Isolation and Identification of Sulfur-oxidizing Bacteria from the Buried Layer Containing Reduced Sulfur Compounds of a Paddy Field on Sado Island in Niigata Prefecture

Hirotomo OHBA[1] and Naoto OWA[2*]

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We isolated sulfur-oxidizing bacteria from the buried layer of a paddy field on Sado Island in Niigata Prefecture, which contained marine reduced sulfur compounds under semi-anaerobic conditions that were oxidized by sulfur-oxidizing bacteria. As a result, 3 sulfur-oxidizing bacterial strains on the medium where the pH was adjusted to 3.5 (named A4-1H, A4-2F and A4-3A) and 3 sulfur-oxidizing bacterial strains on the medium where the pH was adjusted to 6.5 (named N4-1C, N4-1E and N4-1G) were isolated. Based on 16S rDNA analysis, all isolates were found to be phylogenetically identical or very closely related, and related most closely to Uncultured *Thiomonas* sp. C02 (DDBJ accession number AF460988). Furthermore, the bacteria grew in media over a wide range of pH (between 3.5 and 6.5) and oxidized a thiosulfate ion to sulfuric acid during lithoautotrophic growth. These results suggest that one of the sulfur-oxidizing bacteria isolated from buried soil layer was a member of the genus *Thiomonas*.

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Key words: buried layer, genus *Thiomonas*, Sulfur-oxidizing bacteria

Potential acid sulfate soils are rich in pyrite (FeS_2). When these pyrites are exposed to air, as a result of agricultural or other use of the potential acid sulfate soils, they are oxidized to sulfuric acid (H_2SO_4) forming acid sulfate soils (Mathew et al., 2001). Indeed, sulfur-oxidizing bacteria such as *Acidithiobacillus thiooxidans* and *Thiobacillus thioparus* and iron-oxidizing bacteria such as *Acidithiobacillus ferrooxidans* have been detected in actual acid sulfate soils and are considered to promote formation of acid sulfate soils (Bloomfield and Coulter, 1973, Arkesteyn, 1980, Takai *et al*., 1989, Ogawa *et al*., 1990). It has been reported that these sulfur-oxidizing bacteria do not oxidize pyrite directly and that pyrite oxidation undergoes a following process (Wakao *et al*., 1982, Schippers *et al*., 1999, Crundwell, 2003, Rodriguez *et al*., 2003).

Pyrite is leached chemically by ferric ions (Fe^{3+}). This reaction produces ferrous ions (Fe^{2+}) and intermediary sulfur compounds such as thiosulfate ions ($S_2O_3^{2-}$) and tetrathionate ($S_4O_6^{2-}$). These reactions can be written as:

$$FeS_2 + 3H_2O + 6Fe^{3+} \rightarrow 7Fe^{2+} + S_2O_3^{2-} + 6H^+ \quad (1)$$
$$2FeS_2 + 6H_2O + 14Fe^{3+} \rightarrow 16Fe^{2+} + S_4O_6^{2-} + 12H^+ \quad (2)$$

The ferrous ions produced by reactions (1) and (2) are re-oxidized to ferric ions either chemically or by iron-oxidizing bacteria such as *A.ferrooxidans* and *Leptospirillum ferrooxidans*, according to the following reaction:

$$14Fe^{2+} + 3.5O_2 + 14H^+ \rightarrow 14Fe^{3+} + 7H_2O \quad (3)$$

After these reactions, sulfur-oxidizing bacteria oxidize the intermediary sulfur compounds to sulfate ions, according to the following reaction:

$$S_2O_3^{2-} + O_2 \rightarrow 2SO_4^{2-} \quad (4)$$
$$S_4O_6^{2-} + 5O_2 \rightarrow 4SO_4^{2-} \quad (5)$$

