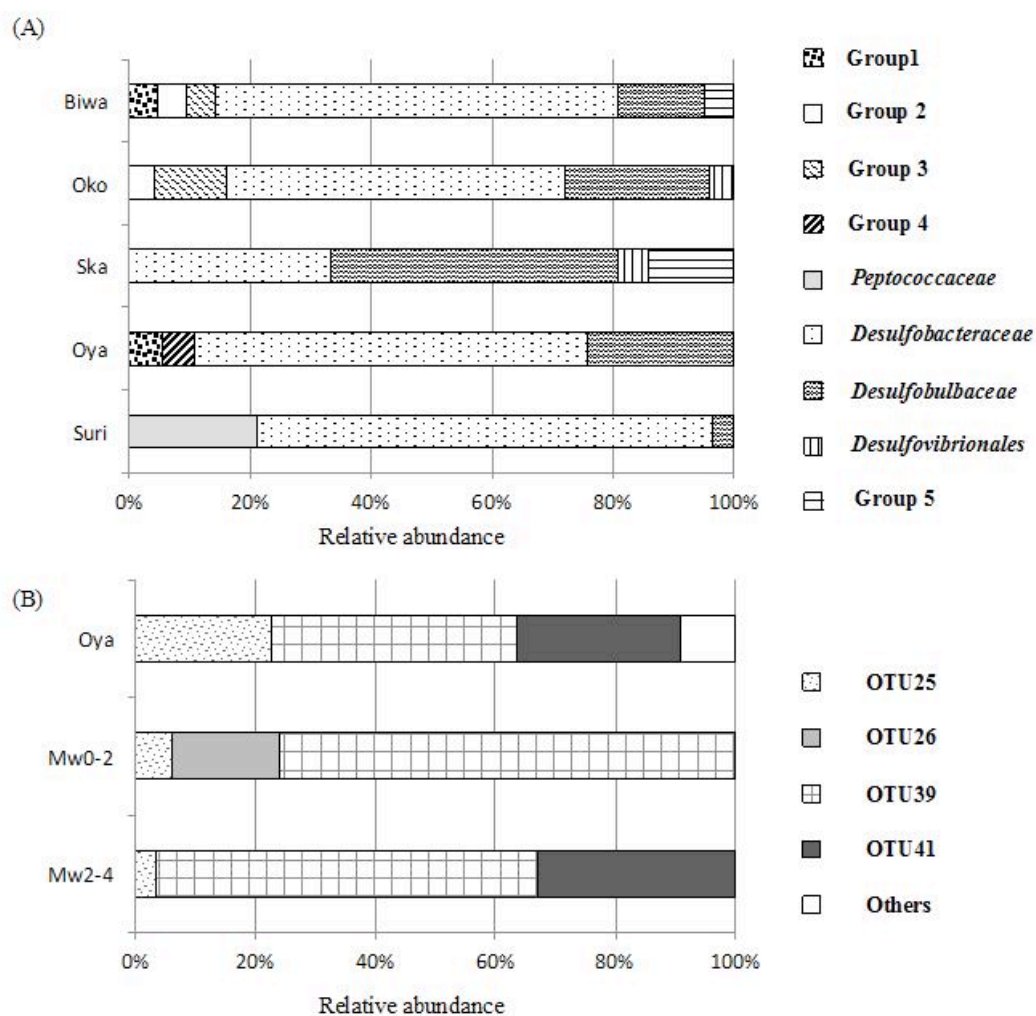


Fig. 2-3. Community composition of *aprA* clone libraries. Relative abundances of phylogenetic groups of SRP-related clones in five libraries (A), and relative abundances of SOP-related OTUs in three libraries (B). The minor SOP-related OTUs (less than 5% of all SOP-related clones) were grouped into “Others”.

OTU36 (Biwa57)	159	E S Y K V I V A E A A K K A	172	L E T N K A A T G M A Q	185	N H Y E R V	190
SRPs							
<i>Thermodesulfovibrio</i> sp. (ex DSM 12270)		E S Y K V I V A E T A K A A		L E F N R K A T G Q A Q		N L Y E R V	
<i>Thermodesulfovibrio islandicus</i>		E S Y K V I V A E T A K A A		L E F N R K A T G Q A Q		N I Y E R V	
<i>Thermodesulfovibrio yellowstonii</i>		E S Y K V I V A E A A K A A		L E F N R K A T G Q A Q		N I Y E R V	
<i>Desulfobacca acetoxidans</i>		E S Y K V I V A E A A K N A		L - - - - - G M D -		N I Y E R V	
<i>Thermodesulfatator indicus</i>		E S Y K C I V A E A A K N A		M E Q - - - L G E N G		E I L E R V	
<i>Thermodesulfobacterium hveragerdense</i>		E S Y K V I V A E A A K N A		L A S - - - Y D K A -		E I I E R C	
<i>Desulfonauticus autotrophicus</i>		E S Y K C I V A E A A K N A		L G E Y R E E D G K G T		G Y L E R I	
<i>Desulfonauticus submarinus</i>		E S Y K C I V A E A A K N A		L G E F R E E D G K A T		G Y I E R L	
<i>Desulfobulbus elongatus</i>		E S Y K C V V A E P A K K A		L G E - - - - - E		N I M E R I	
<i>Archaeoglobus fulgidus</i>		E S Y K P I I A E A A K M A		V - - - - - G - E E		N I Y E R V	
<i>Desulfobacterium anilini</i>		E S Y K I I I A E A A K N A		M K E - - - A G - - G		E I Y E R V	
<i>Desulfotomaculum thermobenzoicum</i>		E S Y K I I V A E A A K N S		I N S - - - L G D K G		E I Y E R V	
<i>Desulfotomaculum kuznetsovii</i>		E S Y K I I V A E A A K N A		L N A - - - L G - P D		N I Y E R V	
<i>Thermodesulforhabdus norvegica</i>		E S Y K V I V A E A A K N A		L A T - - - L G E N G		Q L F E R V	
<i>Desulfotomaculum acetoxidans</i>		E S Y K I I V A E A A K N A		L - - - - - G - T E		N I Y E R V	
<i>Desulfomonile tiedjei</i>		E S Y K V I V A E A A K N A		L A Q - - - N G - - G		E S F E R V	
Green-sulfur bacteria							
<i>Chlorobium chlorochromatii</i>		E S Y K V I V A E A A K K A		L E Y N R K A T G V D Q		N L F E R V	
<i>Chlorobium limicola</i>		E S Y K V I V A E A A K K A		L E Y N R K E T G V D Q		N L F E R V	
<i>Chlorobaculum tepidum</i>		E S Y K V I V A E A A K K A		L E Y N R K E T G V E Q		N L Y E R V	
SOPs							
<i>Thiobacillus denitrificans</i> (aprA lineage 1)		E S Y K P I V A E A A K K S		- - - - - - - - A D		K V F N R I	
<i>Sulfuricella denitrificans</i> (aprA lineage 2)		E S Y K W I V A E A A K K A		L G M - - - - - D		R I Q E R I	

Fig. 2-4. Multiple alignment of AprA sequences of the representative clone of OTU36 (Biwa57) and the selected representatives showing various sequence patterns between positions 172 and 185. Amino acid positions are numbered according to full-length AprA sequence of *Chlorobaculum tepidum*.

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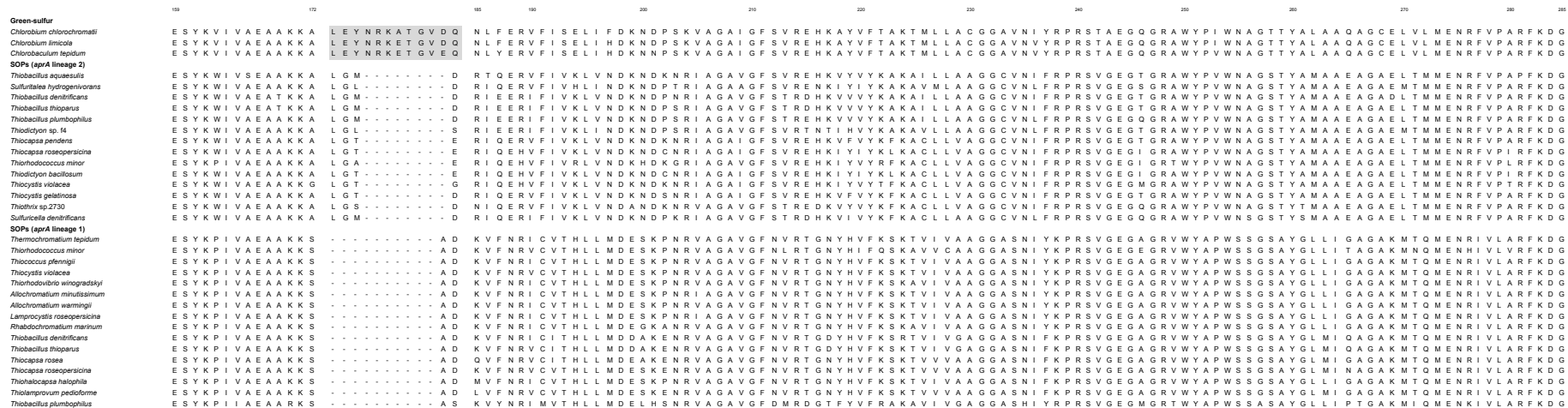


Fig. 2-5. AprA multiple alignment of 122 reference sequences from both SRPs and SOPs. AprA sequences were aligned using T-COFFEE implemented in eBioX software package version 1.5.1. Amino acid positions are numbered in the same manner as Fig. 2-4.

Chapter 3

Identification of major planktonic sulfur oxidizers in a stratified freshwater lake, Lake Mizugaki

Submitted for publication

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3.1 Introduction

One of the major aquatic habitats of sulfur oxidizers is a stratified water body, where the transition zone between the oxic and anoxic realm is developed. In the developed anoxic realms including sediments, one of the major biological activities is the reduction of sulfate to sulfide by sulfate reducers. In general, the generated sulfide is consumed before reaching the upper oxic realm by sulfur oxidizers living in the transition zone and anoxic realm.

Recently, several key sulfur oxidizers living in stratified marine water bodies were identified as gamma- and epsilonproteobacteria (1-3). On the other hand, their counterparts in stratified freshwater lakes are hardly known. In Chapter 2, I could detect *aprA* sequences derived from sulfur oxidizers, but it was difficult to differentiate beta- and gammaproteobacteria, as shown in Fig. 2-2.

Here, assemblages of planktonic sulfur oxidizers were investigated in a stratified freshwater lake, Lake Mizugaki. To overcome the above-mentioned problem, this study constructed clone libraries of 3 functional genes, *dsrA*, *soxB*, and *sqr* in addition to *aprA* gene (details for these genes are given in Chapter 5 and Fig. 5-7). It is well known *soxB* is universally conserved among sulfur oxidizers, and *dsrA* and *sqr* have also been applied to analyze community structure of sulfur oxidizers in environmental samples (4).

3.2 Materials and methods

3.2.1 Sampling site description

Lake Mizugaki is located in Yamanashi Prefecture, and maximum depth is 55 m. A clear stratification is observed through the whole year. Water samples were collected at

pelagic site (water depth, *c.* 43 m) by Kojima et al. (4) in the end of October 2006. At sampling date, dissolved oxygen was depleted in the bottom water (25–43 m). A more detailed description can be found in the paper by Kojima et al (4).

3.2.2 Clone library construction

From DNA samples of 3 selected water depths (25, 35, and 43 m), fragments of *dsrA*, *soxB*, *sqr* and *aprA* were amplified using the primer pairs *dsrA* 625F/877R, *soxB* 704F/1199R, *sqr* 473F/982R, and *AprA*-1-FW/*AprA*-5-RV, respectively. The PCR steps of the primer pairs for *dsrA*, *soxB*, and *sqr* were as follows: initial denaturation for 3 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, 45 s at 72°C; and a final extension for 7 min at 72°C. Those of the primer pair for *aprA* were as described in the chapter 2 with slight change (initial denaturation was 3 min at 94°C). Methods for clone library construction can be found in the chapter 2.

3.2.3 Phylogenetic analysis

The sequence analysis was carried out according to the methods in chapter 2 in most cases. The cutoff value to group the clones in OTUs was changed to 0.07. Phylogenetic trees were constructed by maximum likelihood method.

3.2.4 Nucleotide sequence accession numbers

The nucleotide sequences determined here have been assigned the DDBJ/EMBL/GenBank accession numbers AB754186–AB754337 and AB898344–AB898655.

3.3 Results

3.3.1 *aprA*

Characteristics of 4 clone libraries are listed on Table 3-1. As mentioned in Chapter 2, the primer pair for *aprA* is originally designed for the concomitant detection of both sulfate reducers and sulfur oxidizers. In total, 152 AprA sequences were grouped into 18 OTUs. Of these, 7 OTUs including 84 clones were clustered with sulfate reducers (tree not shown), and 9 OTUs including 64 clones were clustered with sulfur oxidizers (Fig. 3-1). Relative abundance of the clones in the 9 OTUs (related to sulfur oxidizers) decreased along with increasing water depth, from c. 80 (25m) to 5% (43m). All of these OTUs fell within clusters including both beta- and gammaproteobacteria (Fig. 3-1). Among them, aOTU5 shared high sequence identity (97–100%) with an AprA in a sulfur-oxidizing betaproteobacterium, *Sulfuritalea hydrogenivorans* sk43H (5). The other OTUs could not be assigned to specific genera.

3.3.2 *dsrA*

Both sulfate reducers and several sulfur oxidizers harbor *dsrA* encoding dissimilatory sulfite reductase subunit A. Although the used primer pair was designed targeting sulfur oxidizers, 12 clones obtained from the sample of 43 m were assigned to sulfate reducers (tree not shown). In total, 125 clones grouped into 13 OTUs were affiliated with sulfur oxidizers. The majority of them (122 clones) fell into a cluster comprising only betaproteobacteria (Fig. 3-2), and have 17 amino acid insertion, specifically observed in betaproteobacteria (data not shown). It was impossible to accurately assign a majority of OTUs to certain genera, however dOTU7 matched 98–100% of amino acid with *S. hydrogenivorans* sk43H (Fig. 3-2).

3.3.3 *soxB* and *sqr*

Attempts to amplify the fragments of *soxB* and *sqr* genes from the sample of 25 m were unsuccessful, and thus clone libraries from 35 and 43 m were analyzed for these genes. All of the *soxB* and *sqr* clones affiliated with betaproteobacteria (Fig. 3-3 and 3-4). The majority of them clustered with *Sulfuricella denitrificans*, *Sulfuritalea hydrogenivorans*, *Thiobacillus* spp., *Sideroxydans lithotrophicus*, and *Burkholderiales* bacterium JOSHI_001. All of them lack *soxCD* for sulfur dehydrogenase. On the other hand there were no clones clustering with *soxCD*-harboring sulfur oxidizers, *Thiomonas* spp. and *Advenella kashmirensis*. Clones sharing over 97% sequence identities with *S. hydrogenivorans* 43H were detected in both clone libraries.

3.4 Discussion

In total, 297 of 300 sulfur oxidizers-related clones from 7 libraries (*dsrA*, *soxB*, and *sqr*) were assigned to the class *Betaproteobacteria*. This result strongly suggests major sulfur oxidizers in Lake Mizugaki are betaproteobacteria, different from their counterparts in the stratified marine environments. These betaproteobacteria are thought to lack *soxCD*. In general, *soxCD*-lacking sulfur oxidizers with *soxB* or *sqr* oxidize thiosulfate or sulfide to transient form elemental sulfur, which is further oxidized to sulfite by reverse Dsr pathway. At present, all of isolated *dsrAB*-harboring sulfur oxidizers lack *soxCD*, and the majority of them possess *aprBA*. Therefore, candidate betaproteobacteria detected in this study may be capable of completely oxidizing reduced inorganic sulfur compounds to sulfate by the combination of DSR and APR systems.

The close relatives of *Sulfuritalea hydrogenivorans* sk43H were consistently detected in all libraries, and their detection frequencies in 3 libraries (*aprA*, *dsrA*, and *soxB*)

were relatively high. In 16S rRNA clone libraries constructed from the same samples (25, 35, and 43 m), clones corresponding to the genus *Sulfuritalea* (sequence identity > 97%) were frequently detected in 35 and 43m samples, and occupied 12.5% of the whole bacterial community at 43 m (data not shown). Dominance of *Sulfuritalea* spp. has also been observed in anoxic water column of a stratified freshwater lake, Lake Pavin (6). *Sulfuritalea hydrogenivorans* may be a key player in sulfur oxidation in stratified freshwater lake environments.

3.5 References

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Table 3-1. Clone library characteristics.

Depth (m)	No. of clones assigned to sulfur oxidizers/ total clone			
	<i>aprA</i>	<i>dsrA</i>	<i>soxB</i>	<i>sqr</i>
25	43/54	42/42	ND	ND
35	19/54	44/44	18/18	47/47
43	2/44	39/51	49/49	61/61

ND indicates no data.

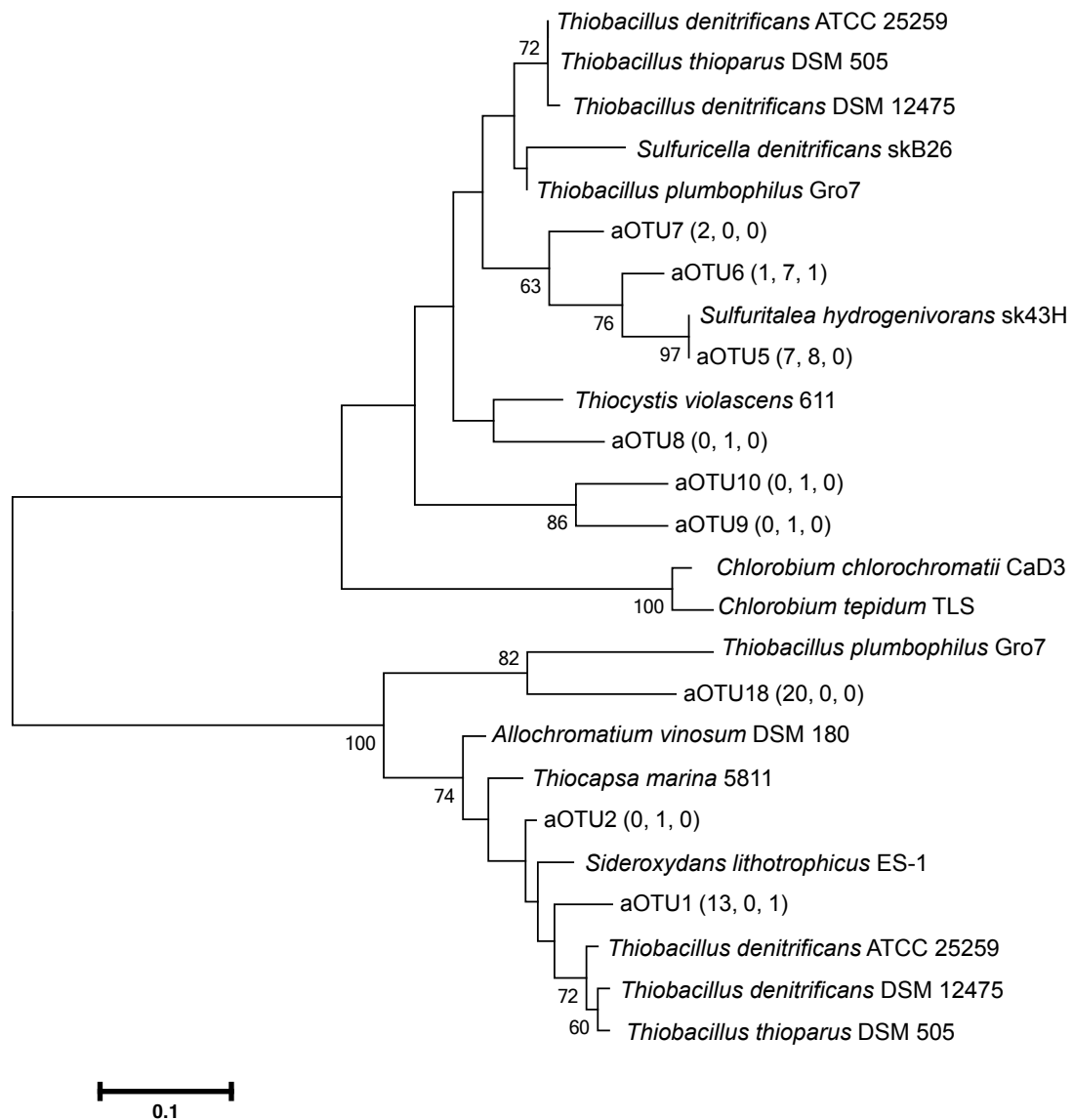


Fig. 3-1. Phylogenetic tree of AprA based on aOTUs assigned to sulfur oxidizers and cultured close relatives. The numbers in parentheses are the number of clones in each OTU in the libraries of 25 m (n=54), 35 m (n=54), and 43 m (n=44), respectively. Boot strap values (100 resamplings) above 50% are shown.