In our previous paper (Ohba and Owa, 2005), we observed the presence of reduced sulfur compounds in the buried soil layer of a paddy field on Sado Island, Niigata Prefecture. We sampled the paddy field soil from a depth of 0 to 300 cm and analyzed the physico-chemical properties of the soil and the number of sulfur-oxidizing bacteria and iron-oxidizing bacteria in order to elucidate both the sulfur oxidizing mechanism and the function of the sulfur-oxidizing bacteria in the buried layer. Based on the physico-chemical properties of the soil, the buried layers, which were located below 1 m in depth, were found to be potential acid sulfate soils under semi-anaerobic conditions. However, the concentrations of water-soluble sulfate ions in the buried layers were higher than those in the surface layers and a significant number of sulfur-oxidizing bacteria (10^{2-6} MPN g^{-1}) were detected in the buried layers. These results suggested that oxidation of the reduced sulfur compounds by sulfur-oxidizing bacteria had occurred in the buried layers. Since no iron-oxidizing bacteria were detected in any of the layers, it was considered that oxidation of the reduced sulfur compounds in the buried layers occurred through

[1]Graduate School of Science and Technology, Niigata University
[2]Faculty of Agriculture, Niigata University
*Corresponding author: owanet@agr.niigata-u.ac.jp

the following processes. At first, reduced sulfur compounds such as pyrite were oxidized chemically by ferric ions to intermediary sulfur compounds such as thiosulfate ions. Subsequently, sulfur-oxidizing bacteria in the buried layers oxidized these intermediary sulfur compounds to sulfate ions.

In the present study, we isolated sulfur-oxidizing bacteria from the buried layers of the paddy field and identified them by 16S rDNA analysis.

MATERIALS AND METHODS

Soil samples. The paddy field soils on Sado Island, Niigata Prefecture used in the study have been described in detail previously (Ohba and Owa 2005). Soil samples for isolation of sulfur-oxidizing bacteria were taken from the following buried layers, namely layer 4-1 (100 − 112.5 cm depth), layer 4-2 (162.5 - 175 cm depth) and layer 4-3 (250 − 260.5 cm depth). These layers were located below 1 m in depth and were found to be potential acid sulfate soils under semi-anaerobic conditions. The physico-chemical properties and the number of sulfur-oxidizing bacteria in the soil analyzed in the previous study (Ohba and Owa, 2005) are shown in Table 1. The soil samples were gently crushed and stored at 4℃ until use.

Isolation of sulfur-oxidizing bacteria. Twenty g of each soil sample and 180 mL of sterilized water was placed in a 300 mL Erlenmeyer flask, the containers were then capped tightly with a rubber cap and shaken at room temperature for 30 min on a reciprocal shaker. The suspension was serially diluted 10-fold and 0.1 mL aliquots were spread on agar plates (20 g L^{-1} agar), which contained NH_4Cl 0.1 g L^{-1}, KH_2PO^4 0.05 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.02 g L^{-1}, $Na_2S_2O_3 \cdot 5H_2O$ 0.4 g L^{-1} and yeast extract 1.0 g L^{-1}. For isolation of acidophilic sulfur-oxidizing bacteria, the pH of the medium was adjusted to 3.5 with 0.05M sulfuric acid. For isolation of neutrophilic sulfur-oxidizing bacteria, the pH of the medium was adjusted to 6.5 with 0.1M sodium hydroxide (Sallah et al., 1993, Ohba *et al*., 2003). The agar plates were cultured at 30℃ for 1 − 2 weeks.

After cultivation, all morphologically different bacterial colonies were picked from each plate. Once the purity of each of these colonies had been verified, each colony was suspended in 10 mL of sterilized water and 0.5 mL of the suspensions were inoculated into 10 mL of liquid medium containing the same components as for the agar plates. After static cultivation at 30 ℃ for 2 weeks, the bacteria were checked to see whether or not they were sulfur-oxidizing bacteria by the consumption amount of thiosulfate ions estimated by manual titration using a 5 mM iodine solution to a starch end-point (Takagi, 1976) and the final pH of the medium measured using an ion meter (IM-40S: TOA Electronics, Tokyo, Japan) fitted with a glass electrode.

DNA extraction from sulfur-oxidizing bacteria. Sulfur-oxidizing bacterial DNA was extracted using the following protocol: Each bacterium was inoculated into 50 mL of the above liquid medium and the medium was shake cultured at 30℃ for one week. Cell cultures were transferred into a micro-tube, centrifuged at 13,000 g for 1 min and the supernatant liquid was removed. The pellet was resuspended in 1.0 mL of sterilized water, centrifuged at 13,000 g for 3 min and the supernatant liquid was once again removed. The pellet was resuspended in 400 μL of 50 mM Tris-HCl/50 mM EDTA, 40 μL of lysozyme solution (250 mM Tris-HCl and 10 g L^{-1} lysozyme) was added and the suspension was incubated at 37℃ for 15 min. Then, 40 μL of 100 g L^{-1} sodium dodecylsulfate (SDS) solution and 10 g L^{-1} proteinase K solution were added and incubated at 55℃ for 60 min with shaking every 15 min. Five hundred μL of phenol equilibrated with TE buffer was added to the suspension. Then the suspension was mixed, left to stand for 10 min and then centrifuged at 13,000 g for 10 min. The supernatant phase (almost 450 μL) was transferred to a new micro-tube, and 5.0 μL of 10 g L^{-1} ribonuclease A (RNase A) was added and incubated at 37℃ for 30 min with shaking every 10 min. Thereafter 450 μL of a chloroform-isoamylalcohol (24:1) mixture was added to the suspension. The suspension was mixed, left to stand for 10 min and centrifuged at 13,000 g for 10 min. The supernatant phase (almost 400 μL) was transferred to a new micro-tube, 40 μL of 3.0 M sodium acetate solution and 1.0 mL of ethanol were added and mixed slowly. After being static at room temperature for 10 min, the precipitated DNA was transferred to a new micro-tube. One mL of 70% cold ethanol (−20℃) was added to the micro-tube, centrifuged at 13,000 g for 10 min and the aqueous phase was removed. This procedure was repeated three times. Then the precipitated DNA was dried and dissolved in 100 μL of TE buffer (10 mM Tris-HCl and 1 mM EDTA). The suspension was used as DNA template for subsequent PCR amplification.

Table 1. Physico-chemical properties and number of sulfur-oxidizing bacteria of the soil samples.

	Soil depth (cm)	Soil texture	pH(H_2O)	pH(H_2O_2)	Reduced sulfur compounds	Sulfate ions	Ferrous ions	Ferric ions	Sulfur-oxidizing bacteria	
									acidophilic	neutrophilic
					(g S kg^{-1})	(mg S kg^{-1})	(g Fe kg^{-1})		(MPN g^{-1})	
Layer 4-1	100 − 112.5	SL	5.18	2.66	2.98	444	0.489	2.25	1.05×10^2	9.39×10^6
Layer 4-2	162.5 − 175	SCL	6.47	2.40	4.82	88.2	1.05	2.54	1.02×10^4	7.13×10^3
Layer 4-3	250 − 260.5	SCL	5.22	2.04	7.74	216	1.27	2.16	2.01×10^3	3.41×10^3

Cited from Ohba and Owa (2005).

PCR amplification. The set of primers used for PCR amplification of 16S rDNA from the isolates were 27f (*Escherichia coil* position 8-27, 5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (*E.coli* position 1510-1492, 5'-GGC TAC CTT GTT ACG ACT T-3'). These primers are complementary to most bacterial 16S rDNA (Lane 1991, Ikenaga *et al.*, 2002). One hundred μL of a PCR mixture contained 2.0 μL of each primer solution (20 pmol each), 0.5 μL of Ex Taq DNA polymerase (2.5U; TAKARA BIO, Shiga, Japan), 10 μL of Ex Taq buffer (Mg^{2+} free), 8.0 μL of 25 mM $MgCl_2$ solution, 8.0 μL of dNTP mixture (2.5 mM each), 10 μL of DNA template and 59.5 μL of sterilized water. The PCR amplification conditions involved an initial denaturation step at 94℃ for 5 min, 30 extension cycles of 94℃, 1 min; 53℃, 1 min; 72℃, 1.5 min; and a final extension step at 72℃ for 10 min. The PCR products were confirmed by visualization on 7.0 g L^{-1} agarose gels and purified using a QIAquick Gel Extraction Kit (QUIAGEN, Valencia, CA, USA) following the manufacturer's instructions.