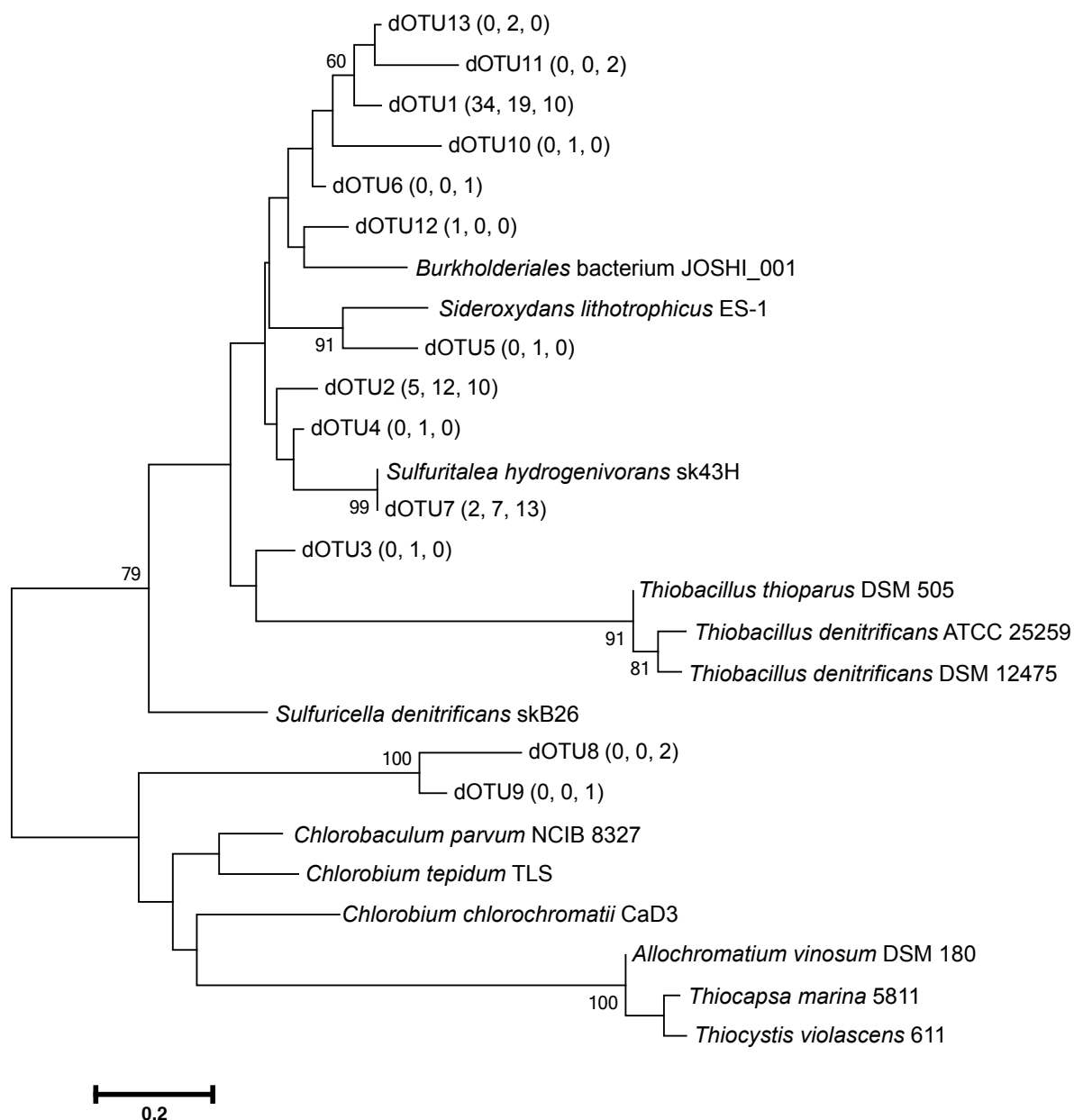


Fig. 3-2. Phylogenetic tree of DsrA based on dOTUs assigned to sulfur oxidizers and cultured close relatives. The numbers in parentheses are the number of clones in each OTU in the libraries of 25 m (n=42), 35 m (n=44), and 43 m (n=51), respectively. Boot strap values (100 resamplings) above 50% are shown.

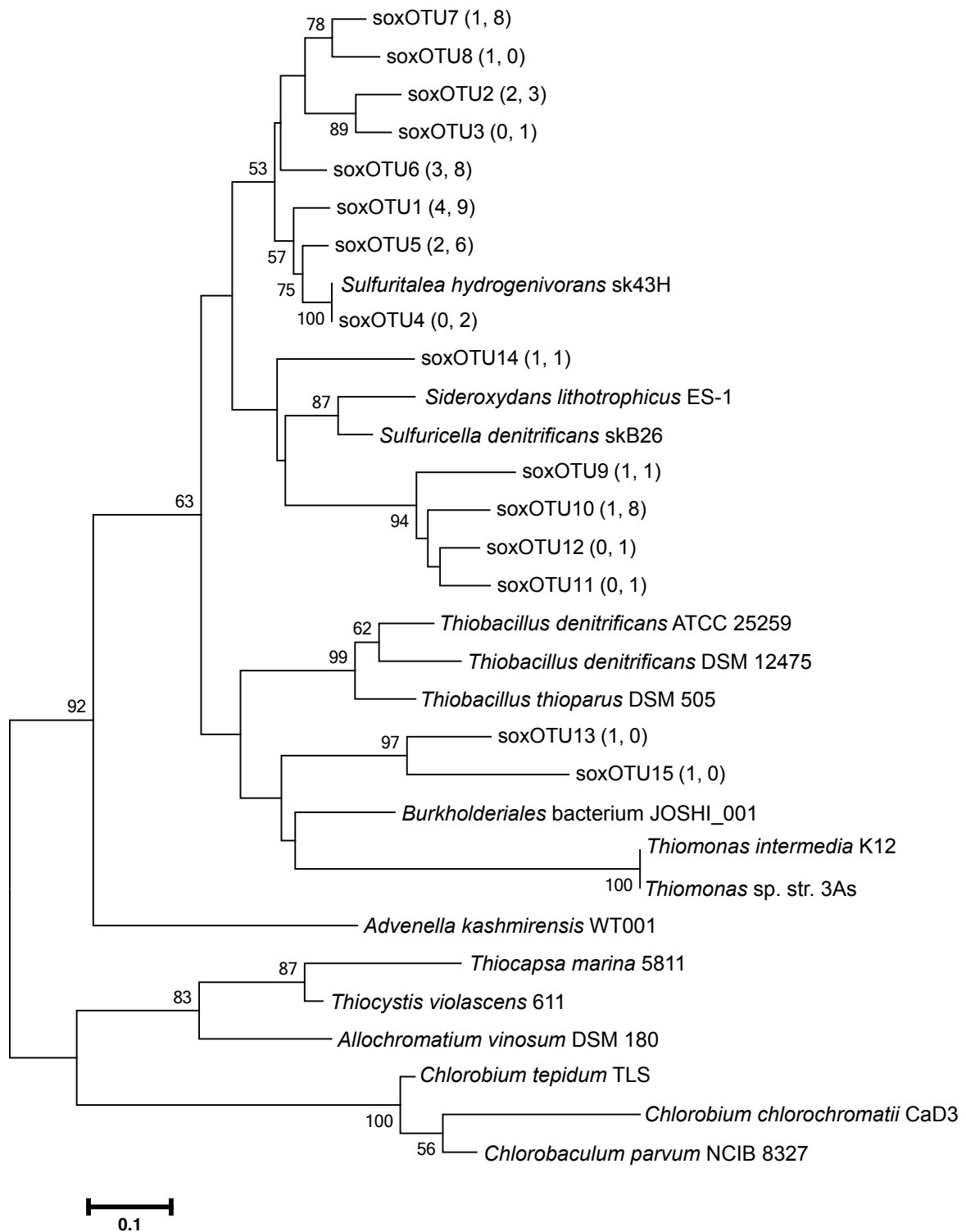


Fig. 3-3. Phylogenetic tree of SoxB based on soxOTUs and cultured close relatives. The numbers in parentheses are the number of clones in each OTU in the libraries of 35 m (n=18), and 43 m (n=49), respectively. Boot strap values (100 resamplings) above 50% are shown.



Fig. 3-4. Phylogenetic tree of Sqr based on sqrOTUs and cultured close relatives. The numbers in parentheses are the number of clones in each OTU in the libraries of 35 m (n=47), and 43 m (n=61), respectively. Boot strap values (100 resamplings) above 50% are shown.

Chapter 4

Draft genome sequence of a psychrotolerant sulfur-oxidizing bacterium, *Sulfuricella denitrificans* skB26 and proteomic insights into cold adaptation

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4.1 Introduction

Chemolithoautotrophic sulfur-oxidizing bacteria, which are widely distributed in aquatic environments, have attracted interest among microbial ecologists. With their functions of inorganic carbon fixation and sulfur oxidation, they thrive as primary components of ecosystems fueled by reduced sulfur species, represented by those in terrestrial hot springs and hydrothermal vents. On the other hand, significant influences of sulfur oxidizers on cycles of nitrogen and phosphorus have been shown for several nitrate-storing bacteria supplied with sulfide from sulfate reduction in organic-rich marine sediments (1-3). Except for these conspicuous cases, however, the ecology of chemolithoautotrophic sulfur oxidizers in natural environments remains largely unknown. In marine environments, several key players in sulfide oxidation were identified only recently, in intertidal sediment (4) and water columns (5-7). A trial to identify major planktonic sulfur oxidizers was also performed in a stratified freshwater lake (8), but very little is known about chemolithotrophic sulfur oxidizers living in general freshwater environments.

Recently, a chemolithotrophic sulfur oxidizer, *Sulfuricella denitrificans* skB26, was isolated from a stratified freshwater lake (9) as a representative of a new genus in the class *Betaproteobacteria*. Occurrences of close relatives of this bacterium have been reported in several studies, in the form of 16S rRNA gene clones detected in libraries constructed with general primer pairs (10-13). Strain skB26 was isolated from a cold anoxic hypolimnion and has the ability to grow at temperatures as low as 0°C. In general aquatic ecosystems, the primary source of reduced sulfur species is sulfate reduction in anoxic nitrate-depleted zones, which are often associated with low temperature. Therefore, cold adaptation may have essential significance for sulfur oxidizers in natural environments for access to sufficient supplies of growth substrate. Although several sulfur oxidizers have been isolated from

low-temperature environments (3, 14, 15), their cold adaptation mechanisms are poorly understood. Here I report the draft genome sequence of this novel psychrotolerant chemolithoautotroph, along with results of a proteomic analysis to gain insight into cold adaptation.

4.2 Materials and methods

4.2.1 Genome sequence and annotation

Sulfuricella denitrificans skB26 was grown in a previously described NaCl-free defined medium (9) containing 20 mM thiosulfate and 20 mM nitrate at 22°C. Genomic DNA was extracted by using an AquaPure genomic isolation kit (Bio-Rad Laboratories, CA) and then fragmented by shearing with a Hydroshear instrument (GeneMachines, CA). From the resulting DNA fragments, an 8-kbp paired-end library was prepared using GS Titanium paired-end library adaptors (Roche Diagnostics). The library was sequenced by using a Genome Sequencer FLX system (Roche Diagnostics). The paired-end reads were assembled by using GS De Novo Assembler, version 2.3, with default parameters. Gaps between contigs belonging to the same scaffold were closed by Sanger sequencing of PCR products. The draft genome sequence was automatically annotated using the Microbial Genome Annotation Pipeline (www.migap.org/). In the pipeline, open reading frames (ORFs) were identified by MetaGene Annotator, and then predicted ORFs were used to search reference databases, including RefSeq, TrEMBL, and the COGs (clusters of orthologous groups of proteins) data set. Genes for tRNAs and rRNAs were identified by tRNAscan-SE and rRNAmmer, respectively (16). Further genome analysis was performed using IMC-GE software (In Silico Biology, Japan). In the draft genome, homologous genes involved in sulfur oxidation, denitrification, and carbon dioxide fixation were identified by a BLASTP search using the

sequence data of *Thiobacillus denitrificans* as the query (threshold, >30%), and normalized gene names were assigned. To predict the functions of two homologous genes encoding Csp family proteins, the amino acid sequences were subjected to PSI-BLAST searches, with an identity threshold of 90%, and then manually annotated.

4.2.2 Protein extraction and SDS-PAGE

S. denitrificans was cultured in the same medium at the temperature for optimum growth (22°C) and the in situ temperature of the isolation source (5°C). Cells were harvested at stationary phase (35 and 68 days at 22°C and 5°C, respectively) and washed once with phosphate-buffered saline by centrifugation at $10,000 \times g$ for 20 min at 4°C. The pellets were frozen at -80°C until protein extraction. Protein extraction was performed using a kit intended to extract total cellular proteins, including membrane proteins, i.e., a ReadyPrep protein extraction kit for total protein (Bio-Rad Laboratories, CA). The cell pellets were suspended in a buffer (included in the kit) which contained a strong chaotropic extraction reagent. After 10 sonications for 10 s each at 30-s intervals, resulting lysates were centrifuged for 20 min at 10,000 rpm at 20°C. The soluble fractions were recovered and used for further analysis. The protein content was quantified by the Bradford method, using a Bio-Rad protein assay kit (Bio-Rad Laboratories). The protein samples were mixed with sodium dodecyl sulfate sample buffer. The resulting samples were incubated at 99°C for 5 min, and then 50 µg of each denatured protein sample was subjected to SDS-PAGE using a 12.5% SuperSep Ace precast gel (Wako Pure Chemical Industries, Japan). After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and destained, and the lanes were cut out for in-gel protein digestion with trypsin.

4.2.3 Trypsin in-gel protein digestion and mass analysis

Proteins were digested with trypsin as previously described (17). Nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nano-LC-ESI-MS/MS) experiment was performed on a multidimensional HPLC Paradigm MS2 (AMR Inc, Japan) and nanospray electrospray ionization device (Michrom Bioresources Inc, CA) connected to a LTQ ion-trap MS (Thermo Fisher Scientific, MA). The digested peptides were loaded on an L-column 2 ODS (Chemicals Evaluation & Research Inst., Japan) packed with C18 (5 μ m, 12 nm pore size). Solvent system consisted of solvent A (2% acetonitrile and 0.1% formic acid in H₂O) and solvent B (90% acetonitrile and 0.1% formic acid in H₂O). Flow rate was maintained at 1 μ l/min, and solvent B was increased in a linear gradient from 5% to 65% over 40 min. Then, it was further increased to 95% and kept at the concentration for 5 min before returning to 5% for analysis of the next sample. Peptide spectra were recorded in a range of m/z 450-1800. Mass spectra were acquired in data-dependent scan mode. One MS/MS spectrum of the one most intense peak was collected following on the full spectrum scan.

4.2.4 MASCOT search and semi-quantitative analysis of proteins

For peptide identification from MS/MS data, a protein sequence database was constructed from the *S. denitrificans* draft genome. By using the MASCOT (ver.2.3.01), MS/MS spectra were searched against the generated database. Search parameters were set as follows; tryptic digest with a maximum of two missed cleavage; fixed modifications, carbamidomethyl cysteine; variable modifications, methionine oxidation; peptide masses, monoisotopic; positive charge (+1, +2, +3) of peptide; mass tolerance of 1.2 Da for the precursor ion and 0.8 Da for productions. To assess false-positive identifications, an automatic decoy search was performed against a randomized database with significance threshold of $p < 0.05$, and the estimated false discovery rate was below 3.4 %. Protein identification was judged as positive when two or more different peptides derived from it

were detected.

To evaluate protein content, exponentially modified protein abundance index (emPAI) was determined as previously described (18). For comparative analysis, normalized protein content (PC) value was calculated as follows: $PC = \text{emPAI} / \Sigma(\text{emPAI}) \times 100 (\%)$, where ΣemPAI is the summation of emPAI values for all identified proteins.

4.3 Results and discussion

4.3.1 Genome sequence and annotation

By pyrosequencing, 77,574,798 bp from 386,840 reads were sequenced. The generated sequences were assembled into 177 contigs, and two scaffolds containing multiple contigs were constructed. There were 124 gaps within the scaffolds, and 117 of them were closed by Sanger sequencing of PCR products. As a result, an *S. denitrificans* skB26 draft genome consisting of 23 contigs was obtained. Basic characteristics of the draft genome are shown in Table 4-1. The functional classification of the identified coding sequences (CDSs) based on the COG designations is shown in Fig. 4-1.

Genes required for sulfur oxidation, denitrification, and carbon fixation are summarized in Table 4-2. As reported previously, strain skB26 oxidizes thiosulfate and elemental sulfur to sulfate. The draft genome contains the *soxXYZAB* gene cluster but lacks *soxCD*, suggesting the generation of elemental sulfur as an intermediate of thiosulfate oxidation (19). In *Allochromatium vinosum*, elemental sulfur is oxidized to sulfite by the dissimilatory sulfite reductase system, encoded by *dsr* genes (20). Accordingly, the gene cluster *dsrABEFHCMKLJOPNR* occurs in the genome of skB26. I also identified a putative *hdrAACB* gene cluster encoding the heterodisulfide reductase complex, hypothesized to oxidize disulfide intermediates to sulfite (21). It has been proposed that

adenosine-5'-phosphosulfate reductase (*apr*) and ATP sulfurylase (*sat*) oxidize sulfite to sulfate (22); the genes for these enzymes were also found in the strain. Furthermore, the gene for sulfide:quinone oxidoreductase (*sqr*), which catalyzes sulfide oxidation (23), occurred in the genome.

Strain skB26 contains genes necessary for denitrification, including nitrite reductase (*nir*), nitric oxide reductase (*nor*), and nitrous oxide reductase (*nos*) genes (24). The draft genome lacks a cytoplasmic membrane nitrate reductase (*nar*) gene, thought to generally be possessed by denitrifiers (24), but contains a periplasmic nitrate reductase (*nap*) gene.

4.3.2 Effects of low temperature on relative protein abundance

For proteomic analysis, *S. denitrificans* was grown on thiosulfate under denitrifying conditions. Cells cultured at 22°C (MT sample) and 5°C (LT sample) were harvested in stationary phase and subjected to comparative proteomic analysis using nano-LC-ESI-MS/MS. Using MASCOT searches, 1,450 and 1,579 proteins were identified in MT and LT, respectively. The detected proteins were classified into functional categories based on COG designations, but an effect of temperature was not apparent in this qualitative comparison (Fig. 4-1). For the comparisons based on semiquantitative analysis, normalized PC values were calculated.

The most abundant protein in LT was the ribosomal protein L5 (PC = 47%), which increased about 5-fold compared to that in MT (Table 4-3). Similarly, a 20- fold increase was observed for the ribosomal protein L7/L12 in LT, and 6 ribosomal proteins were identified only in LT. The protein L7/L12 has been reported to be a cold acclimation protein (25), and increased ribosomal protein abundance at low temperatures has been observed in other prokaryotes (26, 27). The high cellular levels of ribosomal proteins observed in *S. denitrificans* may be associated with cold adaptation.

To eliminate interference from abundant ribosomal proteins, PC values were recalculated after excluding these proteins for comparison of other proteins. In order to examine effects of temperature specifically observed in anaerobic sulfur oxidizers, PC values of proteins putatively involved in sulfur oxidation, denitrification, and carbon dioxide fixation were compared between LT and MT (Fig. 4-2). Among these three functions, differences in relevant proteins were apparent for sulfur oxidation, with generally lower contents in LT (Fig. 4-2A). These sulfur oxidation-related proteins, including heterodisulfide reductase (Hdr), which lacks direct evidence for involvement in thiosulfate oxidation, were the second most abundant proteins (following the ribosomal proteins) in both MT and LT. The sum of the PC values of the proteins shown in Fig. 4-2A reached 40% in MT but decreased to 17% in LT. These results may be relevant to suppressed energy production at low temperatures, as shown for other cold-adapted bacteria (28). It should be noted, however, that the decrease in total sulfur oxidation-related proteins was attributed largely to exceptionally drastic changes in DsrH and SoxZ (Fig. 4-2A). In the process of sulfur oxidation, DsrH and SoxZ work in conjunction with DsrEF and SoxY, respectively (29). The imbalanced changes of DsrH and SoxZ may have no connection to energy production.

As candidates for components of the cold adaptation mechanism, proteins with greater PC values in LT (more than 5 times those in MT) after the recalculation are listed in Table 4-4. One of the candidate proteins for cold adaptation, the cold shock protein CspD, has been reported as a cold-inducible protein in *Bacillus subtilis* (30). Another *cspD* homolog was also found in the genome (SCD_00462), and its product was identified only in LT, as the most abundant LT-specific protein (Table 4-5). One of the major negative effects of low temperature is the inhibition of translation caused by stabilization of the mRNA secondary structure. It has been proposed that Csp proteins work in conjunction with the DEAD/DEAH box helicase to maintain the proper conformation of mRNA in *B. subtilis* (31). In the draft

genome of strain skB26, 3 copies of the DEAD/DEAH box helicase gene were identified. The protein encoded by one of these genes was more abundant (about 9-fold) at 5°C (Table 4-4), and the product of another copy was identified only in LT (Table 4-5). The greatest change (32-fold) was observed in the sulfur relay protein DsrC (SCD_00181), although other putative sulfur relay proteins, encoded by five genes, were not increased in LT (Table 4-6). In *Escherichia coli*, the DsrC homolog is involved in thiolation of tRNA, which facilitates ribosome binding (32). Increased abundances of these proteins and ribosomal proteins consistently suggest that maintenance of proper translation at low temperatures is important for cold adaptation of *S. denitrificans* skB26.

Another candidate protein for cold adaptation was beta-hydroxyacyl-(acyl carrier protein) dehydratase (FabZ), a protein involved in fatty acid biosynthesis (Table 4-4). It is well known that modification of membrane fatty acid composition is critical for maintaining membrane fluidity under cold conditions, and various bacteria tend to increase the content of unsaturated fatty acids at low temperatures (33). In a cycle of hydrocarbon chain elongation for fatty acid biosynthesis, FabZ catalyzes dehydration to generate unsaturated fatty acids (34). Although the fatty acid profile of strain skB26 has not been revealed, it may change in response to low temperatures by involvement of FabZ.

Other candidate proteins shown in Table 4-4 were classified into various functional categories, including cell motility, nucleic acid metabolism, energy production, and signal transduction. For these proteins, it was impossible to deduce how they contribute to growth or survival at low temperatures. Their functional diversity may reflect universal effects of temperature on various biological processes and respective responses of this organism to cope with them.

From the comparative proteomic analysis, it was suggested that mechanisms to maintain proper translation and membrane fluidity play central roles in cold adaptation of *S.*

denitrificans. It should be noted that the protein identification was performed on the basis of the draft genome, and some important genes and proteins may thus still be undiscovered. For further detailed analyses, a project to obtain the complete genome sequence of strain skB26 is ongoing. Considering that studies on sulfur oxidizers tend to place a disproportionate emphasis on extremophiles living in harsh environments, knowledge from studies on *S. denitrificans* will be valuable for a better understanding of the sulfur cycle in general freshwater environments.

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Table 4-1. Basic characteristics of *S. denitrificans* skB26 draft genome.

Feature	Value
Size (bp)	3238714
GC content (%)	56.1
No. of contigs	23
Max contig length (bp)	737595
Total no. of coding sequences	3133
Coding sequence density (gene/kbp)	0.905
Total CDS length (bp)	2931503
Average gene length (bp)	935.7
No. of rRNA operons	1
No. of tRNA genes	43

Table 4-2. Genes for enzymes involved in sulfur oxidation, denitirification, carbon fixation in strain skB26 draft genome.

Function	Locus tag ^a	Normalized gene name	Identification number in Fig.
Sulfur oxidation	SCD_00355	<i>dsrB</i>	1
	SCD_02832	<i>dsrA</i>	2
	SCD_02833	<i>dsrB</i>	3
	SCD_02834	<i>dsrE</i>	4
	SCD_02835	<i>dsrF</i>	5
	SCD_02836	<i>dsrH</i>	6
	SCD_02837	<i>dsrC</i>	7
	SCD_02838	<i>dsrM</i>	8
	SCD_02839	<i>dsrK</i>	9
	SCD_02840	<i>dsrL</i>	10
	SCD_02841	<i>dsrJ</i>	11
	SCD_02842	<i>dsrO</i>	12
	SCD_02843	<i>dsrP</i>	13
	SCD_02844	<i>dsrN</i>	14
	SCD_02845	<i>dsrR</i>	15
	SCD_02617	<i>dsrS</i>	16
	SCD_00231	<i>dsrC</i>	17
	SCD_01513	<i>dsrC</i>	18
	SCD_02829	<i>dsrC</i>	19
	SCD_00181	<i>dsrC</i>	20
	SCD_00147	<i>dsrC</i>	21
	SCD_02369	<i>dsrE</i>	22
	SCD_01721	<i>sqr</i>	23
	SCD_01723	<i>sqr</i>	24
	SCD_00004	<i>aprB</i>	25
	SCD_00005	<i>aprA</i>	26
	SCD_02561	<i>sat</i>	27
	SCD_00003	<i>sat</i>	28
	SCD_00159	<i>hdrB</i>	29
	SCD_00160	<i>hdrC</i>	30
	SCD_00161	<i>hdrA</i>	31
	SCD_00162	<i>hdrA</i>	32
	SCD_00687	<i>soxB</i>	33
	SCD_00688	<i>soxA</i>	34
	SCD_00689	<i>soxZ</i>	35
	SCD_00690	<i>soxY</i>	36
	SCD_00691	<i>soxX</i>	37
	SCD_01652	<i>soxH</i>	38
	SCD_01293	<i>soxH</i>	39
	SCD_02615	<i>soxE</i>	40
	SCD_02132	<i>soxE</i>	41
	SCD_02133	<i>soxF</i>	42
	SCD_01681	<i>soxW</i>	43
Denitirification	SCD_02134	<i>napB</i>	44
	SCD_02135	<i>napA</i>	45
	SCD_02146	<i>napC</i>	46
	SCD_02149	<i>napD</i>	47
	SCD_02150	<i>napG</i>	48
	SCD_02151	<i>napH</i>	49
	SCD_02159	<i>napH</i>	50
	SCD_00392	<i>nirS</i>	51
	SCD_02137	<i>nirN</i>	52
	SCD_02148	<i>nirS</i>	53
	SCD_02055	<i>norC</i>	54
	SCD_02056	<i>norB</i>	55
	SCD_02165	<i>nosZ</i>	56
Carbon fixation	SCD_00311	<i>cbbL</i>	57
	SCD_00312	<i>cbbS</i>	58
	SCD_00313	<i>cbbQ</i>	59
	SCD_00314	<i>cbbO</i>	60
	SCD_02697	<i>cbbO</i>	61
	SCD_02698	<i>cbbQ</i>	62
	SCD_02699	<i>cbbM</i>	63

^aLocus tags indicate gene localizations in the draft genome BAFJ01000001–BAFJ01000023.

The version reported here is the first version.

Table 4-3. List of all ribosomal proteins encoded in strain skB26 draft genome and their normalized protein content (PC).

	Locus tag ^a	Description	Normalized protein content (PC) ^b		Ratio of PC (5/22°C)
			22°C	5°C	
Large subunit	SCD_01882	50S ribosomal protein L2	0.11833	0.14038	1.19
	SCD_03128	50S ribosomal protein L2	0	0	-
	SCD_01885	50S ribosomal protein L3	0.12849	0.02732	0.21
	SCD_03131	50S ribosomal protein L3	0	0.02628	-
	SCD_02968	Ribosomal protein L3	0	0	-
	SCD_01884	50S ribosomal protein L4	0.18097	0.07378	0.41
	SCD_03130	50S ribosomal protein L4	0	0	-
	SCD_01873	50S ribosomal protein L5	10.44615	47.22850	4.52
	SCD_01870	50S ribosomal protein L6	3.23236	2.38270	0.74
	SCD_01537	Ribosomal protein L6P/L9E	0	0	-
	SCD_01893	50S ribosomal protein L7/L12	0.05313	1.07925	20.31
	SCD_00066	Ribosomal protein L9	0.05484	0.02366	0.43
	SCD_00065	Ribosomal protein L9	0	0.01552	-
	SCD_01894	50S ribosomal protein L10	0.65125	0.33134	0.51
	SCD_03029	50S ribosomal protein L13	0.46360	0.89188	1.92
	SCD_01875	50S ribosomal protein L14	0.04655	0.07716	1.66
	SCD_01866	Ribosomal protein L15	0.42319	0.10500	0.25
	SCD_01878	50S ribosomal protein L16	0.06831	0.05728	0.84
	SCD_01859	50S ribosomal protein L17	0.27723	0.12174	0.44
	SCD_01869	50S ribosomal protein L18	0.05933	0.02651	0.45
	SCD_01527	Ribosomal protein L18E	0	0	-
	SCD_00738	50S ribosomal protein L19	0.09658	0.06279	0.65
	SCD_01895	50S ribosomal protein L1P	0.10812	0.22317	2.06
	SCD_01397	50S ribosomal protein L20	0.03020	0.01572	0.52
	SCD_00387	50S ribosomal protein L21	0.00962	0.02628	2.73
	SCD_01880	50S ribosomal protein L22P	0.07333	0.10616	1.45
	SCD_01883	50S ribosomal protein L23	0.18119	0.10397	0.57
	SCD_03129	50S ribosomal protein L23	0	0	-
	SCD_01874	50S ribosomal protein L24P	0.10999	0.12213	1.11
	SCD_02931	Ribosomal protein L25	0.02346	0.05161	2.20
	SCD_00388	50S ribosomal protein L27	0.01881	0.03255	1.73
	SCD_01761	50S ribosomal protein L28	0.10037	0.08688	0.87
	SCD_01877	Ribosomal protein L29	0.03004	0.04373	1.46
	SCD_01867	Ribosomal protein L30	0.04848	0.07840	1.62
	SCD_01778	50S ribosomal protein L31	0	0.00488	-
	SCD_00963	50S ribosomal protein L32	0	0	-
	SCD_01760	50S ribosomal protein L33	0	0.00352	-
	SCD_01398	50S ribosomal protein L35	0.01523	0.01664	1.09
Small subunit	SCD_01474	30S ribosomal protein S1	0.07600	0.04603	0.61
	SCD_00865	30S ribosomal protein S2	0.26242	0.20925	0.80
	SCD_01879	30S ribosomal protein S3	0.04773	0.04998	1.05
	SCD_01861	30S ribosomal protein S4	0.15516	0.13200	0.85
	SCD_01868	Ribosomal protein S5	0.98401	0.84027	0.85
	SCD_00069	30S ribosomal protein S6	0.15836	0.10834	0.68
	SCD_01889	30S ribosomal protein S7	0.62886	0.48299	0.77
	SCD_01871	30S ribosomal protein S8	0.21005	0.13371	0.64
	SCD_03030	Ribosomal protein S9	0.01587	0.01192	0.75
	SCD_01886	30S ribosomal protein S10	0.03816	0.06625	1.74
	SCD_03132	30S ribosomal protein S10	0	0	-
	SCD_01862	30S ribosomal protein S11	0.02603	0.01635	0.63
	SCD_01890	30S ribosomal protein S12	0.07915	0.01782	0.23
	SCD_01863	30S ribosomal protein S13	0.04762	0.02884	0.61
	SCD_01872	30S ribosomal protein S14	0.05681	0.03474	0.61
	SCD_00788	30S ribosomal protein S15	0.02373	0.01220	0.51
	SCD_00741	30S ribosomal protein S16	0.00593	0.01648	2.78
	SCD_01876	Ribosomal protein S17	0.10593	0.30661	2.89
	SCD_00067	30S ribosomal protein S18	0	0.01003	-
	SCD_01881	30S ribosomal protein S19	0.02480	0.02146	0.87
	SCD_00465	30S ribosomal protein S20	0.00540	0.00962	1.78
	SCD_00729	30S ribosomal protein S21	0	0.01914	-

^aLocus tags indicate gene localization in the draft genome BAFJ01000001–BAFJ01000023.

The version reported here is the first version.

^bNormalized protein content (PC) was calculated as described in the materials and methods.

Table 4-4. List of protein more abundant (fold change>5) at 5°C.

Locus tag ^a	Description	Functional category ^b	Normalized protein content (PC) ^c		Ratio of PC (5°C/22°C)
			22°C	5°C	
SCD_00181	Sulfur relay protein, TusE/DsrC/DsvC family	P	0.03548	1.12914	31.8
SCD_01927	Glutaredoxin-like protein	O	0.03040	0.57336	18.9
SCD_00850	Flagellin and related hook-associated proteins	N	0.00107	0.01660	15.5
SCD_01247	FeS cluster assembly scaffold IscU	C	0.00835	0.08731	10.5
SCD_00930	DEAD/DEAH box helicase domain-containing protein	LKJ	0.00281	0.02504	8.9
SCD_01712	Hypothetical protein	S	0.17117	1.39561	8.2
SCD_02129	Anaerobic ribonucleoside-triphosphate reductase	F	0.00207	0.01552	7.5
SCD_01704	Hydrogenase maturation factor	O	1.45679	10.89136	7.5
SCD_02710	Fe(II) trafficking protein YggX	CO	0.01884	0.12361	6.6
SCD_01982	Nitroreductase	C	0.00247	0.01570	6.4
SCD_00192	Guanylate kinase	F	0.00909	0.05573	6.1
SCD_02164	Cytochrome c553	C	0.09928	0.60598	6.1
SCD_03038	Protein tyrosine phosphatase	X	0.00882	0.05151	5.8
SCD_00637	RNA chaperone (cspD: CspA family protein)	K	0.01951	0.10436	5.3
SCD_02624	Pilus protein	T	0.01971	0.10504	5.3
SCD_00876	Beta-hydroxyacyl-(acyl-carrier-protein) dehydratase FabZ	I	0.00354	0.01772	5.0

^aLocus tags indicate gene localizations in the first version of the draft genome BAFJ0100001- BAFJ01000023.

^bOne-letter abbreviations indicate the COG functional category. ^cPC values were calculated excluding all ribosomal proteins.

Table 4-5. List of all proteins identified only in 5°C. PC value was calculated except for all ribosomal proteins.

Locus tag ^a	Description	Normalized protein content ^b
SCD_00462	RNA chaperone (cspD: CspA family protein)	0.1093425
SCD_01985	Uncharacterized conserved protein	0.0624558
SCD_01918	Phosphoribosyl-AMP cyclohydrolase	0.0395284
SCD_03080	Alkylphosphonate utilization operon proteinPhnA	0.036298
SCD_00603	Actin and related proteins	0.0264719
SCD_00929	Superfamily II DNA and RNA helicases	0.0251259
SCD_01928	BolA family protein	0.0244978
SCD_02000	DNA-directed RNA polymerase subunit omega	0.0219403
SCD_03039	Thiol-disulfide isomerase/thioredoxin	0.021716
SCD_00984	Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain	0.0143128
SCD_00430	Protein tyrosine phosphatase	0.0136398
SCD_01785	Uncharacterized conserved protein	0.013236
SCD_00660	Peptide methionine sulfoxide reductase	0.0125629
SCD_01588	Predicted RNA-binding protein containing KH domain, possibly ribosomal protein	0.0122937
SCD_00342	Beta-galactosidase	0.0115759
SCD_02076	Predicted proteasome-type protease	0.0104542
SCD_00294	Rhodanese-related sulfurtransferase	0.0100055
SCD_00447	Rubryerythrin	0.0093773
SCD_02831	Predicted nucleic acid-binding protein, contains PIN domain	0.0092427
SCD_02762	3-dehydroquinate dehydratase II	0.0085697
SCD_02430	Phosphocarrier HPr protein	0.0083005
SCD_02455	Uncharacterized homolog of plant Iojap protein	0.0082108
SCD_00944	Alkylphosphonate utilization operon proteinPhnA	0.0080762
SCD_01014	Arsenate reductase and related proteins, glutaredoxin family	0.0079416
SCD_01101	Hypothetical protein	0.0078967
SCD_03000	Transcriptional regulator containing GAF, AAA-type ATPase, and DNA binding domains	0.0076724
SCD_01532	DnaK suppressor protein	0.0074032
SCD_02750	Thioredoxin-related protein	0.0073583
SCD_00790	Anaerobic dehydrogenases, typically selenocysteine-containing	0.0069096
SCD_02858	Rhodanese-related sulfurtransferase	0.006775
SCD_01097	Hypothetical protein	0.0067302
SCD_00428	Hypothetical protein	0.0065507
SCD_02410	Class IV aminotransferase	0.0065058
SCD_02071	Predicted transcriptional regulator	0.0064161
SCD_02936	Peptide methionine sulfoxide reductase	0.0064161
SCD_01246	Iron-sulfur cluster assembly protein IscA	0.0062366
SCD_01661	Protocatechuate 3,4-dioxygenase beta subunit	0.0058777
SCD_00934	FOG: Ankyrin repeat	0.0058328
SCD_02015	Uncharacterized protein conserved in bacteria	0.0058328
SCD_00704	Transcriptional regulators	0.0054739
SCD_01120	Micrococcal nuclease (thermonuclease) homologs	0.0053393
SCD_02453	Nucleotide-binding protein implicated in inhibition of septum formation	0.0053393
SCD_00852	Flagellar capping protein	0.00507
SCD_01905	Stringent starvation protein B	0.0050252
SCD_02140	AsnC family transcriptional regulator	0.0049803
SCD_02359	Hypothetical protein	0.0049803
SCD_02431	Phosphotransferase system, mannose/fructose-specific component IIA	0.0049803
SCD_02210	Hypothetical protein	0.0049354
SCD_02956	Hypothetical protein	0.0049354
SCD_00164	Inorganic pyrophosphatase	0.0048906
SCD_01636	Isopropylmalate isomerase small subunit	0.0048457
SCD_00431	Thiol-disulfide isomerase and thioredoxinsfamily protein	0.004756
SCD_01585	Dihydropteroate synthase and related enzymes	0.004756
SCD_02817	Uncharacterized protein involved in biosynthesis of c-type cytochromes	0.0046662
SCD_00877	Acyl-(acyl-carrier-protein)-UDP-N-acetylglucosamine O-acyltransferase	0.0046214
SCD_02305	Predicted transcriptional regulator	0.0042176
SCD_00458	Hypothetical protein	0.0041727
SCD_00612	Glycosyltransferase	0.0040381
SCD_00435	Hypothetical protein	0.0039035
SCD_01668	Transcription termination factor	0.0038586
SCD_01931	Predicted NTP binding protein (contains STAS domain)	0.0038586
SCD_02432	Membrane-associated lipoprotein involved in thiamine biosynthesis	0.0038586
SCD_01664	Uncharacterized protein (competence- and mitomycin-induced)	0.0037689
SCD_01966	Histidyl-tRNA synthetase	0.0037689
SCD_02254	Predicted membrane protein	0.0037689
SCD_02082	Putative protease	0.003724
SCD_00217	Cytochrome c553	0.0036791
SCD_02213	Glucoamylase and related glycosyl hydrolases	0.0036343
SCD_01064	Outer membrane protein	0.0035894
SCD_02341	Protein implicated in iron transport, frataxin homolog	0.0035894
SCD_01716	ATP-dependent Clp protease adaptor protein ClpS	0.0035445
SCD_01757	Predicted transcriptional regulators containing the CopG/Arc/MetJ DNA-binding domain and a metal-binding domain	0.0035445
SCD_01762	DNA repair protein RadC	0.0035445
SCD_01917	Phosphoribosyl-ATP pyrophosphohydrolase	0.0035445
SCD_02373	ArsR family transcriptional regulator	0.0035445
SCD_01717	ATP-dependent Clp protease ATP-binding subunitClpA	0.0034099

Table 4-5. *continued.*

SCD_02229	Uncharacterized protein conserved in bacteria	0.0034099
SCD_00327	Cobalamin biosynthesis protein CobD/CbiB	0.0033651
SCD_00702	Predicted proteasome-type protease	0.0033651
SCD_01987	Hypothetical protein	0.0033651
SCD_00033	2C-methyl-D-erythritol 2,4-cyclodiphosphatesynthase	0.0033202
SCD_00595	Response regulator containing CheY-like receiver domain and AraC-type DNA-binding domain	0.0033202
SCD_00167	UDP-N-acetylglucosamine 2-epimerase	0.0030959
SCD_00389	GTP-binding protein Obg/CgtA	0.0030959
SCD_01338	Putative protein-S-isoprenylcysteine methyltransferase	0.003051
SCD_01910	Uncharacterized conserved protein	0.003051
SCD_00175	Biotin carboxylase	0.0030061
SCD_01245	DnaJ-domain-containing proteins 1	0.0030061
SCD_00255	Hypothetical protein	0.0030061
SCD_00486	Non-ribosomal peptide synthetase modules and related proteins	0.0029613
SCD_02647	Methionyl-tRNA formyltransferase	0.0029613
SCD_00043	Predicted peroxiredoxins	0.0029164
SCD_00350	Predicted SAM-dependent methyltransferases	0.0029164
SCD_01034	Response regulator receiver	0.0028267
SCD_02729	cAMP-binding proteins - catabolite gene activator and regulatory subunit of cAMP-dependent protein kinases	0.0028267
SCD_01170	UDP-glucose 4-epimerase	0.0027818
SCD_02961	PhoH-like protein	0.0027369
SCD_00457	Translation initiation factor 1 (eIF-1/SUI1) and related proteins	0.0026921
SCD_00978	FOG: Ankyrin repeat	0.0026921
SCD_00473	Predicted aspartyl protease	0.0026472
SCD_01848	Uncharacterized conserved protein	0.0026472
SCD_01125	Uncharacterized conserved protein related to C-terminal domain of eukaryotic chaperone, SACSIN	0.0026023
SCD_01528	Response regulator containing a CheY-like receiver domain and a GGDEF domain	0.0026023
SCD_00378	Negative regulator of beta-lactamase expression	0.0025575
SCD_01727	Predicted dehydrogenases and related proteins	0.0025575
SCD_02069	Molecular chaperone	0.0025575
SCD_00611	Protein chain release factor B	0.0025126
SCD_00919	Predicted ATPase	0.0025126
SCD_02822	Methyl-accepting chemotaxis protein	0.0024677
SCD_02882	Hypothetical protein	0.0023331
SCD_01436	Predicted transcriptional regulators	0.0022883
SCD_02820	Uncharacterized protein conserved in bacteria	0.0022883
SCD_02932	Peptidyl-tRNA hydrolase	0.0022883
SCD_00329	Sugar phosphate isomerases/epimerases	0.0022434
SCD_02454	Uncharacterized conserved protein	0.0022434
SCD_00111	Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain	0.0021985
SCD_02581	FOG: TPR repeat	0.0021985
SCD_00154	HD-GYP domain	0.0021536
SCD_00362	Hypothetical protein	0.0021536
SCD_01111	Negative regulator of sigma E activity	0.0021536
SCD_01385	Predicted hydrolase (HAD superfamily)	0.0021536
SCD_01971	UBA/THIF-type NAD/FAD binding protein	0.0021536
SCD_02322	Monofunctional biosynthetic peptidoglycantransglycosylase	0.0021536
SCD_03070	Uncharacterized conserved protein	0.0021536
SCD_03112	Outer membrane protein	0.0021536
SCD_00221	Transcriptional regulator containing GAF, AAA-type ATPase, and DNA binding domains	0.0021088
SCD_00474	Thioredoxin domain-containing protein	0.0021088
SCD_02167	Thiol-disulfide isomerase and thioredoxins	0.0021088
SCD_02459	DNA polymerase III, delta subunit	0.0021088
SCD_01144	FOG: TPR repeat, SEL1 subfamily	0.002019
SCD_02115	TRNA nucleotidyltransferase/poly(A) polymerase	0.002019
SCD_02752	Uroporphyrinogen-III synthase	0.002019
SCD_02962	Predicted metal-dependent hydrolase	0.002019
SCD_01004	Succinate dehydrogenase and fumarate reductaseiron-sulfur protein	0.0019742
SCD_01040	Methyl-accepting chemotaxis protein	0.0019742
SCD_01471	Predicted N-acetylglucosaminyl transferase	0.0019742
SCD_01543	Hypothetical protein	0.0019742
SCD_02194	ATP-dependent protease ATP-binding subunit HslU	0.0019742
SCD_02970	Outer membrane protein and related peptidoglycan-associated (lipo)proteins	0.0019742
SCD_03065	Protein involved in initiation of plasmid replication	0.0019742
SCD_02380	Hypothetical protein	0.0019293
SCD_00390	Gamma-glutamyl kinase	0.0019293