Cloning. The purified 16S rDNA PCR products were ligated into a pT7Blue vector (Novagen, California, USA) using a DNA Ligation Kit (TAKARA BIO), and transformed into *E.coli* JM109 Competent Cells (TAKARA BIO) following the manufacturer's instructions. The transformed Competent Cell suspensions were spread on Luria-Bertani (LB) agar plates (10 g L^{-1} bacto tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl and 15 g L^{-1} agar) containing 50 mg L^{-1} ampicillin, 100 μmol L^{-1} isopropyl-β-D-thiogalactoside (IPTG) and 40mg L^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and cultured at 37℃ overnight. White colonies on the plates were replicated onto 10 mL of LB liquid medium and grown overnight. Plasmid preparation from the transformed Competent Cells was performed using a common alkaline lysis method (Birnboim, 1983).

Sequencing and phylogenetic analysis. The plasmid-cloned PCR products were sequenced using a Thermo Sequenase Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences, Pscataway, NJ, USA) and LI-COR Model4000L DNA sequencer (LI-COR, Nebraska, USA) following the manufacturer's instructions. Sequences of the PCR products were aligned to the 16S rDNA sequences obtained from the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/Welcome-j.html) with the FASTA search program (Lipman and Pearson 1985, Pearson and Lipman 1988). Sequences of PCR products and their closest relatives were aligned using the CLUSTAL W analysis program (Thompson *et al.*, 1994) form DDBJ and a phylogenetic tree was constructed using Tree View.

RESULTS AND DISCUSSION

Isolation of sulfur-oxidizing bacteria and measurement of their sulfur-oxidizing activity

We isolated 10 bacterial strains on the medium where the pH was adjusted to 3.5 (the medium for acidophilic bacteria) and 8 bacterial strains on the medium where the pH was adjusted to 6.5 (the medium for neutrophilic bacteria) from Layer 4-1, 6 bacterial strains on the medium for acidophilic bacteria and 6 bacterial strains on the medium for neutrophilic bacteria from Layer 4-2 and 1 bacterial strain on the medium for acidophilic bacteria and 1 bacterial strain on the medium for neutrophilic bacteria from Layer 4-3.

Based on measuring the amounts of thiosulfate ions consumed by the isolated bacteria and the final pH of the medium, it was confirmed that 6 strains, which consisted of 1 bacterial strain on the medium for acidophilic bacteria (named A4-1H) and 3 bacterial strains on the medium for neutrophilic bacteria (named N4-1C, N4-1E and N4-1G) from layer 4-1, 1 bacterial strain on the medium for acidophilic bacteria from layer 4-2 (named A4-2F) and 1 bacterial strain on the medium for acidophilic bacteria from layer 4-3 (named A4-3A), were sulfur-oxidizing bacteria. The amounts of thiosulfate ions consumed by these bacteria and the initial and final pH of the liquid media are shown in Table 2.

The N4-1C and N4-1E colonies were 4 mm in diameter and were larger than the other colonies (A4-1H, N4-1G, A4-2F and A4-3A; 1 -1.5 mm in diameter). All isolated sulfur-oxidizing bacteria formed lustrous colonies with a yellow or orange color. Apart from N4-1C, the form of the isolated sulfur-oxidizing bacterial colonies was round and that of N4-1C was irregular. The amount of thiosulfate ions consumed by the isolated sulfur-oxidizing bacteria ranged

Table 2. Consumption amount of thiosulfate ion by isolated sulfur-oxidizing bacteria and initial and final pH of the liquid media.

Isolate designation		Consumption amount of thiosulfate ion (mM)	Initial pH	Final pH
Isolates on the medium for acidophilic bacteria	A4-1H	1.51	3.5	3.3
	A4-2F	1.54	3.5	3.0
	A4-3A	1.54	3.5	3.1
Isolates on the medium for neutrophilic bacteria	N4-1C	1.59	6.5	4.3
	N4-1E	1.60	6.5	4.3
	N4-1G	1.60	6.5	3.4

from 1.51 to 1.60 mM and thiosulfate ions in liquid medium (initial concentration: 1.61 mM) were consumed almost complementary. The pH of the liquid medium in which A4-1H, A4-2F and A4-3A were inoculated changed from 3.5 to 3.1 - 3.3. The pH of the liquid medium in which N4-1C, N4-1E and N4-1G were inoculated changed from 6.5 to 3.4 - 4.3.

Sequencing of 16S rDNA and phylogenetic analysis of the isolated sulfur-oxidizing bacteria

The 16S rDNA of the isolated sulfur-oxidizing bacteria obtained in this study are available in the DDBJ database. The accession numbers are from AB219367 to AB219372.