Table 4-5. *continued.*

SCD_01963	Hypothetical protein	0.0019293
SCD_01770	Acyl-CoA thioesterase	0.0018844
SCD_00242	Predicted signal transduction protein	0.0018396
SCD_00600	Disulfide bond chaperones of the HSP33 family	0.0018396
SCD_00207	Predicted signal transduction protein	0.0017947
SCD_00914	Peroxiredoxin	0.0017947
SCD_01020	Chromosome segregation and condensation protein	0.0017947
SCD_01696	Hypothetical protein	0.0017947
SCD_02125	RIO-like kinase	0.0017947
SCD_02269	FOG: TPR repeat	0.0017947
SCD_00661	Phosphatidylserine/phosphatidylglycerophosphate /cardiolipin synthases and related enzymes	0.0017498
SCD_00871	1-deoxy-D-xylulose 5-phosphate reductoisomerase	0.0017498
SCD_03179	Putative PAS/PAC sensor protein	0.0017498
SCD_01016	Methylase of polypeptide chain release factors	0.0016601
SCD_02012	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	0.0016601
SCD_02401	Hypothetical protein	0.0016601
SCD_03058	ABC-type phosphate/phosphonate transport system, periplasmic component	0.0016601
SCD_00262	Predicted NADH:ubiquinone oxidoreductase, subunit RnfG	0.0016152
SCD_00318	Fructose/tagatose bisphosphate aldolase	0.0016152
SCD_01025	Zn-dependent proteases	0.0016152
SCD_01614	Uncharacterized protein conserved in bacteria	0.0015704
SCD_00672	Glycosyltransferases involved in cell wall biogenesis	0.0015704
SCD_01063	Membrane-fusion protein	0.0015704
SCD_02238	Small-conductance mechanosensitive channel	0.0015704
SCD_02351	TetR family transcriptional regulator	0.0015704
SCD_00923	Lysophospholipase L1 and related esterases	0.0015255
SCD_01386	CheR-type MCP methyltransferase	0.0015255
SCD_02334	ABC-type multidrug transport system, ATPase component	0.0015255
SCD_03003	Hypothetical protein	0.0015255
SCD_00208	6-phosphogluconolactonase/Glucosamine-6-phosphate isomerase/deaminase	0.0014806
SCD_00779	FAD linked oxidase domain-containing protein	0.0014806
SCD_00823	Succinyldiaminopimelate transaminase	0.0014806
SCD_02297	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	0.0014806
SCD_00588	Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain	0.0014358
SCD_01370	FAD/FMN-containing dehydrogenases	0.0014358
SCD_01709	Uncharacterized homolog of biotin synthetase	0.0014358
SCD_01865	Preprotein translocase subunit SecY	0.0014358
SCD_02434	Glutamate--cysteine ligase GshA	0.0014358
SCD_02821	Putative homoserine kinase type II (protein kinase fold)	0.0014358
SCD_00533	Two component heavy metal response transcriptional regulator	0.0013909
SCD_00641	Hypothetical protein	0.0013909
SCD_00651	Thiamine pyrophosphate-requiring enzymes	0.0013909
SCD_00886	RRNA methylases	0.0013909
SCD_00954	Pseudouridylate synthases, 23S RNA-specific	0.001346
SCD_02755	Hypothetical protein	0.001346
SCD_02779	Superfamily I DNA and RNA helicases	0.001346
SCD_00810	ABC transporter-like protein	0.001346
SCD_01845	Quinolinate synthetase	0.001346
SCD_02184	Predicted hydrolase (HAD superfamily)	0.001346
SCD_00077	GTPases	0.0013012
SCD_00764	UDP-N-acetylglucosamine:LPS N-acetylglucosamine transferase	0.0013012
SCD_00996	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases	0.0013012
SCD_02320	Predicted Co/Zn/Cd cation transporters	0.0013012
SCD_03042	Arsenite S-adenosylmethyltransferase	0.0013012
SCD_00395	Spermidine synthase	0.0012563
SCD_00556	16S rRNA uridine-516 pseudouridylate synthase and related pseudouridylate synthases	0.0012563
SCD_01011	2-methylcitrate synthase/citrate synthase II	0.0012563
SCD_01249	Cysteine sulfinate desulfinase/cysteine desulfurase and related enzymes	0.0012563
SCD_02033	DNA polymerase III, epsilon subunit and related 3'-5' exonucleases	0.0012563
SCD_03086	SAM-dependent methyltransferases	0.0012563
SCD_00677	Glycosyltransferases involved in cell wall biogenesis	0.0012114
SCD_00842	Trans-aconitate methyltransferase	0.0012114
SCD_01993	Coproporphyrinogen III oxidase and related Fe-S oxidoreductases	0.0012114
SCD_00229	Predicted membrane protein	0.0011666
SCD_00760	Cell division septal protein	0.0011666
SCD_01166	Glycosyltransferase	0.0011666
SCD_02673	Glycosyltransferases involved in cell wall biogenesis	0.0011666
SCD_00680	ABC-type polysaccharide/polyol phosphate transport system, ATPase component	0.0011217
SCD_02515	Predicted GTPase	0.0011217
SCD_02496	Aminopeptidase N	0.0010768
SCD_01435	Uncharacterized protein related to capsule biosynthesis enzymes	0.0010768
SCD_01654	Restriction endonuclease	0.0010768
SCD_02198	Periplasmic solute-binding protein	0.0010768

Table 4-5. *continued.*

SCD_02264	Dimethyladenosine transferase (rRNA methylation)	0.0010768
SCD_03103	ATP synthase subunit beta	0.0010768
SCD_00453	DnaJ-class molecular chaperone	0.001032
SCD_02668	Hypothetical protein	0.001032
SCD_00694	Uncharacterized conserved protein	0.0009871
SCD_02348	DEAD/DEAH box helicase domain protein	0.0009871
SCD_02356	ABC-type multidrug transport system, ATPase component	0.0009871
SCD_02407	Lipoic acid synthetase	0.0009871
SCD_01344	ATPase components of various ABC-type transport systems, contain duplicated ATPase	0.0009422
SCD_00857	Glutathione S-transferase	0.0009422
SCD_00875	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	0.0009422
SCD_00972	Predicted periplasmic solute-binding protein	0.0009422
SCD_00257	Alanine dehydrogenase	0.0008974
SCD_00663	Uncharacterized conserved protein	0.0008974
SCD_00846	CDP-glucose 4,6-dehydratase	0.0008974
SCD_00975	ATPase involved in DNA replication	0.0008974
SCD_01839	Holliday junction DNA helicase RuvB	0.0008974
SCD_02288	FOG: GGDEF domain	0.0008974
SCD_02507	Predicted Rossmann fold nucleotide-binding protein involved in DNA uptake	0.0008974
SCD_02756	2-polyphenylphenol hydroxylase and related flavodoxin oxidoreductases	0.0008974
SCD_03111	Membrane-fusion protein	0.0008974
SCD_00445	Uncharacterized NAD(FAD)-dependent dehydrogenases	0.0008525
SCD_00482	Type II secretory pathway, component ExeA (predicted ATPase)	0.0008525
SCD_00826	Nitroreductase	0.0008525
SCD_01493	Filamentation induced by cAMP protein fic	0.0008525
SCD_01740	Hydrogenase formation HypD protein	0.0008525
SCD_01991	Uncharacterized conserved protein	0.0008525
SCD_02092	Predicted signal transduction protein containing a membrane domain, an EAL and a GGDEF domain	0.0008525
SCD_02396	Hypothetical protein	0.0008525
SCD_00321	Phosphoribosylaminoimidazole carboxylase (NCAIR synthetase)	0.0008076
SCD_00695	Uncharacterized protein conserved in bacteria	0.0008076
SCD_01159	Glycosyltransferase	0.0008076
SCD_01187	Type II secretory pathway, component PulF	0.0008076
SCD_01368	Serine--pyruvate transaminase	0.0008076
SCD_02237	Hypothetical protein	0.0008076
SCD_02642	Putative polysaccharide biosynthesis protein	0.0008076
SCD_02791	3-oxoacyl-(acyl carrier protein) synthase I	0.0008076
SCD_03014	Membrane-bound lytic murein transglycosylase	0.0008076
SCD_00062	DNA repair protein RadA	0.0007179
SCD_00534	Signal transduction histidine kinase	0.0007179
SCD_00705	1,4-alpha-glucan-branching enzyme	0.0007179
SCD_00948	Hypothetical protein	0.0007179
SCD_01411	LoI/C/E family lipoprotein releasing system,transmembrane protein	0.0007179
SCD_02558	Signal transduction histidine kinase	0.000673
SCD_00861	Aromatic-L-amino-acid decarboxylase	0.000673
SCD_02316	Nitrogen metabolism transcriptional regulatorNtrC	0.000673
SCD_01068	Signal transduction histidine kinase	0.0006281
SCD_01809	F0F1 ATP synthase subunit beta	0.0006281
SCD_02906	Polyferredoxin	0.0006281
SCD_00915	Fe-S oxidoreductase	0.0005833
SCD_02084	Predicted signal transduction protein containing a membrane domain, an EAL and a GGDEF domain	0.0005833
SCD_00270	Hypothetical protein	0.0005384
SCD_00806	Flagellin and related hook-associated proteins	0.0005384
SCD_00950	Excinuclease ABC, B subunit	0.0004487
SCD_01309	FOG: GGDEF domain	0.0004487
SCD_01428	ATPase involved in DNA repair	0.0004487
SCD_01540	Cytochrome c, mono- and diheme variants	0.0004487
SCD_02096	Predicted signal transduction protein containing a membrane domain, an EAL and a GGDEF domain	0.0004487
SCD_02483	Highly conserved protein containing a thioredoxin domain	0.0004487
SCD_02676	Predicted nucleoside-diphosphate sugar epimerases	0.0004487
SCD_01165	Phosphate uptake regulator	0.0004038
SCD_00814	Transporter, hydrophobe/amphiphile efflux-1(HAE1) family	0.0003589
SCD_01361	Predicted periplasmic ligand-binding sensor domain	0.0002692
SCD_01774	ATP-dependent exoDNAse (exonuclease V) beta subunit (contains helicase and exonuclease domains)	0.0002692

^aLocus tags indicate gene localizations in the draft genome BAFJ01000001–BAFJ01000023.

The version reported here is the first version.

^bNormalized protein content (PC) was calculated by excluding all ribosomal proteins.

Table 4-6. All genes encoding the sulfur relay protein in strain skB26 draft genome and normalized protein content.

Locus tag ^a	Description	Normalized protein content (PC) ^b	
		22°C	5°C
SCD_00181	sulfur relay protein, TusE/DsrC/DsvC family	0.03548	1.12914
SCD_01513	sulfur relay protein, TusE/DsrC/DsvC family	0.22228	0.07968
SCD_02834	sulfur relay protein TusD/DsrE	0.98357	0.20603
SCD_02835	sulfur relay protein TusC/DsrF	0.40961	0.19423
SCD_03153	sulfur relay protein TusD/DsrE	0	0
SCD_03152	sulfur relay protein TusC/DsrF	0.22181	0.02746

^aLocus tags indicate gene localizations in the draft genomeBAFJ01000001–BAFJ01000023.

The version reported here is the first version.

^bNormalized protein content (PC) was calculated by excluding all ribosomal proteins.

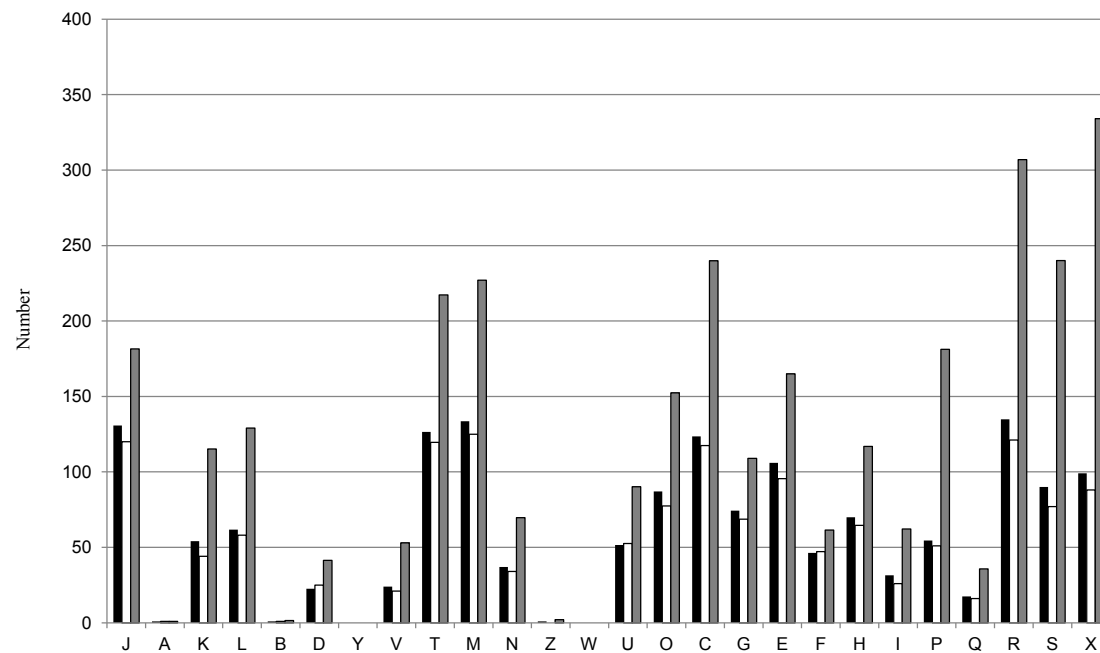


Fig. 4-1. COG distribution of proteins identified from LT (black bar), MT (white bar), and that of all CDSs found in *S. denitrificans* draft genome (gray bar). One-letter abbreviations indicate the functional categories: J, translation, ribosomal structure and biogenesis; A, RNA processing and modification; K, transcription; L, replication, recombination and repair; B, chromatin structure and dynamics; D, cell cycle control, cell division, chromosome partitioning; Y, nuclear structure; V, defense mechanisms; T, signal transduction mechanisms; M, cell wall/membrane/envelope biogenesis; N, cell motility; Z, cytoskeleton; W, extracellular structure; U, intracellular trafficking, secretion, and vesicular transport; O, posttranslational modification, protein turnover, chaperones; C, energy production and conversion; G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown. In addition, X indicates CDSs that were not assigned in any category.

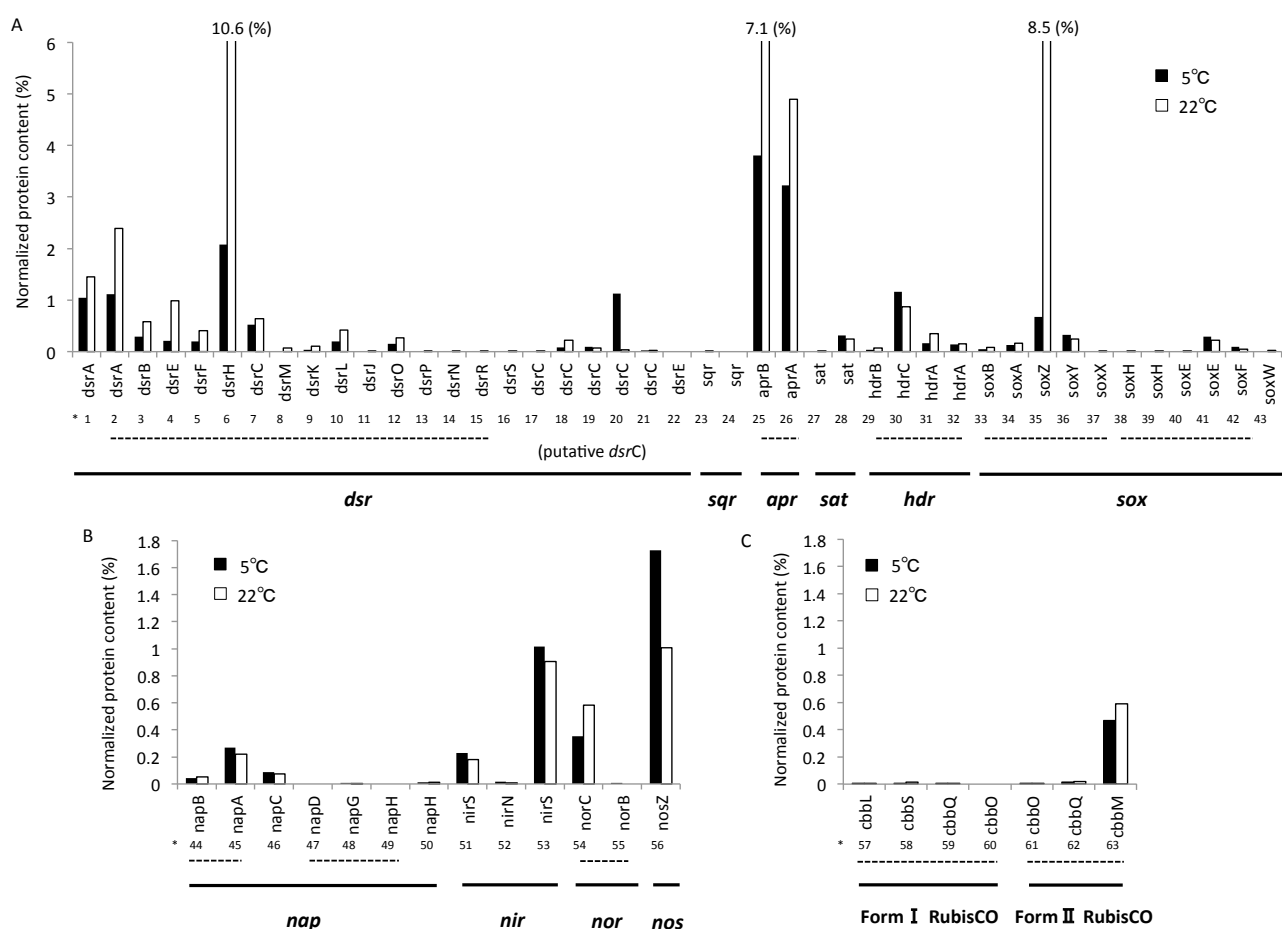


Figure 1

Fig. 4-2. Normalized protein contents of products of genes associated with sulfur oxidation (A), denitrification (B), and carbon fixation via RubisCO (C), calculated by excluding all ribosomal proteins. Normalized gene names are shown with identification numbers corresponding to those in Table 4-2. Dashed lines indicate putative gene clusters.

Chapter 5

**Complete genomes of freshwater sulfur oxidizers *Sulfuricella*
denitrificans skB26 and *Sulfuritalea hydrogenivorans* sk43H:
genetic insights into the sulfur oxidation pathway of
betaproteobacteria**

Submitted for publication

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5.1 Introduction

Phylogenetically diverse chemolithotrophic sulfur-oxidizing bacteria (SOB) are widespread in aquatic environments. Key SOB identified in marine environments, such as coastal sediments, water columns of oxygen minimum zones, and hydrothermal vents, include alpha-, gamma-, and epsilon- proteobacteria. Their genomes have been analyzed, which has provided important insights into the genetic basis of how these organisms derive energy by the oxidation of inorganic sulfur compounds (1-4).

In contrast to the breadth of information related to the marine SOB, knowledge about their counterparts in freshwater lake ecosystems is relatively limited. However, recent cloning analysis of the composition of SOB present in freshwater lakes showed major clone groups were phylogenetically distinct from marine SOB, and were classified as *Betaproteobacteria* (H. Kojima, T. Watanabe, T. Iwata, M. Fukui, submitted for publication; 5). Although the majority of these clone groups could not be classified into any specific genera, in part due to the absence of isolates, two clone groups were found to be closely related to the obligate and facultative sulfur chemolithoautotrophs, *Sulfuricella denitrificans* skB26 and *Sulfuritalea hydrogenivorans* sk43H. Strains skB26 and sk43H were recently isolated from hypolimnion water of Lake Mizugaki, a freshwater lake, and were described as type strains of their respective novel genera and species within the *Betaproteobacteria* (6, 7). Close relatives of strain skB26 have been reported from soil environments (8, 9) and various aquatic environments, such as freshwater lake sediments (5, 10, 11), drinking water (12), and biofilm developed on pipe transporting drinking water (13). Dominance of *Sulfuritalea* spp. has been observed in anoxic water columns of stratified freshwater lakes (H. Kojima, T. Watanabe, T. Iwata, M. Fukui, submitted for publication; 14). Occurrences of *Sulfuritalea* spp. in freshwater lake sediments (11, 15), coal tar waste-contaminated groundwater (16), and

nitrifying–denitrifying activated sludge (17) have also been reported. Dominancy of the genera *Sulfuricella* and *Sulfuritalea* has been shown in a study of groundwater microbiota, but two genera were predominant in the different samples respectively, collected from wells within a radius of 50 m (18). These observations suggest that their environmental distribution does not overlap, and they are most likely to occur in freshwater environments.