The following 16S rRNA bacterial sequences were obtained from DDBJ for inclusion in the phylogenic analysis: Uncultured *Thiomonas sp.* C02 (AF460988), *Thiomonas perometabolis* (AY455808), *Caenibacterium thermophilum* (AJ512945) and *Pseudomonas saccharophila* (AF368755). These strains are all close relatives of the isolated sulfur-oxidizing bacteria. Also included are *Thiomonas cuprina* (U67162), *Thiomonas thermosulfata* (U27839), *Thiomonas intermedia* (AY455809), *Acidithiobacillus thiooxidans* (Y11596), *Acdithiobacillus ferrooxidans* (AF362022), *Thiobacillus thioparus* (M79426), *Starkeya novella* (D32247), *Acidiphilium multivorum* (AB006711), *Halothiobacillus halophilus* (U58020), *Thermithiobacillus tepidarius* (AJ459801), *Thiomicrospira thermophila* (AB166731), *Paracoccus versutus* (AY014174) and *Escherichia coli* (AB045730; as the outgroup). The phylogenic tree for the isolated sulfur-oxidizing bacteria, their close relatives and some of sulfur-oxidizing bacteria strains is shown in Fig.1.

Regardless of medium pH, the closest relative of the isolated sulfur-oxidizing bacteria was an Uncultured *Thiomonas sp.* C02 (AF460988) belonging to the β-proteobactertia (Similarity: 98.3－100 %), and none of the isolated sulfur-oxidizing bacteria were members of the genus *Thiobacillus*.

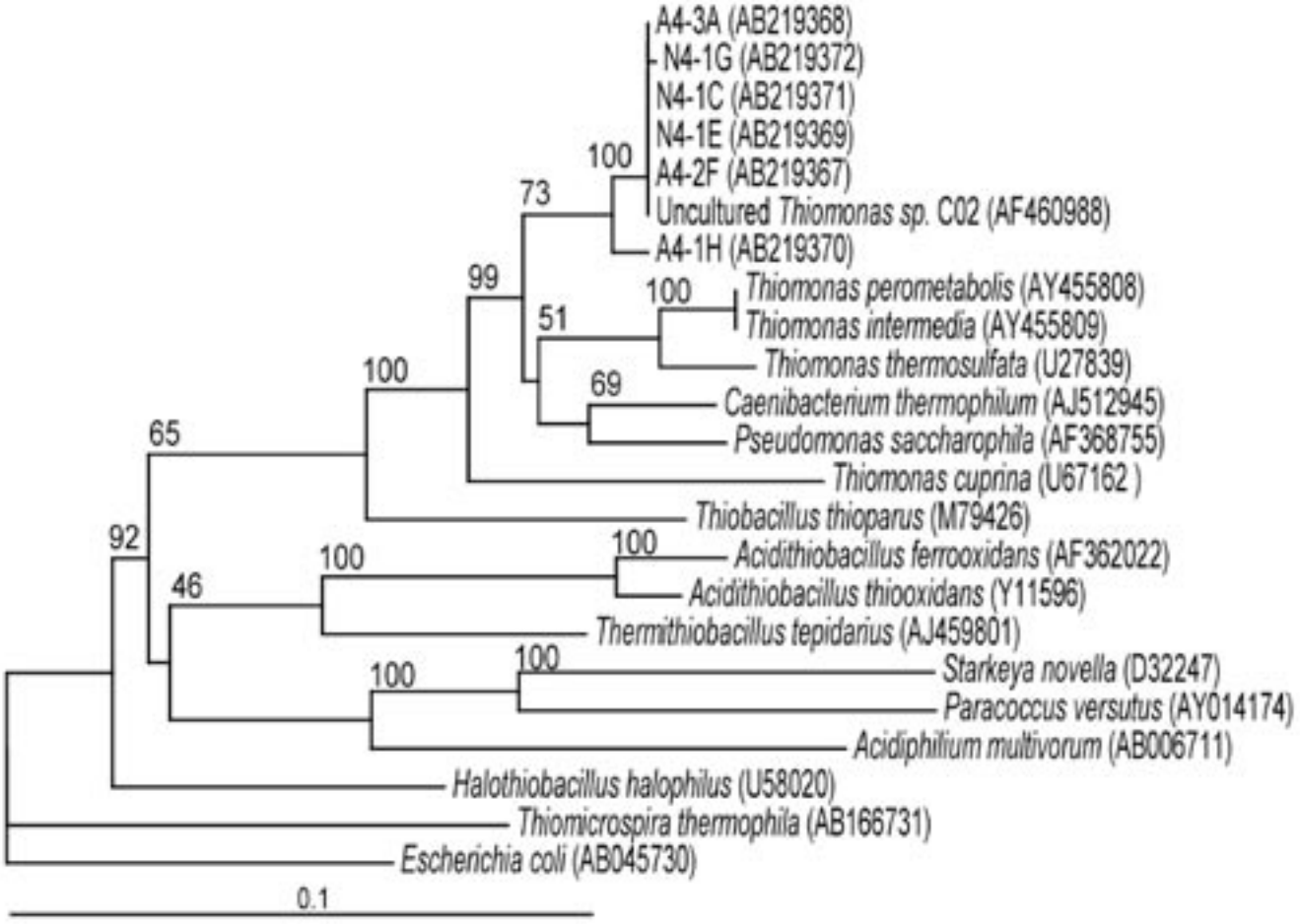

Fig.1. Phylogenic tree among the isolated sulfur-oxidizing bacteria, their close relatives and some of sulfur-oxidizing bacteria strains. The branching pattern, which is rooted by using Escherichia coli as the out group, was generated by the neighbor-joining method. Bootstrap values are given for each node. Bar = 0.1 nucleotide substitution per site. The accession number is shown in parentheses.

Table 3. Consumption amount of thiosulfate ion and final pH of the liquid media by isolated sulfur-oxidizing bacteria inoculated into the liquid media that pH was adjusted to 3.5 and 6.5.

Isolates designation	Initial medium pH			
	3.5		6.5	
	Consumption amount of thiosulfate ion (mM)	Final pH	Consumption amount of thiosulfate ion (mM)	Final pH
A4-1H	1.60	3.3	1.58	4.3
A4-2F	1.59	3.0	1.52	3.4
A4-3A	1.61	3.1	1.56	3.3
N4-1C	1.61	3.3	1.56	4.4
N4-1E	1.60	3.2	1.57	3.4
N4-1G	1.61	3.3	1.56	4.2

Differences in sulfur-oxidizing activity of the isolated sulfur-oxidizing bacteria together with medium pH

Based on phylogenetic analysis of the isolated sulfur-oxidizing bacteria, it was found that all isolated sulfur-oxidizing bacteria were phylogenetically the same or very closely related regardless of their optimum pH. Thus, in order to investigate the differences in sulfur-oxidizing activity of the isolated sulfur-oxidizing bacteria in different pH medium, we inoculated the bacteria into the same liquid medium adjusted the pH (3.5 and 6.5). We then measured the consumption amount of thiosulfate ions and the final pH of the medium after static cultivation at 30℃ for 2 weeks. The results are shown in Table 3.

The amount of thiosulfate ions consumed by these sulfur-oxidizing bacteria when the media was adjusted to pH 3.5 ranged from 1.59 to 1.61 mM and the final pH of the medium ranged from 3.0 to 3.3. In the case of media adjusted to pH 6.5, the consumption amount of thiosulfate ions ranged from 1.52 to 1.58 mM and the final pH ranged from 3.3 to 4.4. Since no significant differences were found for either the consumption amount of thiosulfate ions or the final pH for these bacteria grown in media with varying pHs, and since thiosulfate ion consumption in liquid medium (initial concentration: 1.61 mM) was almost complementary, it is found that all the isolated sulfur-oxidizing bacteria were the same or very closely related and can grow in a wide range of pH medium (between 3.5 and 6.5).