Numerically abundant bacteria in natural environments often cannot be cultured in the laboratory. In recent years, advanced culture-independent methods, including metagenomics and single-cell genomics, have allowed us to better understand the physiological potential and ecology of these bacteria. However, interpretation of these data still depends largely on the information derived from pure culture studies. Here, I report the complete genomes of strains skB26 and sk43H.

The genomic information of these strains further advances our understanding of their taxonomy. Furthermore, a comparison of genome sequences of skB26 and sk43H, with available complete genome sequences of other betaproteobacterial SOB, including *Thiobacillus denitrificans* ATCC 25259 (19), *Thiomonas* spp. (20), *Advenella kashmirensis* WT 001(21), *Sideroxydans lithotrophicus* ES-1 (22), provided the genetic basis of diverse sulfur oxidation pathways. Finally, I also discuss the genetic basis of carbon fixation and arsenic metabolism.

5.2 Materials and methods

5.2.1 Genome sequence and annotation

Sulfuricella denitrificans skB26 and *Sulfuritalea hydrogenivorans* sk43H were anaerobically grown in the defined medium (7), containing 20 mM nitrate as an electron acceptor. As an electron donor, 20 mM thiosulfate and hydrogen gas were used for strains

skB26 and sk43H, respectively. Genomic DNA was extracted using AquaPure genomic isolation kit (Bio-Rad Laboratories; Hercules, CA). Methods for library construction, sequencing, and gap closing have been published previously (23). To fill the gaps left open, genomic fosmid libraries were constructed. Fosmid clones covering the gap regions were sequenced by using the Sanger's method. Finally, the complete genomes of strains skB26 and sk43H were obtained.

The genomes were annotated automatically using the Microbial Genome Annotation Pipeline (24). In the pipeline, genes for tRNAs and rRNAs were predicted by tRNAscan-SE and rRNAmmer, respectively. Open reading frames that are likely to encode proteins [coding sequence (CDS)] were predicted by using MetaGene Annotator, and the putative CDSs were annotated with the help of reference databases, including Reference Sequence (RefSeq), TrEMBL, and the Clusters of Orthologous Groups (COGs) databases. Further manual annotation and genome analysis were performed using IMC-GE software (In Silico Biology; Yokohama, Japan). The translations of the putative CDSs were searched again against the database of RefSeq database in GenBank using the BLASTP tool. The putative CDSs with more than 70% coverage and 35% identity, and an e -value less than 1×10^{-5} were retained. Wrongly predicted start sites were corrected based on multiple sequence alignments. Short putative CDSs (basically less than 300 bp) with no sequence similarity or overlap to longer one were eliminated. If a BLAST match was not returned, the longest model was retained. The revised CDS set was searched against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and then annotated. Clustered regularly interspaced short palindromic repeats (CRISPR) were identified with the CRISPR Recognition Tool (25). The candidate genes homologous to those involved in sulfur oxidation were identified by conducting a BLASTP search using the previously published draft genome sequence data of *Sulfuricella denitrificans* skB26 as query. The identified candidates were searched using

BLASTP against the RefSeq database, and normalized gene names were assigned for the candidates that shared more than 70% coverage and 35% identity, and an e -value less than 10^{-5} to the best BLAST hits.

5.2.2 Comparative genomics

The strains skB26 and sk43H complete genomes were compared with the genomes of the following betaproteobacteria: *Thiobacillus denitrificans* strains ATCC 25259 (NC_007404) and DSM 12475 (NZ_AQWL000000000), *Thiobacillus thioparus* DSM 505 (NZ_ARDU000000000), *Thiomonas intermedia* K12 (NC_014153), *Thiomonas* sp. 3As (NC_014145), *Advenella kashmirensis* WT 001 (NC_017964), *Sideroxydans lithotrophicus* ES-1 (NC_013959), *Burkholderiales* bacterium JOSHI_001 (NZ_AHLZ000000000), *Dechloromonas agitata* strains CKB (GOLD ID in IMG Database: Gi10479) and is5 (NCBI Project ID: 195888), and *Dechloromonas aromatica* RCB (NC_007298). Using the IMC-GE software, homologous genes involved in sulfur oxidation were identified by BLAST searches.

5.2.3 Plasmid preparation, restriction enzyme digestion, and pulsed-field gel electrophoresis

To confirm the presence of the plasmid, plasmid DNA was prepared from strain skB26 using a NucleoBond PC 20 plasmid DNA purification kit (Macherey-Nagel; Düren, Germany). One microgram DNA was incubated for 15 min at 37°C with a mixture containing 20 units of *Bam*HI-HF (New England Biolabs; Ipswich, MA) and 1× NE buffer. The total volume of the reaction was 50 µl. Digestion was stopped by incubating the reaction mixture at 65°C for 20 min. The digested DNA sample was loaded onto 1.5% Pulsed field Certified Agarose (Bio-Rad Laboratories, CA) gel in 0.5× Tris-borate-EDTA (TBE) buffer for electrophoresis. Pulsed-field gel electrophoresis (PFGE) was performed by clamped

homogeneous electric field (CHEF) electrophoresis for 15 h using the CHEF-DRII system (Bio-Rad Laboratories). The gel was subjected to electrophoresis in 0.5× TBE buffer (pH 8.3) at 6 V/cm and 13°C. The pulse time was ramped from 1 to 7 s. The gels were stained with ethidium bromide and photographed under ultraviolet light. The sizes of DNA fragments were estimated by comparison with molecular weight marker, CHEF DNA size standard 8–48 kb (Bio-Rad Laboratories).

5.2.4 Phylogenetic analysis

Reference sequences for phylogenetic analyses were obtained from GenBank and the Integrated Microbial Genomes (IMG) platform (26) developed by the DOE Joint Genome Institute (<http://img.jgi.doe.gov>). The sequences were aligned using T-COFFEE or ClustalW implemented in the eBioX software package version 1.5.1 (<http://www.ebioinformatics.org/ebiox/>). A phylogenetic tree was constructed using MEGA software version 5.05.

5.2.5 Fatty acid analysis

The cellular fatty acid profile of strain skB26 was constructed using cells grown in the defined medium containing thiosulfate and nitrate as an electron donor and acceptor, respectively. Fatty acid methyl esters were prepared and analyzed using the Sherlock Microbial Identification System (version 6; MIDI) according to the manufacturer's instructions.

5.2.6 Nucleotide sequence accession numbers

The complete genome sequence data of strains skB26 and sk43H have been deposited in the DDBJ/EMBL/GenBank database under accession numbers AP013066 and

AP013067 for strain skB26, and AP012547 for strain sk43H.

5.3 Results and discussion

5.3.1 Genome organization

The complete genome of strain sk43H was constructed as a single circular chromosome consisting of 3,802,648-bp by assembling sequence data from pyrosequencing and Sanger sequencing of PCR products and fosmid clones. As for strain skB26, an 86,619-bp plasmid was constructed in addition to a circular chromosome of 3,130,594-bp. The PFGE profile of the *Bam*HI-HF-digested plasmid suggested that three expected bands of 33-, 40-, and 13-kb were originated from about 86-kb circular plasmid (Fig. 5-1). A total of 2982 sequences in the skB26 chromosome, 91 in the skB26 plasmid, and 3587 in the sk43H chromosome were predicted as protein-coding genes. The position of nucleotide 1 was assigned to the predicted origin of replication, flanked on one side by the *dnaA*, *dnaN*, and *gyrB* genes. In the plasmid, *repA* gene was arbitrarily assigned as the first gene. Basic characteristics of the genomes are shown in Table 5-1. When the genes were classified into functional categories based on the COG database, the COG category with a great number of genes was ‘energy production’ (Table 5-2). A CRISPR locus with 15 repeats of a unique 37-bp sequence was found in the genome of strain sk43H. The sequence of the repeats was very similar to that of the CRISPR locus in the genome of the freshwater iron oxidizer *Acidovorax ebreus* TPSY, sharing 34-bp. The length of the spacers was 30-bp in most cases, but one of the spacers was 31-bp exceptionally. Three CRISPR-associated proteins (Cas2, Cas1, and Csn1: SUTH_01319–01321) were encoded adjacent to the CRISPR locus.

5.3.2 Phylogeny

BLAST searches matched 2681 chromosomal genes from skB26 and 3279 from sk43H to a database of RefSeq based on the criteria of genes with more than 70% coverage, 35% identity, and an e -value less than $1e^{-5}$. Analysis of the best BLAST hits for these genes established the taxonomic breakdown as shown in Table 5-3. More than half of the skB26 genes (1820) showed best BLAST hits to 21 betaproteobacteria. The top five of these bacteria were *Sideroxydans lithotrophicus* ES-1 (312 genes), *Thiobacillus denitrificans* ATCC 25259 (247 genes), *Nitrosospira multiformis* ATCC 25196 (236 genes), *Gallionella capsiferriformans* ES-2 (137 genes), and *Nitrosomonas* sp. Is79A3 (127 genes). These bacteria belong to three different families, namely *Gallionellaceae*, *Hydrogenophilaceae*, and *Nitrosomonadaceae*, which are all known as chemolithoautotrophs, as is strain skB26. In the sk43H genome, 2409 genes showed best BLAST hits to 33 betaproteobacteria. The top five of these bacteria were *Azospira suillum* PS (344 genes), *Dechloromonas aromatica* RCB (292 genes), *Accumulibacter phosphatis* UW-1 (261 genes), *Azoarcus* sp. KH32C (206 genes), and *Aromatoleum aromaticum* EbN1 (174 genes). These bacteria belong to the family *Rhodocyclaceae*, a physiologically versatile group encompassing bacteria with diverse functions. The results of the best BLAST hit analysis reflected their respective physiological features.

Phylogenetic analysis of the 16S rRNA gene sequence from representative betaproteobacteria revealed that the family *Rhodocyclaceae* formed five distinct clades, and strain sk43H was classified within *Rhodocyclaceae* group I (Fig. 5-2). Strain skB26 could not be assigned to any known family or order, and formed a clade distinct from the other betaproteobacteria.

I also carried out multi-locus sequence analysis (MLSA) of six concatenated conserved genes, *rpoA*, *rpoB*, *recA*, *gyrB*, *fusA*, and *ileS* (Fig. 5-3). As a result, strain sk43H fell into the cluster of *Rhodocyclaceae*, which included the genera of the *Rhodocyclaceae*

groups II and IV on the basis of 16S rRNA phylogeny. This result suggested that strain sk43H can be placed in the family *Rhodocyclaceae*. *Tepidiphilus margaritifer* of the family *Hydrogenophilaceae* was included in this cluster. Other members of the *Hydrogenophilaceae* (*Thiobacillus* spp. and strain skB26) were located at distant positions from *T. margaritifer* (Fig. 5-3), in accordance with the 16S rRNA-based phylogenetic tree (Fig. 5-2). These results indicated the necessity of reclassification of the order *Hydrogenophiales*. MLSA also confirmed the distinct phylogenetic position of strain skB26 from other betaproteobacteria and its clear separation from the genus *Thiobacillus*. Use of other algorithm to derive the phylogenetic tree (neighbor-joining) yielded the same tree topology (tree not shown). The unique phylogenetic position of strain skB26 suggested that it is representative of a novel family and order within the class *Betaproteobacteria*. Therefore, the designations *Sulfuricellales* ord. nov. and *Sulfuricellaceae* fam. nov. are proposed for the genus *Sulfuricella*. The fatty acid profile of strain skB26 was characterized by high concentration of summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c; 62.2%), C_{16:0} (23.5%), and summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c; 8.4%) (Table 5-4).

5.3.3 Genes for thiosulfate oxidation

Strains skB26 and sk43H were originally isolated from anoxic water samples of a freshwater lake using the defined medium with thiosulfate as an electron donor. Their genomes encode Sox proteins that catalyze the thiosulfate oxidation. To date the extensive *sox* clusters have been observed in *Alphaproteobacteria*, such as *soxRSVWXYZABCDEFGH* of *Paracoccus pantotrophus*. Seven out of the 15 genes (*soxXYZABCD*) encode proteins essential for the complete oxidation of thiosulfate to sulfate (27). In betaproteobacterial SOB, the largest *sox* gene cluster consists of *soxXYZAB* in all of the genome-sequenced *Thiobacillus* spp. (*T. thioparus* DSM 505, *T. denitrificans* strains ATCC 25259 and DSM

12475) and *Sideroxydans lithotrophicus*, *soxRCDYZAXB* in *Thiomonas* spp. and *Dechloromonas agitata*, and *soxCDYZAXWB* in *Advenella kashmirensis* (Table 5-5). The largest *sox* gene cluster in the genome of strain skB26 was identical to that of the genome-sequenced *Thiobacillus* spp. (*soxXYZAB*), and that of strain sk43H was *soxYZAXB*, which is identical to that of *Burkholderiales* bacterium JOSHI_001, whose sulfur-oxidizing ability has not been reported. The majority of *sox* gene containing-alpha-, epsilon-, gamma-, and betaproteobacteria harbor seven essential *sox* genes (28); however, strains skB26 and sk43H lack *soxCD*, encoding sulfur dehydrogenase, indicating that elemental sulfur is the final product. In *Allochromatium vinosum*, the elemental sulfur generated is enclosed in a protein envelope consisting of the sulfur globule proteins SgpA, SgpB, and SgpC (29). However, their homologous genes were not found in the genomes of *soxCD*-lacking betaproteobacterial SOB (Table 5-5). The lack of *sgp* genes is consistent with *Chlorobium tepidum*, which deposits sulfur globule outside the cells.

5.3.4 Genes for elemental sulfur oxidation

For the oxidation of elemental sulfur, both genomes harbor a gene cluster *dsrABEFHCMKLJOPNR* that is very similar to a cluster *dsrABEFHCMKLJOPNRS* in *A. vinosum*. The gene organization of this cluster is conserved in the genome-sequenced *Thiobacillus* spp., and a very similar cluster is also present in the genomes of *S. lithotrophicus* (*dsrABEFHCMKLJOPN*) and *Burkholderiales* bacterium JOSHI_001 (*dsrABEFHCMKxLJOPN*). In the class *Betaproteobacteria*, occurrence of the *dsr* gene cluster is restricted to *soxCD*-lacking SOB (Table 5-5). These betaproteobacteria generate sulfite via a reverse-acting dissimilatory sulfite reductase (DsrAB).

5.3.5 Betaproteobacteria-specific gene cluster putatively involved in the reverse Dsr

pathway

A gene cluster that is conserved among these betaproteobacteria was located immediately upstream of the *dsr* operon (Fig. 5-4A). Because a similar gene cluster is not found in other SOB genomes, this cluster was named betaproteobacteria-specific gene cluster (BSGC) in this study. All of BSGCs had four homologous genes in common, encoding hypothetical protein 1, CRISPR-associated protein Cas6, DsrC, and hypothetical protein 2.

The Cas 6 is one of the six well-conserved core Cas proteins, which are always found near the CRISPR locus (30). However, the sk43H *cas6* gene is located at a distant position from its CRISPR locus. Furthermore, the others of BSGC-harboring members listed in Fig. 5-4 have no CRISPR locus. Thus, the Cas6 encoded in BSGC may have a function different from that in the CRISPR system, as an endoribonuclease generating CRISPR-derived guide RNAs for invader defense in prokaryotes (31).

Although *dsrC* is replicated multiple times (up to 8 times) in each genome of *dsrAB*-harboring betaproteobacteria (Table 5-5), DsrC encoded in BSGC formed a phylogenetic cluster distinct from the others (Fig. 5-4B). These DsrC sequences lack the carboxy-terminal cysteine residues that have a central importance in a postulated function of DsrC encoded in the *dsr* operon (32). Compared to DsrC encoded in the *dsr* operon, DsrC encoded in BSGC is more similar to TusE of *Escherichia coli* (data not shown). Among the 8 *dsrC* copies present in *T. denitrificans*, transcription of Tbd_2488 in BSGC was previously described to be most upregulated when oxidizing thiosulfate compared to when oxidizing Fe(II) (19). This observation indicated that the DsrC encoded in BSGC plays a role in sulfur oxidation process.

The gene for hypothetical protein 1 is found only in *Alphaproteobacteria* and *Ectothiorhodospiraceae*, and the gene for hypothetical protein 2 is found only in *Chromatiaceae*, *Ectothiorhodospiraceae*, *Thiothrix*, and sulfur-oxidizing

gammaproteobacterial symbionts. All of these gamma- and alphaproteobacteria are *dsrAB*-harboring SOB (the only one exception is *Ectothiorhodospira* sp. PHS-1).

From the restricted distribution of BSGC and that of the hypothetical proteins, it appears that the proteins encoded in BSGC have restricted importance in *dsrAB*-harboring SOB, and may be involved in the reverse Dsr pathway. Homologous genes encoding taurine catabolism dioxygenase are also found in BSGC (only strain skB26 encodes the homologous gene at a distant position: SCD_n00462). Recently, these homologous genes have been designated *dsrQ*, and are postulated to catalyze the release of sulfite during the oxygenolytic breakdown of sulfonates (1).

5.3.6 Genes for sulfite oxidation

For the oxidation of sulfite to sulfate as the end product, strains skB26 and sk43H employ the same genetic machinery in two different pathways. One is an indirect pathway in which, adenosine-5'-phosphosulfate (APS) is formed as an intermediate and is catalyzed by cytoplasmic APS reductase (AprBA) and ATP sulfurylase (Sat), which are encoded by a gene cluster, *sat-aprBA*. The AprBA sequences of SOB diverge into two distinct lineages, described by Meyer and Kuever as *apr* lineages I and II (33). A previously reported phylogenetic analysis of AprBA revealed that *apr* lineage I is the 'authentic' SOB lineage, and that *apr* lineage II is laterally transferred genes from SRPs. SRP-related *apr* genes (*apr* lineage II) are present in some members of the *Chlorobiaceae*, *Gammaproteobacteria*, and *Thiobacillus*. Several of these gammaproteobacteria and *Thiobacillus* spp. also possess the 'authentic' *apr* genes, and this two-gene state is thought to be an intermediate during the replacement (33). Strains skB26 and sk43H possess only SRP-related *apr* genes.

Lineage II *aprBA*-harboring SOB also contain an *hdr* gene cluster and *sat*, which are thought to have been acquired from SRPs as well as *aprBA*. The genomes of strains skB26

and sk43H harbor a *hdrA1A2CB* gene cluster located at a distant position from the *sat-aprBA* gene cluster (Fig. 5-5). Their HdrA1 and HdrA2 sequences share greater than 50% identity with QmoA and QmoB of SRPs, respectively (data not shown). In SOB, the *hdrA1A2CB* cluster is found only in the genome-sequenced *Thiobacillus* spp., but another gene cluster *qmoABhdrCB* is conserved in several SRPs, such as deltaproteobacterium NaphS2, *Desulfarculus baarsii*, and *Desulfurispora thermophila*. The postulated SRP origin of the *sat-aprBA* and *hdrA1A2CB* loci in the betaproteobacteria is supported by the restricted distribution of these loci among the SOB, and the close relationship of these gene organizations between the betaproteobacterial SOB and the aforementioned SRPs (Fig. 5-5). Although the role of the Hdr complex remains unclear, its expression has been observed in strain skB26 grown on thiosulfate (23). By analogy with the postulated interaction of DsrAB and transmembrane complex DsrMKJOP via the conserved cysteine residues of DsrC, it has been proposed that the SRP-related reverse APS reductase and the QmoAB-HdrCB complex couple with the cytoplasmic sulfite/APS redox process to the electron transport chain via reversible disulfide/thiol exchanges (33). Actually, *hdr* gene cluster of betaproteobacterial SOB is only found in the lineage II *aprBA*-harboring members, and other candidate electron accepting unit for AprBA, such as AprM, was not identified in the genomes of strains skB26 and sk43H (Table 5-5).

The second sulfite-oxidation pathway is direct pathway catalyzed by cytoplasmic sulfite oxidizing enzymes (SoeABC). In the *Betaproteobacteria*, the distribution of the *aprBA* gene locus is restricted to *Thiobacillus* spp. and *S. lithotrophicus*, whereas the *soeABC* gene locus is conserved in *Thiomonas* spp., *Burkholderiales* sp. JOSHI_001, and *aprBA*-harboring SOB (Table 5-5). As pointed out by Dahl et al. (34), the *soeABC* locus is more widely distributed in the *Betaproteobacteria* than the APS reductase and extracytoplasmic sulfite oxidizing enzymes SorAB (Table 5-5).

The previous proteomic analysis of strain skB26 confirmed a greater amount of AprBA and Sat than SoeABC during chemolithoautotrophic growth on thiosulfate (23). Transcriptional analysis of *Thiobacillus denitrificans* ATCC 25259 also showed high expression of *sat-aprBA* (35). These findings indicated that sulfite is oxidized by the indirect pathway (AprBA and Sat) in these obligate sulfur chemolithoautotrophs. The combination of the Dsr system and the indirect pathway (AprBA and Sat) appears to have significant energetic advantages for chemolithotrophic growth (33). This sulfur oxidation pathway may also be a feature of SOB living in freshwater lake environments, because the available amount of reduced sulfur compounds is limited in such environments, which differs from that found in acid mine drainage where *Thiomonas* spp. are ubiquitous.

5.3.7 Genes for sulfide oxidation

Although the previous attempts to grow strains skB26 and sk43H on sulfide were unsuccessful, both strains have two catalytic systems for sulfide oxidation. One is a flavocytochrome *c*, consisting of a di-heme cytochrome *c* subunit (SoxE) and a flavin-binding subunit SoxF catalyzing the oxidation of sulfide to sulfur or polysulfides using SoxE as electron acceptors (36). The other is *sqr*, which encodes sulfide:quinone oxidoreductase (37).

5.3.8 Differences in the genetic components involved in sulfur oxidation among obligate and facultative sulfur chemolithotrophs

Strain skB26 is an obligate sulfur oxidizer (6), whereas strain sk43H is a facultative sulfur oxidizer which can also grow on hydrogen gas or carboxylic acids (7). Their genetic components for sulfur oxidation were slightly different from each other. The *sox* genes in both genomes do not occur in one cluster, but in different parts of the genomes. In addition to

the largest *sox* gene cluster (*soxYZAXB*), strain sk43H harbors an additional cluster, *soxXAYZ*, at a distant position from the other *sox* genes (Table 5-5). At present, four distinct phylogenetic groups of SoxAX are known: Type I–IV (38). Phylogenetic analysis revealed that translated *soxA* sequences in *soxYZAXB* and *soxXAYZ* of strain sk43H belong to the Type II and IV groups, respectively (tree not shown). In the *Betaproteobacteria*, SoxA sequences of the facultative sulfur chemolithotrophs *Advenella kashmirensis*, *Thiomonas* spp., and *Dechloromonas agitata* CKB are classified into the Type II group, indicating that the Type II SoxAX of strain sk43H is ‘canonical’ (Table 5-6). The Type IV group consists of members of the class *Aquificae*, *Epsilonproteobacteria*, and *Gammaproteobacteria* and *Thiobacillus denitrificans* DSM 12475 (38). The restricted distribution of Type IV SoxAX in the *Betaproteobacteria* suggests lateral gene transfer.

The comparative genomic analysis of 8 betaproteobacteria revealed the presence of genetic features shared among strain skB26 and the genome-sequenced *Thiobacillus* spp., which are obligately dependent on reduced sulfur compounds as energy source. In contrast to facultative sulfur chemolithotrophs, the skB26 SoxA sequence belongs to the Type III group. In the *Betaproteobacteria*, Type III SoxAX is found only in obligate chemolithotrophs *Thiobacillus* spp. and *Sideroxydans lithotrophicus* (Table 5-6). In most cases members of the Type III group harbor a homologous gene encoding putative SoxAX binding protein (SoxK) (39). The strain skB26 genome also contains *soxK*. The distribution of *soxK* among the *Betaproteobacteria* is strictly restricted to strain skB26 and the genome-sequenced *Thiobacillus* spp. (Table 5-5 and data not shown). It has been demonstrated that SoxK is required for efficient thiosulfate oxidation through the formation of a tight complex with SoxA and SoxX in *Chlorobium tepidum* (39).

Strain skB26 harbors *dsrS*, which is not part of the *dsr* gene cluster, as is the case in genome-sequenced *Thiobacillus* spp. (Table 5-5 and data not shown). The homologous gene

of *dsrS* is not present in the other betaproteobacterial genomes. Although the function of DsrS remains unknown, the previous proteomic analysis of strain skB26 detected a relatively small amount of DsrS in comparison with Dsr proteins encoded in *dsrABEFHCMKLJOPNR* (Chapter 4). It has also been reported that the presence of *dsrS* is not required for sulfur oxidation, but transcription of the *A. vinosum dsrS* gene increased under sulfur-oxidizing conditions (40).

Common in the genome of *T. denitrificans* ATCC 25259, the genes encoding DsrA, Sqr, and Sat occur more than once in the strain skB26 genome, while those of strain sk43H occur only once (Table 5-5). As listed in Table 5-5, a total of 54 genes in strain skB26 and 60 genes in *T. denitrificans* ATCC 25259 are putatively involved in dissimilatory sulfur oxidation. These numbers are higher than that for other betaproteobacterial SOB, including strain sk43H, underscoring the importance of sulfur oxidation for these obligate sulfur chemolithoautotrophs.

5.3.9 Genes for carbon fixation

Both strains skB26 and sk43H contain genes encoding key enzymes in the Calvin-Benson-Bassham cycle, i.e., ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) and phosphoribulokinase. Strain sk43H has a gene encoding a large subunit of RubisCO, referred to as Form II, in a *cbbMQO2* cluster (SUTH_00757–00759). This cluster is identical in gene order to that of strain skB26 (SCD_n02850–02852). In addition, strain skB26 possesses a gene encoding a second large subunit of RubisCO, referred to as Form I, in a *cbbLSQO* cluster (SCD_n02031–02034). The second large subunit of RubisCO is in the Form IAq group (41). This type of RubisCO is not known to be associated with the presence of carboxysomes, which is consistent with the lack of evidence for the presence of carboxysome genes in strain skB26.

Carboxylase activity of Form I RubisCO is higher than that of Form II RubisCO in the presence of oxygen. This would allow strain skB26 to fix CO₂ at higher O₂ pressures than strain sk43H, which possesses only Form II RubisCO. Indeed, chemolithoautotrophic growth of strain sk43H has been observed only under anoxic conditions (7). Consistent with these observations, the bacterial 16S rRNA clone library from the water column of Lake Mizugaki suggested that the relative abundance of the genus *Sulfuritalea* is greater in anoxic zones (H. Kojima, T. Watanabe, T. Iwata, M. Fukui, submitted for publication). In Lake Pavin, a stratified freshwater lake, 16S rRNA clones closely related to strain sk43H (>97% sequence identities) have been detected only in the anoxic hypolimnion (14). These findings are also indicative of the adaptation of strain sk43H to anoxic conditions. Thus, for this facultative autotroph, it may not be advantageous to possess form I RubisCO, which requires additional subunits to sustain carbon fixation in the presence of oxygen.

5.3.10 Putative genes for arsenic metabolism

A notable genomic feature of strain skB26 is the presence of a circular plasmid. The plasmid was similar to the chromosome in GC content (Table 5-1) as well as in codon usage (data not shown). Of the genes located on the plasmid, BLAST searches matched 78 to the database of RefSeq based on the above-mentioned criteria. Only 11 genes showed the best BLAST hits to the chromosomal genes of strain skB26. Discounting the skB26 chromosomal genes, the best BLAST hits of 33 genes were gammaproteobacteria and those of 32 genes were betaproteobacteria. A large percentage of genes present in the plasmid (20.1%) belonged to the category of energy production and conversion (Table 5-2). Among these, an arsenate reductase-like gene (SCD_n03116) was found. However, phylogenetic analysis showed a closer relationship of SCD_n03116 with a new clade of anaerobic arsenite oxidase, recently designated as ArxA by Zargar et al. (42), than arsenate reductase (Fig. 5-6A). The *arxA*-like

gene SCD_n03116 is located in a cluster that appears to be syntenic with *Alkalilimnicola ehrlichii* MLHE-1 and *Ectothiorhodospira* sp. PHS-1 (Fig. 5-6B). Strain MLHE-1 is the only known ArxA-dependent anaerobic arsenite oxidizer (42), and strain PHS-1 is capable of anoxygenic photosynthesis using arsenite, most likely catalyzed by ArxA (43). At present, 11 ArxA-like sequences from 4 gammaproteobacterial genera cluster with ArxA sequences of strains MLHE-1 and PHS-1 (Fig. 5-6A). These gammaproteobacteria are haloalkaliphilic (one of them is slightly halophilic), and were isolated from arsenic-rich environments. SCD_n03116 is the first report of an *arxA*-like sequence outside of the *Gammaproteobacteria*. It is clear that the *arx*-like cluster located on the plasmid was acquired by horizontal transfer. This finding suggests that plasmids play a key role in the horizontal transfer of the *arxA* gene among proteobacteria. It has been demonstrated that ArxA from *A. ehrlichii* exhibits both arsenite oxidase and arsenate reductase activities, similar to ArrA from the arsenate reducers *Alkaliphilus oremlandii* and *Shewanella* sp. ANA-3 (44). The translated sequence of SCD_n03116 has a unique 105-amino acid insertion between positions 338 and 444 (Fig. 5-6C). Further experiments are required to determine the precise role of the ArxA-like protein with this unique signature.

Some genes for arsenic metabolisms were found in the chromosomes of strains skB26 and sk43H. Strain sk43H carries the genetic machinery for arsenic resistance, i.e., *arsR* (regulatory element), *arsC* (arsenate reductase), and *arsB* (arsenite-specific-efflux pump) (SUTH_00386–00388), in addition to the genes for respiratory arsenate reduction (*arrAB*: SUTH_00437–00438). In the chromosome of strain skB26, *arsR* (SCD_n00140), *arsC* (SCD_n00657), and *arsB* (SCD_n01458 and n02155) were also found, in addition to the gene for S-adenosylmethionine methyltransferase (SCD_n02152) involved in arsenite resistance through methylation and subsequent volatilization.

5.4 Conclusion

Despite the distant phylogenetic positions of strains skB26 and sk43H from one another (Fig. 5-3), their deduced sulfur oxidation pathways are identical (Fig. 5-7). This pathway is also shared by the other freshwater SOB, *Thiobacillus* spp. and *S. lithotrophicus*, and is different from those of the *soxCD*-harboring betaproteobacteria *Thiomonas* spp. and *Advenella kashmirensis*. The previous cloning analysis (Chapter 3) suggests that the candidate sulfur-oxidizing betaproteobacteria without SoxCD dominate the *sqr* and *soxB* gene libraries that were constructed from anoxic water samples of Lake Mizugaki. Therefore, complete thiosulfate or sulfide oxidation via the cytoplasmic enzyme system (Dsr, Apr, Sat) may be a feature of SOB living in freshwater lake environments. As the first comprehensive comparative genomic analysis of betaproteobacterial SOB, the results of this study suggest that: (1) BSGC located immediately upstream of the *dsr* cluster may involve in the reverse Dsr pathway, (2) the betaproteobacteria-specific *hdrA1A2CB* locus was most likely acquired from SRP together with the *sat-aprBA* gene locus, supporting the postulated functional relatedness of Hdr and Apr in analogy with that of the Dsr components, and (3) distribution of *soxK* and *dsrS* in the *Betaproteobacteria* is strictly restricted to obligate sulfur chemolithoautotrophs. Further experiments using pure cultures of strains skB26 and sk43H will contribute to demonstrate these suggested features.

In addition to the sets of arsenic metabolism genes present in the chromosomes of strains skB26 and sk43H, I also described an *arx*-like operon that is located on the skB26 plasmid. Sulfur and arsenic often coexist in the environment and undergo similar microbial redox transformations. However, the geochemical environment from which strain skB26 was isolated (Lake Mizugaki) (45) is completely different from those of *Alkalilimnicola ehrlichii* and *Ectothiorhodospira* sp. PHS-1 (Mono Lake and Paoha Island hot spring, respectively) (46,

47). The *arxA*-like sequences may be widely distributed in diverse geochemical environments. Further culture-independent and -dependent approaches should address the phylogenetic and functional relationships of *arxA*-like sequences within freshwater lake environments, as well as pure culture studies of strain skB26.

5.5 Taxonomic description

Description of *Sulfuricellales* ord. nov.

Sulfuricellales (Sul.fu.ri.cel.la'les. N.L. fem. n. *Sulfuricella* type genus of the order; -ales ending to denote order; N.L. fem. pl. n. *Sulfuricellales* the order of the genus *Sulfuricella*)

The description has been previously published Kojima and Fukui (6). The family is a member of the class *Betaproteobacteria*. The type genus is *Sulfuricella*.

Description of *Sulfuricellaceae* fam. nov.

Sulfuricellaceae (Sul.fu.ri.cel.la'ce.ae. N.L. fem. n. *Sulfuricella* type genus of the family; -aceae ending to denote family; N.L. fem. pl. n. *Sulfuricellaceae* the family of the genus *Sulfuricella*)

The description is the same as for the order *Sulfuricellales*. The family is a member of the order *Sulfuricellales*. Type genus is *Sulfuricella*.

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Table 5-1. Basic characteristics of the strains skB26 and sk43H genomes.

Feature	skB26		sk43H
	Chromosome	Plasmid	Chromosome
Size (bp)	3,130,594	86,619	3,802,648
GC content (%)	56.1	56.7	63.3
No. of CDSs	2982	91	3587
Total CDS length (bp)	2,846,291	76,284	3,530,988
CDS density (%)	90.9	88.1	92.9
No. of rRNA operons	2	0	2
No. of tRNA genes	45	0	44
No. of genes assigned to KEGG	1566	30	1791
No. of genes assigned to COGs	3246	82	2669
CRISPR count	0	0	1

Table 5-2. Classification of genes into COG functional categories.

Functional category	skB26 (%)		sk43H (%)
	Chromosome	Plasmid	Chromosome
General function prediction only	9.89	10.98	11.18
Not in COGs	10.50	0	9.51
Energy production and conversion	7.32	20.12	8.25
Function unknown	7.88	7.32	7.92
Signal transduction mechanisms	7.07	4.27	7.35
Amino acid transport and metabolism	5.36	2.44	6.51
Cell wall/membrane/envelope biogenesis	7.38	5.49	5.32
Translation, ribosomal structure and biogenesis	5.82	1.22	4.72
Transcription	3.56	7.32	4.71
Inorganic ion transport and metabolism	5.47	9.76	4.57
Replication, recombination and repair	4.11	6.71	4.47
Posttranslational modification, protein turnover, chaperones	4.71	8.54	4.41
Lipid transport and metabolism	1.95	2.44	4.05
Coenzyme transport and metabolism	3.73	4.27	3.67
Carbohydrate transport and metabolism	3.60	0.61	3.05
Intracellular trafficking, secretion, and vesicular transport	2.91	2.44	2.27
Secondary metabolites biosynthesis, transport and catabolism	1.16	1.22	2.12
Cell motility	2.34	0	1.99
Nucleotide transport and metabolism	2.00	1.22	1.40
Defense mechanisms	1.78	2.44	1.34
Cell cycle control, cell division, chromosome partitioning	1.32	1.22	1.07
Chromatin structure and dynamics	0.05	0	0.06
RNA processing and modification	0.03	0	0.06
Cytoskeleton	0.07	0	0
Extracellular structures	0	0	0
Nuclear structure	0	0	0

Table 5-3. Phylogenetic classification of the Best BLAST hits for 2681 chromosomal genes from skB26 and 3279 from sk43H matched to a database of RedSeq (see text for the criteria).

Class	Number of genes	
	skB26	sk43H
<i>Betaproteobacteria</i>	2130	2788
<i>Gammaproteobacteria</i>	325	246
<i>Alphaproteobacteria</i>	45	88
<i>Delta/Epsilonproteobacteria</i>	78	71
others	103	86

Table 5-4. Cellular fatty acid content (percentage of total) of strain skB26 and selected members of the family *Hydrogenophilaceae*.

Strains: 1, skB26^T (this study); 2, *Tepidiphilus margaritifer* N2-214^T (Manaia et al., 2003); 3, *Thiobacillus denitrificans* ATCC 23644^T (Katayama-Fujimura et al., 1982); 4, *Thiobacillus thioparus* ATCC 8158^T (Katayama-Fujimura et al., 1982); 5, *Hydrogenophilus islandicus* 16C^T (Vésteinsdóttir et al., 2011); *Hydrogenophilus hirshii* DSM 11420^T (Stöhr et al., 2001).

Fatty acid	1	2	3	4	5	6
C _{8:0} 3-OH	2.4	-	-	-	-	-
C _{10:0}	-	-	tr	3	0.4	NA
C _{10:0} 3-OH	-	1.4	-	-	2.96	2.58
C _{12:0}	-	-	4	6	1.95	1.66
C _{13:1} at 12-13	0.12	-	-	-	-	-
C _{14:0}	0.36	2.7	tr	tr	2.35	2.49
C _{15:0}	-	-	1	1	-	-
Summed feature 3	62.24	-	-	-	-	-
Summed feature 4	-	3.7	-	-	9.89	3.12
C _{16:1}	-	-	29	8	-	-
C _{16:1} ω5c	1.4	-	-	-	-	-
C _{16:0}	23.52	36.8	44	41	44.98	55.34
C _{16:0} 3-OH	0.25	2.9	-	-	-	-
C _{17:0}	-	-	-	1	-	-
cyclo-C _{17:0}	-	6.2	17	34	13.9	17.92
Summed feature 7	-	15.6	-	-	-	-
C _{18:0}	-	-	tr	2	-	-
C _{18:1}	-	-	3	3	-	-
C _{18:1} ω9c	0.89	-	-	-	-	-
C _{18:1} ω7c	-	-	-	-	17.93	2.27
Summed feature 8	8.35	-	-	-	-	-
C _{18:1} ω5c	0.13	-	-	-	-	-
C _{18:0}	0.35	1.6	-	-	0.98	1.17
cyclo-C _{19:0} (ω8c)	-	25.9	-	-	4.67	12.98

Summed feature 3: C_{16:1} ω7c and/or C_{16:1} ω6c

Summed feature 4: C_{16:1} ω7c and/or iso-C₁₅ 2-OH

Summed feature 7: C_{18:1} ω7c, ω9t and/or ω12t

Summed feature 8: C_{18:1} ω7c and/or C_{18:1} ω6c

tr: trace amount (less than 1%)

NA: No data available

Table 5-4. *continued.*

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Stöhr R, Waberski A, Liesack W, Völker H, Wehmeyer U, Thomm M. 2001. *Hydrogenophilus hirschii* sp. nov., a novel thermophilic hydrogen-oxidizing beta-proteobacterium isolated from Yellowstone National Park. Int. J. Syst. Evol. Microbiol. **51**: 481-488

Vésteinsdóttir H, Reynisdóttir DB, Örlygsson J. 2011. *Hydrogenophilus islandicus* sp. nov., a thermophilic hydrogen-oxidizing bacterium isolated from an Icelandic hot spring. Int. J. Syst. Evol. Microbiol. **61**: 290–294

Table 5-5. Sulfur oxidation-related genes identified in the genomes of selected betaproteobacteria.