Sulfur-oxidizing bacteria in the buried layer of the paddy field on Sado Island, Niigata Prefecture

In our previous study (Ohba and Owa, 2005), it was found that the oxidation of reduced sulfur compounds by sulfur-oxidizing bacteria occurred in the buried layer of the paddy field on Sado Island, Niigata Prefecture. Furthermore, it was found that the closest relative of all sulfur-oxidizing bacteria isolated from the buried layer was an Uncultured *Thiomonas sp.* C02 (AF460988), which could grow over a wide range of pH medium. These results suggest that one of the sulfur-oxidizing bacteria isolated from the buried soil layer was a member of the genus *Thiomonas* belonging to the β-proteobactertia. It is clear that the genus *Thiomonas* are facultative chemolithoautotrophs where lithoautotrophic growth occurs on thiosulfate, tetrathionate, hydrogen sulfide and elemental sulfur, resulting in production of sulfuric acid. Furthermore, 4 species of the genus *Thiomonas* namely *Thiomonas cuprina*, *T intermedia*, *T perometabolis* and *T thermosulfate*, were once classified into the genus *Thiobacillus* since the phenotypic description of the genus *Thiomonas* is the same as that for the species belonging to group Ⅱ of the genus *Thiobacillus* in Bergey's Manual of Systematic Bacteriology (Kuenen 1984), such as *Acidithiobacillus thiooxidans* (Moreira and Amils, 1997). However, Moreira and Amils (1997) proposed that these organisms should be reclassified into a new genus, the genus *Thiomonas*, because of their phylogenetic similarity and physiological differences from other groups belonging to the Proteobacteria, including other Thiobacilli. As a result, they were transferred to the genus *Thiomonas*, and therefore only a few reports about the genus *Thiomonas* exist. Furthermore, most of these reports describe their function and movement in acid mine drainages (Baker and Banfield, 2003; Casiot *et al.*, 2003; Hallberg and Johnson, 2005; Johnson and Hallberg, 2005) but not in acid sulfate soils.

However, Uncultured *Thiomonas sp.* C02 (AF460988), which is the closest relative of all the sulfur-oxidizing bacteria isolated from the buried layer on Sado Island, was one of the most reprehensive strains of an autotrophic arsenic (Ⅲ)-oxidizing population isolated from a mine residue in a disused Cheni gold mine site in Limousin, France (Battaglia-Brunet *et al.*, 2002). Since the buried layer used for the present study is alluvial soil that contains marine-reduced sulfur compounds and is under semi-anaerobic condition, it was considered that the buried layer and the disused Cheni gold mine site differed remarkably from each other in environmental conditions. Nevertheless, the bacteria from both sites were phylogenetically similar. These results suggest that isolated sulfur-oxidizing bacteria from the buried layers are widely distributed in nature and can grow under

various conditions.

In order to clarify the function of these bacteria with regards to their role in oxidation of reduced sulfur compounds in the buried layers, it is necessary to measure their sulfur-oxidation activity in different medium or under different oxygen pressures. Furthermore, it has been reported that 0.1－15 % of viable cells in the environment form visible colonies on plates (Amann *et al.* 1995). Thus it is necessary to clarify the microbial community of the buried layers using molecular methods such as PCR-DGGE.

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還元型硫黄化合物を含む新潟県佐渡島水田土壌の埋没層中の硫黄酸化細菌の単離・同定

大峡広智[1]・尾和尚人[2*]

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要　約

海成の還元型硫黄化合物を含み、半嫌気的条件下にあり、硫黄酸化細菌による還元型硫黄化合物の酸化が起こっていることが明らかになった新潟県佐渡島の水田土壌の埋没層中の硫黄酸化細菌の単離を行った。その結果、pH を3.5に調節した寒天平板培地から３菌株、pH を6.5に調節した寒天平板培地から３菌株の硫黄酸化細菌を単離した。次に、この単離した６菌株について、16S rDNA の解析による同定と系統解析を行った。その結果、単離した6菌株は全てβ-プロテオバクテリアの Uncultured *Thiomonas sp.* C02（AF460988）と高い相同性が認められ、硫黄酸化細菌として一般的に認められている *Thiobacillus* 属の近縁種ではなかった。また単離した硫黄酸化細菌は全て中性域から酸性域までの広い培地 pH で高い硫黄酸化能力を示した。以上の結果から、今回供試した埋没層中の硫黄酸化細菌のひとつは、*Thiomonas* 属に属する細菌であることが明らかになった。

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[1] 新潟大学大学院自然科学研究科

[2] 新潟大学農学部

*代表著者 : owanet@agr.niigata-u.ac.jp