Organism		<i>S. denitrificans</i> skB26	<i>S. hydrogenivorans</i> sk43H	<i>T. denitrificans</i> ATCC 25259	<i>A. kashmirensis</i> WT 001	<i>T. intermedia</i> K12	<i>Thiomonas</i> sp. 3As	<i>S. lithotrophicus</i> ES-1	<i>Burkholderiales</i> sp. JOSHI_001
<i>sqr</i> (alone)	<i>sqr</i>	SCD_n00788	SUTH_02888	Tbd_2225	-	Tint_2084	THI_2411	Slit_1728	BurJ1DRAFT_3328
		SCD_n00790	-	Tbd_1407	-	-	-	Slit_1145	-
<i>sox</i> (largest cluster)	<i>soxX</i>	SCD_n02412	SUTH_02729	Tbd_0567	TKWG_08960-08995: <i>soxCDYZAXWB</i>	Tint_2301-2294: <i>soxRCDYZAXB</i>	THI_2674-2667: <i>soxRCDYZAXB</i>	Slit_1700	BurJ1DRAFT_3352
	<i>soxY</i>	SCD_n02411	SUTH_02726	Tbd_0566				Slit_1699	BurJ1DRAFT_3349
	<i>soxZ</i>	SCD_n02410	SUTH_02727	Tbd_0565				Slit_1698	BurJ1DRAFT_3350
	<i>soxA</i>	SCD_n02409	SUTH_02728	Tbd_0564				Slit_1697	BurJ1DRAFT_3351
	<i>soxB</i>	SCD_n02408	SUTH_02730	Tbd_0563	-	-	-	Slit_1696	BurJ1DRAFT_3353
<i>sox</i> (cluster)	<i>soxE</i>	SCD_n00381	SUTH_01080	Tbd_2034	-	Tint_2898	THI_3461	-	BurJ1DRAFT_0865
					-	Tint_2078	THI_2405	-	-
	<i>soxF</i>	SCD_n00382	SUTH_01079	Tbd_2035	-	Tint_2899	THI_3462	-	BurJ1DRAFT_0866
					-	Tint_2079	THI_2406	-	-
<i>sox</i> (cluster)	<i>soxX</i>	-	SUTH_03438	-	-	-	-	-	BurJ1DRAFT_0774
	<i>soxA</i>	-	SUTH_03439	-	-	-	-	-	BurJ1DRAFT_0777
	<i>soxY</i>	-	SUTH_03440	-	-	-	-	-	BurJ1DRAFT_0775
	<i>soxZ</i>	-	SUTH_03441	-	-	-	-	-	BurJ1DRAFT_0776
<i>sox</i> (cluster)	<i>soxX</i>	-	-	Tbd_0917	-	-	-	-	-
	<i>soxA</i>	-	-	Tbd_0918	-	-	-	-	-
<i>sox</i> (alone)	<i>soxE</i>	SCD_n02934	SUTH_00334	Tbd_2027	TKWG_23985	Tint_0653	THI_0840	Slit_1877	BurJ1DRAFT_4397
		SCD_n02935	SUTH_01078	Tbd_0187	TKWG_13705	Tint_2893	THI_3455	-	BurJ1DRAFT_0165
		SCD_n00334	SUTH_02821	Tbd_0064	-	-	-	-	-
		SCD_n00367	-	-	-	-	-	-	-
	<i>soxK</i>	SCD_n01244	-	Tbd_1753	-	-	-	-	-
					-	-	-	-	-
	<i>soxH</i>	SCD_n00859	-	Tbd_1041	TKWG_22645	Tint_2995	THI_3546	-	BurJ1DRAFT_2133
		SCD_n01426	-	Tbd_1103	-	Tint_3153	THI_3253	-	BurJ1DRAFT_3037
		SCD_n02222	-	-	-	Tint_3155	-	-	BurJ1DRAFT_2773
	<i>soxW</i>	SCD_n00830	SUTH_03095	Tbd_2117	-	Tint_0828	THI_1072	-	-
		SCD_n02799	-	Tbd_2569	-	-	-	-	-
<i>dsr</i> (cluster)	<i>dsrA</i>	SCD_n02718	SUTH_01444	Tbd_2485	-	-	-	Slit_1686	BurJ1DRAFT_3336
	<i>dsrB</i>	SCD_n02717	SUTH_01443	Tbd_2484	-	-	-	Slit_1685	BurJ1DRAFT_3335
	<i>dsrE</i>	SCD_n02716	SUTH_01442	Tbd_2483	-	-	-	Slit_1684	BurJ1DRAFT_3334
	<i>dsrF</i>	SCD_n02715	SUTH_01441	Tbd_2482	-	-	-	Slit_1683	BurJ1DRAFT_3333
	<i>dsrH</i>	SCD_n02714	SUTH_01440	Tbd_2481	-	-	-	Slit_1682	BurJ1DRAFT_3332
	<i>dsrC</i>	SCD_n02713	SUTH_01439	Tbd_2480	-	-	-	Slit_1681	BurJ1DRAFT_3331
	<i>dsrM</i>	SCD_n02712	SUTH_01438	Tbd_2479	-	-	-	Slit_1680	BurJ1DRAFT_3330
	<i>dsrK</i>	SCD_n02711	SUTH_01437	Tbd_2478	-	Tint_2963	THI_3514	Slit_1679	BurJ1DRAFT_3329
	<i>dsrL</i>	SCD_n02710	SUTH_01436	Tbd_2477	TKWG_03095	Tint_0696	THI_0885	Slit_1678	BurJ1DRAFT_3327
	<i>dsrJ</i>	SCD_n02709	SUTH_01435	Tbd_2476	-	-	-	Slit_1677	BurJ1DRAFT_3326
	<i>dsrO</i>	SCD_n02708	SUTH_01434	Tbd_2475	-	Tint_3054	THI_1629	Slit_1676	BurJ1DRAFT_3325
	<i>dsrP</i>	SCD_n02707	SUTH_01433	Tbd_2474	-	-	-	Slit_1675	BurJ1DRAFT_3324
	<i>dsrN</i>	SCD_n02706	SUTH_01432	Tbd_2473	-	-	-	Slit_1674	BurJ1DRAFT_3323
	<i>dsrR</i>	SCD_n02705	SUTH_01431	Tbd_2472	TKWG_06410	Tint_1059	THI_1342	-	-
<i>dsr</i> (alone)	<i>dsrA</i>	SCD_n02074	-	Tbd_1369	-	-	-	-	-
				Tbd_1309	-	-	-	-	-
	<i>dsrC</i>	SCD_n00996	SUTH_00991	Tbd_2326	-	-	-	Slit_2831	BurJ1DRAFT_3339
		SCD_n01903	SUTH_02986	Tbd_1408	-	-	-	Slit_1131	-
		SCD_n02062	SUTH_01448	Tbd_2327	-	-	-	Slit_1248	-
		SCD_n02721	-	Tbd_2488	-	-	-	Slit_1495	-
		SCD_n01952	-	Tbd_1926	-	-	-	Slit_1689	-
				Tbd_1365	-	-	-	-	-
				Tbd_2658	-	-	-	-	-
	<i>dsrE</i>	SCD_n00144	SUTH_00979	Tbd_2483	-	-	-	-	-
		SCD_n02573	-	-	-	-	-	-	-
	<i>dsrS</i>	SCD_n02932	-	Tbd_2558	-	-	-	-	-
	<i>dsrQ</i>	SCD_n00462	SUTH_01450	Tbd_2491	-	-	-	Slit_1692	BurJ1DRAFT_3341
<i>hdr</i> (cluster)	<i>hdrA1</i>	SCD_n01884	SUTH_02766	Tbd_1648	-	-	-	-	-
	<i>hdrA2</i>	SCD_n01883	SUTH_02767	Tbd_1647	-	-	-	-	-
	<i>hdrC</i>	SCD_n01882	SUTH_02768	Tbd_1646	-	-	-	-	-
	<i>hdrB</i>	SCD_n01881	SUTH_02769	Tbd_1645	-	-	-	-	-
<i>soe</i> (cluster)	<i>soeA</i>	SCD_n01209	SUTH_02761	Tbd_0570	-	Tint_1304	THI_1628	Slit_1711	BurJ1DRAFT_3313
	<i>soeB</i>	SCD_n01210	SUTH_02760	Tbd_0571	-	Tint_1305	THI_1629	Slit_1710	BurJ1DRAFT_3312
	<i>soeC</i>	SCD_n01211	SUTH_02759	Tbd_0572	-	Tint_1306	THI_1630	Slit_1709	BurJ1DRAFT_3311
<i>sor</i> (cluster)	<i>sorA</i> (1)	-	-	-	TKWG_16985	Tint_1468	THI_1866	-	-
	<i>sorA</i> (2)	-	-	-	TKWG_06475	Tint_2510	THI_2909	-	-
	<i>sorA</i> (3)	-	-	-	TKWG_22140	-	-	-	-
	<i>sorB</i> (1)	-	-	-	TKWG_16980	Tint_1467	THI_1865	-	-
	<i>sorB</i> (2)	-	-	-	TKWG_06470	Tint_2511	THI_2910	-	-
	<i>sorB</i> (3)	-	-	-	TKWG_22135	-	-	-	-
<i>apr</i> lineage I (cluster)	<i>aprM</i>	-	-	Tbd_2284	-	-	-	Slit_1713	-
	<i>aprB</i>	-	-	Tbd_2283	-	-	-	Slit_1714	-
	<i>aprA</i>	-	-	Tbd_2282	-	-	-	Slit_1715	-
<i>apr</i> lineage II (cluster)	<i>sat</i>	SCD_n01725	SUTH_02773	Tbd_0874	-	-	-	-	-
	<i>aprB</i>	SCD_n01726	SUTH_02772	Tbd_0873	-	-	-	-	-
	<i>aprA</i>	SCD_n01727	SUTH_02771	Tbd_0872	-	-	-	-	-
<i>sat</i> (alone)	<i>sat</i>	SCD_n02987	-	Tbd_0210	TKWG_15270	Tint_0316	THI_0352	*Slit_1712	BurJ1DRAFT_2207
					-	-	-	Slit_1035	-
APAT (alone)	APAT	-	-	Tbd_0601	-	-	-	-	-
Total gene number		54	45	60	20	31	30	35	36

* Slit_1712 is in a cluster *aprMBA*.

Table 5-6. SoxAX types in the class *Betaproteobacteria*. The classification was based on the SoxA sequences. Sulfur oxidizers are colored to represent either obligate (green) or facultative (blue) chemolithotrophs. Sulfur-oxidizing ability of the others has not been reported.

Type	Accession no./ Locus tag	Organism
I	YP_001789103.1	<i>Leptothrix cholodnii</i> SP-6 (1)
	BurJ1DRAFT_0777	<i>Burkholderiales</i> bacterium JOSHI_001 (2)
	YP_001155934.1	<i>Polynucleobacter necessarius</i> subsp. <i>asymbioticus</i> QLW-P1DMWA-1 (2)
	YP_003981441.1	<i>Achromobacter xylosoxidans</i> A8
	ZP_09481398.1	<i>Advenella kashmirensis</i> WT 001
	BurJ1DRAFT_3351	<i>Burkholderiales</i> bacterium JOSHI_001 (1)
	EHN65667.1	<i>Comamonas testosteroni</i> ATCC 11996
	YP_003278911.1	<i>Comamonas testosteroni</i> CNB-2
	ZP_03542526.1	<i>Comamonas testosteroni</i> KF-1
	ZP_07043457.1	<i>Comamonas testosteroni</i> S44
	YP_585561.1	<i>Cupriavidus metallidurans</i> CH34
	YP_004687290.1	<i>Cupriavidus necator</i> N-1
	YP_002007001.1	<i>Cupriavidus taiwanensis</i> LMG 19424
	CKB_00005700	<i>Dechloromonas agitata</i> CKB
	K420DRAFT_1378	<i>Dechloromonas agitata</i> is5
	YP_286330.1	<i>Dechloromonas aromatica</i> RCB
	YP_001099497.1	<i>Hermiimonas arsenicoxydans</i>
	BAF34123.1	<i>Hydrogenophilus thermoluteolus</i>
	YP_001352141.1	<i>Janthinobacterium</i> sp. Marseille
	YP_001792799.1	<i>Leptothrix cholodnii</i> SP-6 (2)
	ZP_01915189.1	<i>Limnobacter</i> sp. MED105
	YP_001021624.1	<i>Methylibium petroleiphilum</i> PM1
	YP_549441.1	<i>Polaromonas</i> sp. JS666
II	YP_001155587.1	<i>Polynucleobacter necessarius</i> subsp. <i>asymbioticus</i> QLW-P1DMWA-1 (1)
	YP_001797823.1	<i>Polynucleobacter necessarius</i> subsp. <i>necessarius</i> STIR1
	YP_727992.1	<i>Ralstonia eutropha</i> H16
	YP_297455.1	<i>Ralstonia eutropha</i> JMP134
	YP_002983065.1	<i>Ralstonia pickettii</i> 12D
	YP_001901006.1	<i>Ralstonia pickettii</i> 12J
	YP_003744186.1	<i>Ralstonia solanacearum</i> CFBP2957
	CBJ36425.1	<i>Ralstonia solanacearum</i> CMR15
	NP_521375.1	<i>Ralstonia solanacearum</i> GMI1000
	YP_002261131.1	<i>Ralstonia solanacearum</i> IPO1609
	CAQ17926.1	<i>Ralstonia solanacearum</i> MolK2
	AEG67512.1	<i>Ralstonia solanacearum</i> Po82
	YP_003750891.1	<i>Ralstonia solanacearum</i> PSI07
	ZP_00944483.1	<i>Ralstonia solanacearum</i> UW551
	ZP_08893729.1	<i>Ralstonia</i> sp. 5_2_56FAA
	ZP_07675234.1	<i>Ralstonia</i> sp. 5_7_47FAA
	CCA85634.1	<i>Ralstonia syzygii</i> R24
	YP_004618487.1	<i>Ramlibacter tataouinensis</i> TTB310
	SUTH_02728	<i>Sulfuritalea hydrogenivorans</i> sk43H (1)
	YP_003643975.1	<i>Thiomonas intermedia</i> K12 (1)
	YP_003644808.1	<i>Thiomonas intermedia</i> K12 (2)
	CAZ89285.1	<i>Thiomonas</i> sp. 3As
III	YP_003524317.1	<i>Sideroxydans lithotrophicus</i> ES-1
	SCD_n02409	<i>Sulfuricella denitrificans</i> skB26
	YP_314322.1	<i>Thiobacillus denitrificans</i> ATCC 25259 (1)
	YP_314676.1	<i>Thiobacillus denitrificans</i> ATCC 25259 (2)
	B059DRAFT_01620	<i>Thiobacillus denitrificans</i> DSM 12475 (1)
	B059DRAFT_00577	<i>Thiobacillus denitrificans</i> DSM 12475 (2)
	B058DRAFT_02105	<i>Thiobacillus thioparus</i> DSM 505 (1)
	B058DRAFT_03048	<i>Thiobacillus thioparus</i> DSM 505 (2)
IV	SUTH_03439	<i>Sulfuritalea hydrogenivorans</i> sk43H (2)
	B059DRAFT_01507	<i>Thiobacillus denitrificans</i> DSM 12475 (3)

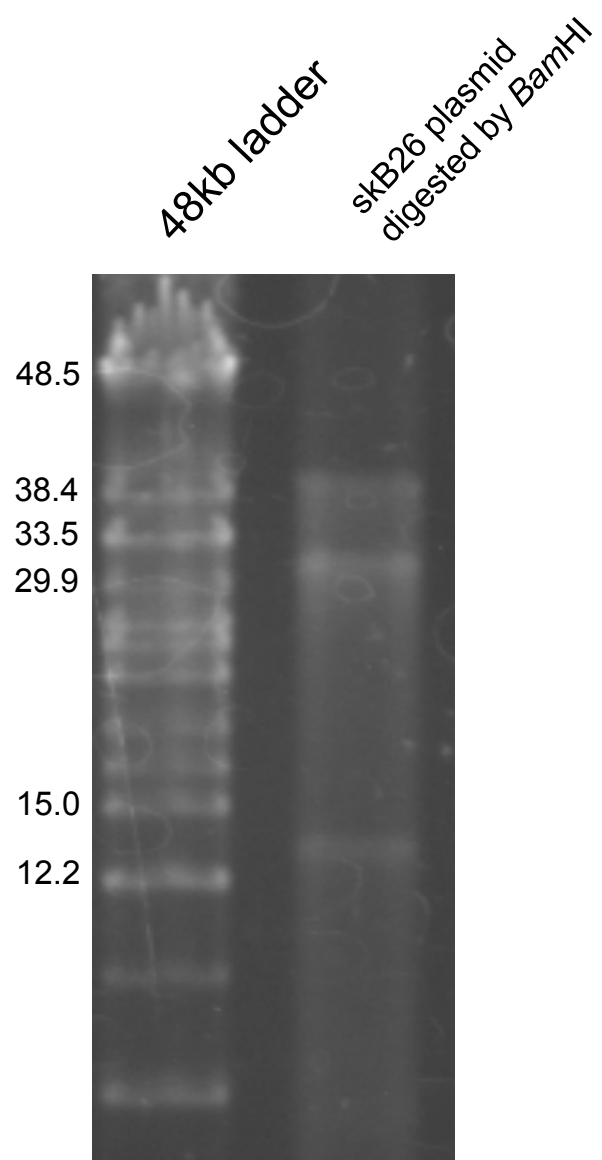


Fig. 5-1. PFGE pattern of *Bam*HI-HF digested plasmid.

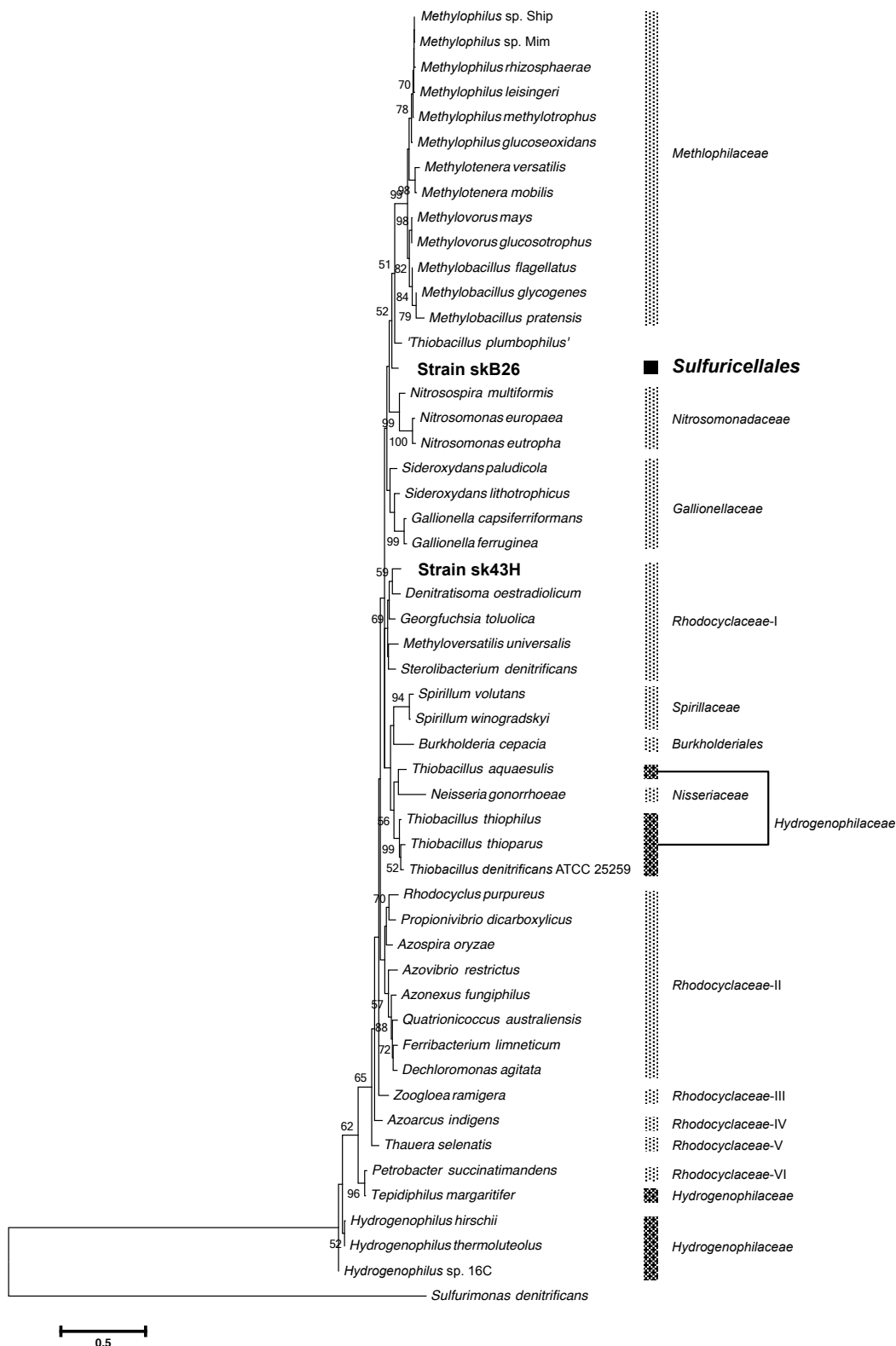


Fig. 5-2. Phylogenetic tree based on 16S rRNA sequences of selected representatives from all betaproteobacterial families. The sequence of *Sulfurimonas denitrificans* was included as an outgroup. The sequences were aligned by T-COFFEE, and the tree was constructed by maximum likelihood method with 1000 bootstrap resamplings (values above 50% are shown).

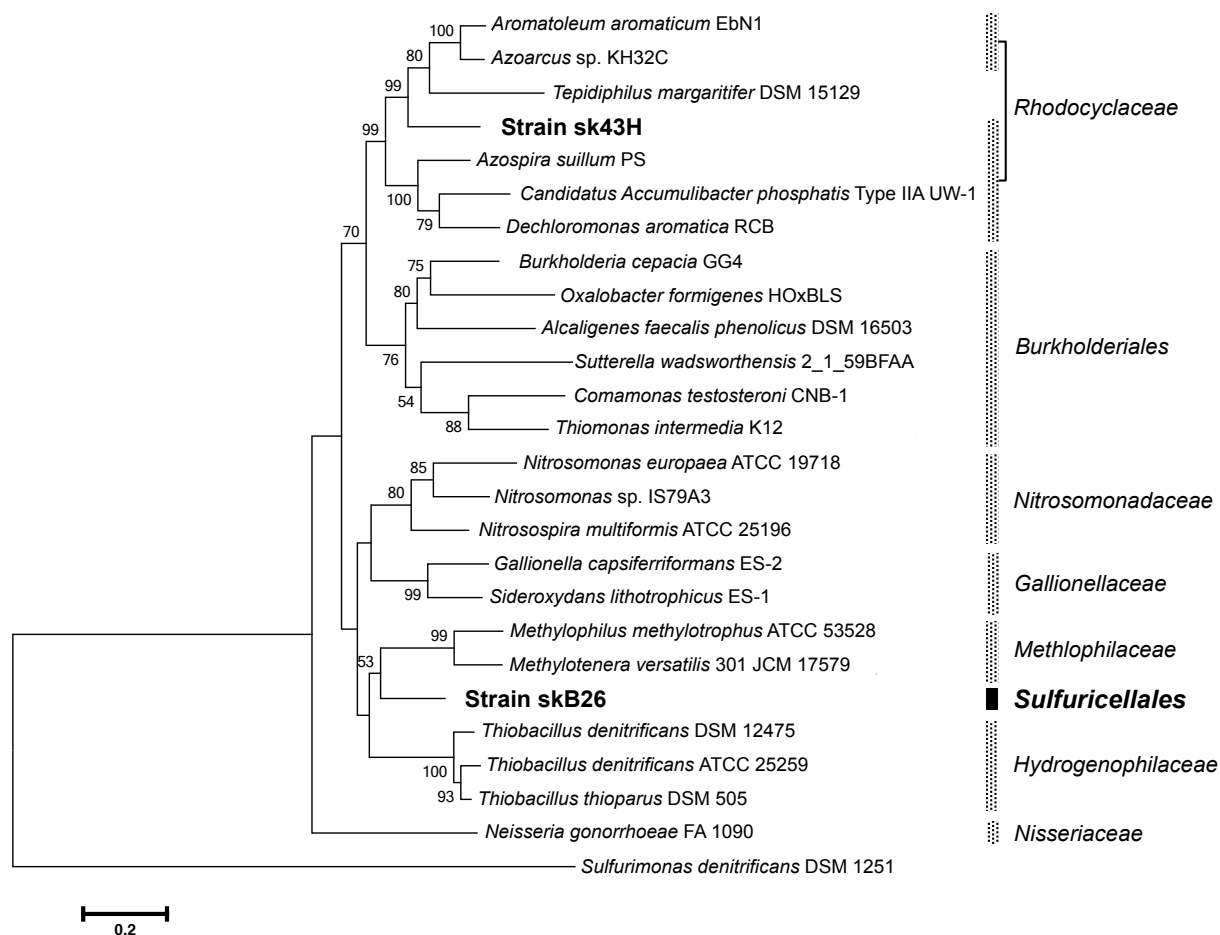


Fig. 5-3. Phylogenetic positions of strains skB26 and sk43H within the class *Betaproteobacteria*, based on the multi-locus sequence analysis of six concatenated conserved protein sequences aligned by T-COFFEE. Tree was constructed by the maximum likelihood method with 1000 bootstrap resamplings (bootstrap values > 50% are shown at the nodes). The scale bar indicates the branch length corresponding to 0.2 substitutions per amino acid position.

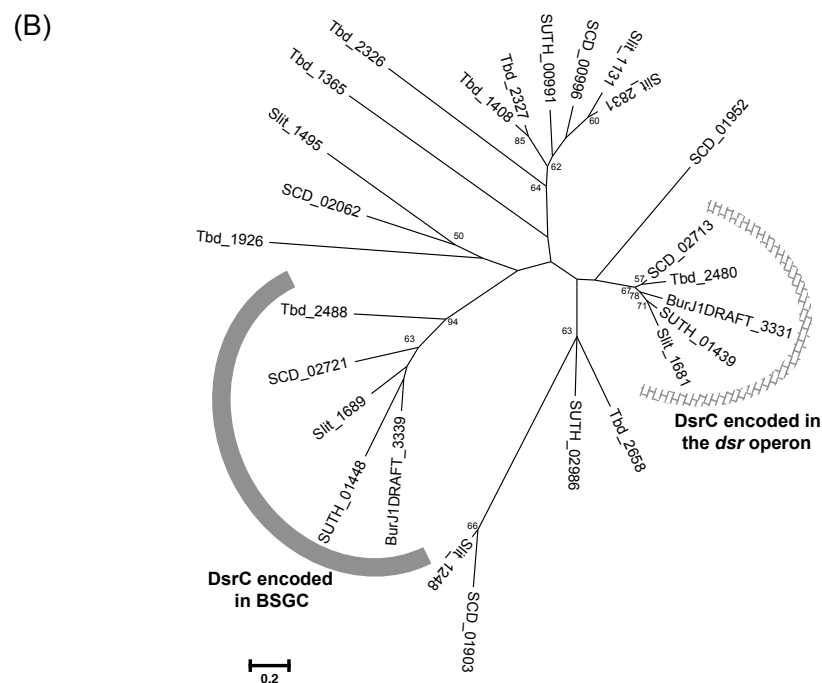
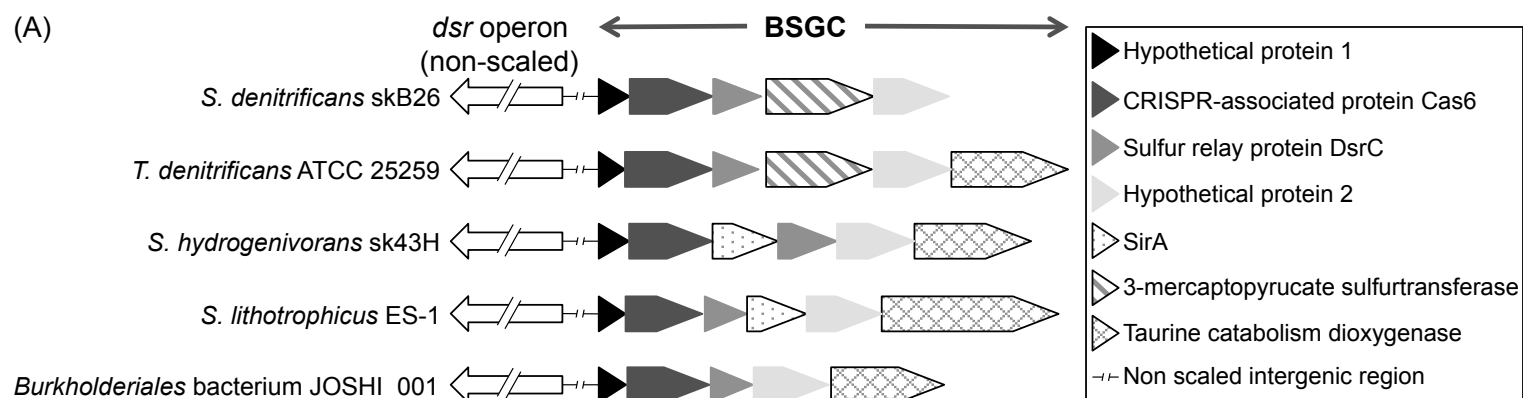


Fig. 5-4. Schematic comparison of betaproteobacteria-specific gene cluster (BSGC) located immediately upstream of the *dsr* operon (A), and phylogenetic relationship of the putative DsrC proteins encoded in the genomes of *dsrAB*-harboring betaproteobacteria (B). Protein sequences were aligned using ClustalW, and the tree was constructed by the maximum likelihood method with 1000 bootstrap resamplings (bootstrap values > 50% are shown at the nodes). Limbs show locus tags, and locus tag prefix corresponds to as follows: SCD, *Sulfuricella denitrificans* skB26; SUTH, *Sulfuritalea hydrogenivorans* sk43H; Tbd, *Thiobacillus denitrificans* ATCC 25259; Slit, *Sideroxydans lithotrophicus* ES-1; BurJ1DRAFT, *Burkholderiales* bacterium JOSHI_001.

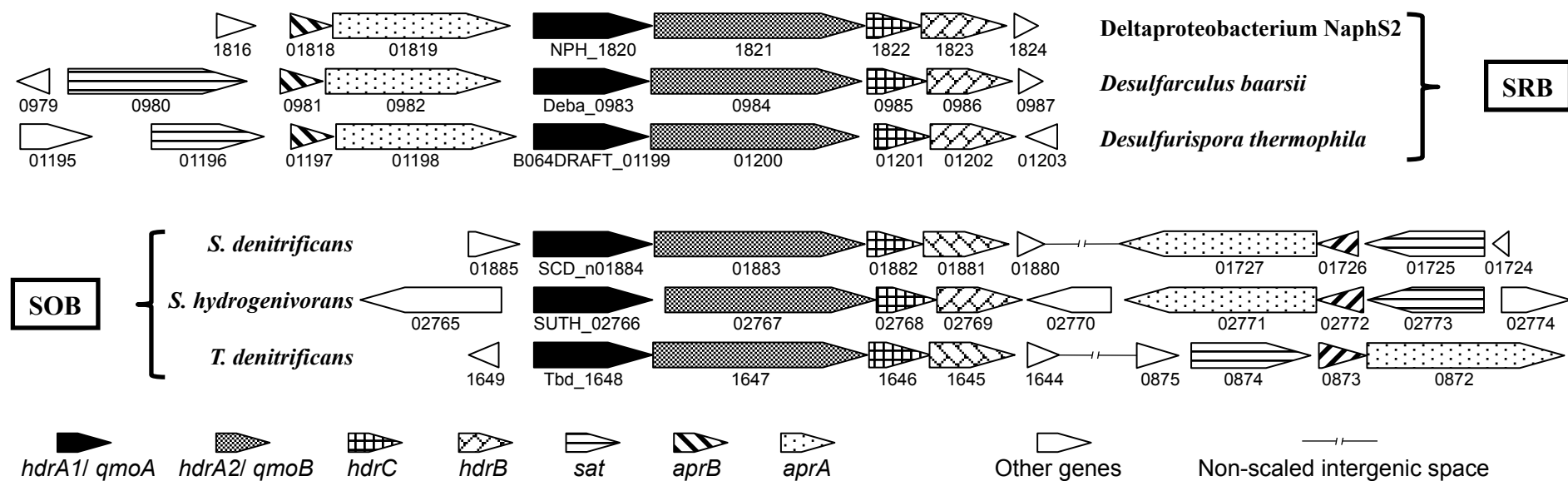


Fig. 5-5. Schematic comparison of gene arrangement of *hdrA1A2/qmoABhdrCB*, *aprBA*, and *sat* in selected SOB and sulfate-reducing bacteria (SRB).

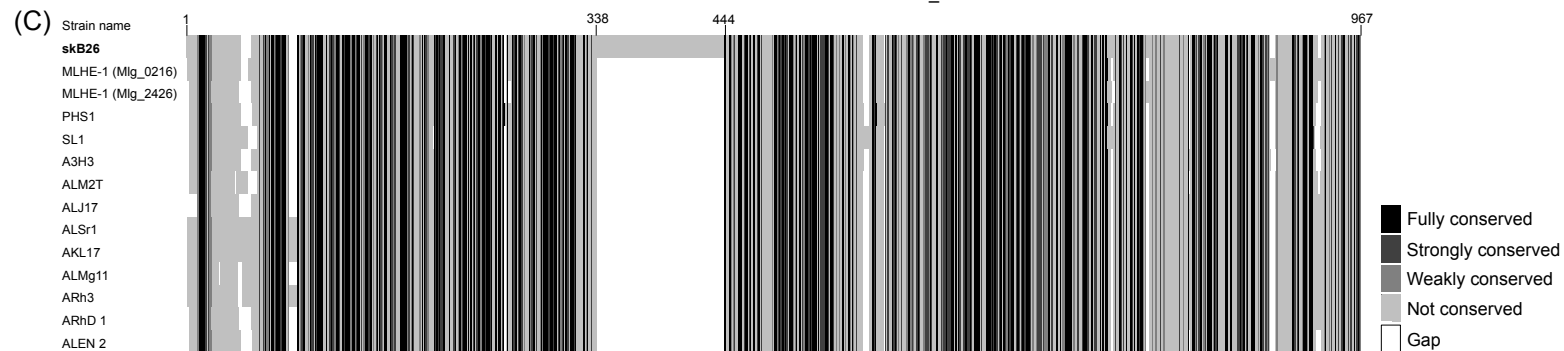
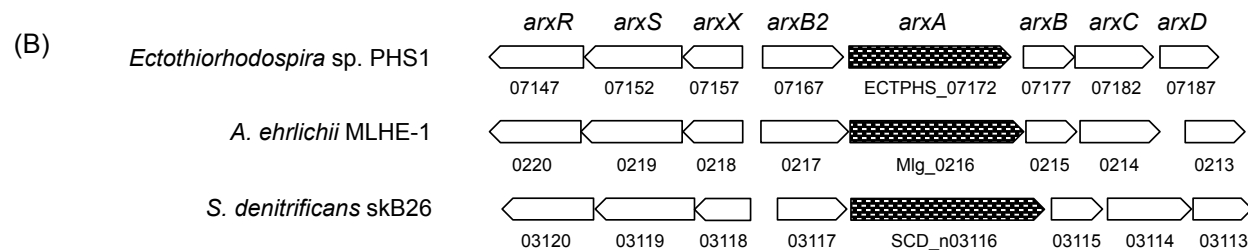


Fig. 5-6. Comparative analyses of *arxA*-like sequence located on the plasmid of strain skB26. (A) Phylogenetic tree of arsenate respiratory reductases (ArrA), AoxB-type arsenite oxidases, and ArxA-like sequences. The ArxA-like sequence of strain skB26 is highlighted in bold type. Anaerobic arsenite oxidizers are marked with an asterisk. The protein sequences were aligned using ClustalW, and the tree was constructed by the maximum likelihood method with 1000 bootstrap resamplings (bootstrap values > 50% are shown at nodes). The scale bar indicates the branch length corresponding to 1 substitutions per amino acid position. Four *Thioalkalivibrio* sp. strains include ARh3 (WP_018864019), ALMg11 (WP_018950173), ALSr1 (WP_019643167), and AKL17 (WP_018946174); 2 *Thioalkalivibrio* spp. indicates *Thioalkalivibrio* sp. ALJ17 (WP_018952916) and *T. thiocyanodenitrificans* (WP_018231946). (B) Schematic comparison of *arx* locus in *Ectothiorhodospira* sp. PHS-1 and *Alkalilimnicola ehrlichii* MLHE-1, and *arx*-like locus in strain skB26. (C) Multiple alignment of ArxA homologous sequences using ClustalW. The colored regions indicate their conservation levels, and colorless regions indicate gap positions. ‘Fully conserved’ indicates fully conserved; ‘Strongly conserved’ indicates strong group conserved; ‘Weakly conserved’ indicates weak group conserved sites, respectively. Strong and weak groups are defined in ClustalW. Amino acid positions are numbered according to full-length ArxA-like sequence of strain skB26. Strain names correspond to those of Fig. 5-6A.

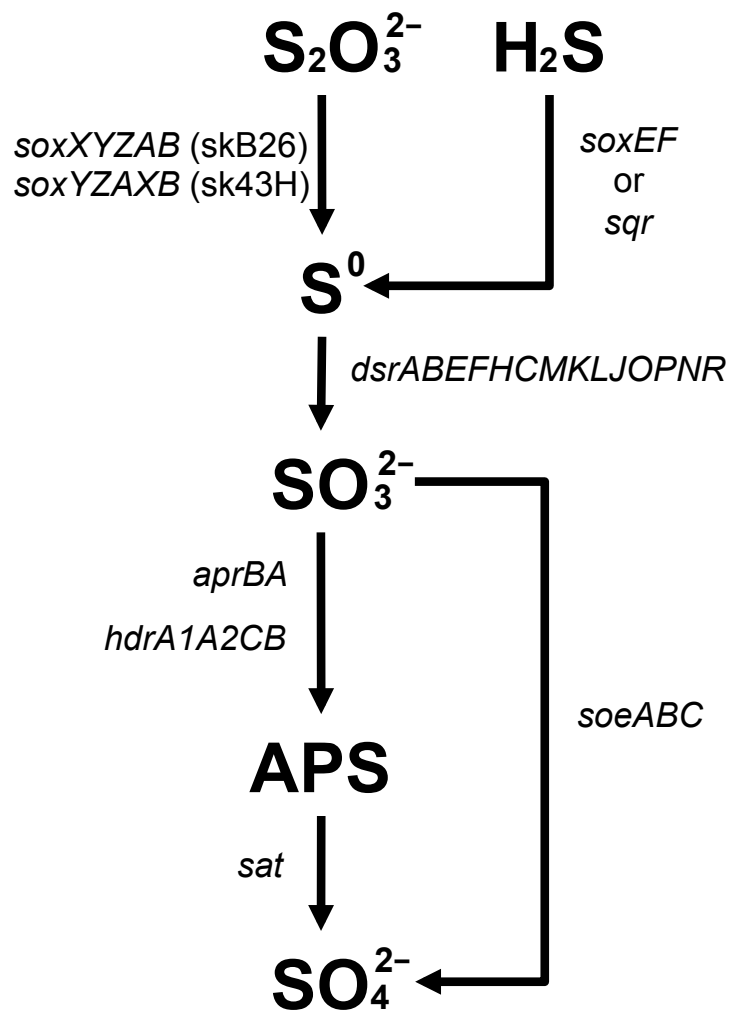


Fig. 5-7. Schematic overview of key genes putatively involved in sulfur oxidation in both strains skB26 and sk43H.

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Abstract in Japanese

淡水湖沼環境で優占する硫黄酸化細菌のゲノミクスおよびプロテオミクス (英文)

硫黄、窒素、および炭素は、生体を構成・駆動する上で重要な元素であり、その自然環境中での挙動は微生物活動に強く依存する。系統的に極めて多様な化学合成硫黄酸化細菌 (SOB) は、これら諸元素の循環をリンクする微生物として地球上に普遍的に生息する。このような特性に加え、その豊富な存在量から、海洋環境に生息する SOB は全球規模での元素循環に大きく貢献すると考えられている。このため、その生態を解明するために主要種の特定ならびにゲノム解析が成されてきた。蓄積してきた SOB のゲノム情報からは、系統ごとに異なる機能が解明されつつある一方で、多様な硫黄酸化経路の存在が見出されてきた。しかし、機能が類似する複数の経路や酵素の存在意義は十分に理解されておらず、重要な研究テーマの一つとなっている。一方で、陸域の元素循環に寄与する淡水湖沼の SOB は、海洋と比べて存在量が少ないことから研究が容易ではなく、現場に生息する系統すら不明瞭であった。一般的に、同じ機能群の細菌であっても、海水と淡水環境では主要種が異なる系統に属することが知られている。このことから類推すると、淡水湖沼には独自の系統の SOB が生息し、特有の性質を有することが推察される。このような SOB のゲノムが解明されることによって、他の環境由来の SOB との比較から、SOB 全体を俯瞰した進化過程を考察することが可能になると期待される。本研究では、淡水湖沼に生息する SOB の系統を特定し、主要種の硫黄酸化経路の特徴をゲノムから見出すことを目的とした。また、プロテオーム解析によりその発現ならびに淡水湖沼の SOB に特有の性質を検討した。

淡水湖沼の主要な SOB を特定するために、部分循環湖の塩川ダム湖 (山梨県) の嫌気水層の水試料、地理的位置の異なる 5 つの淡水湖沼 (琵琶湖、オコタンペ湖、スカーレン大池、親子池、丸湾大池) の堆積物試料を対象として 4 つの硫黄酸化関連遺伝子のクローニング解析を行った。この結果、いずれの淡水湖沼においても海洋環境とは異なり *Betaproteobacteria* 綱の SOB が優占することが示され、これらは不完全チオ硫酸酸化型の SOB であることが推定された。そして、嫌気水層と堆積物の主要種の 1 つを、それぞれ *Sulfiritalea hydrogenivorans* と *Sulfuricella denitrificans* であると同定した。これらは塩川ダム湖の嫌気水層から単離された SOB であり、本研究の調査地以外の淡水環境からもその存在が見出されている。以上より、これら 2 種を淡水湖沼のモデル SOB として、それぞれの基準株である sk43H と skB26 の完全ゲノム配列を対象にゲノム解析を行った。

sk43H のゲノムは約 3.8 Mb の染色体、skB26 のゲノムは約 3.1 Mb の染色体と約 87 kb のプラスミドから構成されていた。それぞれのゲノムから推定された 3587 と 3073 遺伝子の BLAST 解析を行った結果、それぞれの染色体上の 44 と 53 遺伝子が硫黄酸化に関与するものと推定された。その遺伝子構成から硫黄酸化経路を構築した結果、両株とも同じ硫黄酸化経路を有することが明らかとなった。これは、クローニングで解析した各硫黄酸化遺伝子の系統関係から推定された不完全チオ硫酸酸化型の経路であった。また、他の *Betaproteobacteria* 綱の硫黄酸化細菌のゲノムを含めた比較解析によって、この硫黄酸化経路が淡水性 SOB にのみ保存されていることが明らかになった。一方で、亜硫酸を酸化する 2 つの経路の存在が明らかとなった。

次いで、硫黄酸化関連遺伝子の発現を確認するために skB26 を用いてプロテオーム解析を行った。解析を行う際、skB26 が耐冷性 SOB であることに着目した。湖沼環境における SOB の基質の供給源は湖底で起こる硫酸還元が主であり、湖底付近はしばし低温になる。従って、低温への適応は湖沼環境において SOB が基質にアクセスする上で重要な性質であると考えられる。skB26 を至適温度 (22°C) と分離源の温度 (5°C) で培養し、タンパク質量を比較することで低温への応答を硫黄酸化経路の発現と共に検討した。この結果、5°C においてリボソームタンパク質、RNA シャペロン、RNA ヘリカーゼ、Sulfur relay タンパク質、そして脂肪酸合成酵素の 1 つ FabZ の著しい増加が認められた。これらから推定される機能より、正確な翻訳の進行と膜流動性の維持が低温への主な応答であると結論した。その他、12 タンパク質の著しい増加が確認されたが、今回の解析だけでは低温耐性との関連付けはできなかった。一方で、硫黄酸化関連タンパク質はリボソームタンパク質に次いで 2 番目に存在量が多い機能群であることが明らかとなった。そして、チオ硫酸を硫酸まで酸化する一連の酵素群の発現が確認された。これは、Sox によるチオ硫酸の不完全酸化、Dsr による元素状硫黄酸化、そして ATP 生成を伴う APS 経路を介した亜硫酸酸化によって構成されていた。

本研究で明らかとなった硫黄酸化経路は、クローニング解析において種を特定することができなかった他のベータプロテオバクテリアでも共通すると考えられる。実際にこのような経路を有する SOB が優占していたことを鑑みると、この硫黄酸化経路は、淡水湖沼環境に適応した SOB が共有する性質であると考えられる。Dsr および ATP 生成を伴う APS 経路を介するチオ硫酸の酸化は、完全チオ硫酸酸化型の経路よりもエネルギー獲得効率が良いと考えられる。従って、この硫黄酸化経路は、無機硫黄化合物の供給が制限される淡水湖沼環境での生存に有利なものと考えられる。