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Community Composition of Autotrophic Thiosulfate Oxidizers in Tidal Flat Sediment from the Ariake Sea during Growth in Continuous Culture

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Thiosulfate is one of the most abundant energy sources for various lithoautotrophic sulfur bacteria in tidal flat sediment. Thiosulfate-oxidizing bacteria are frequently isolated via continuous culture techniques. However, the community composition within these cultures is not clear. Accordingly, the composition of the community during growth in continuous culture was examined. Selective enrichment for lithoautotrophic thiosulfate-oxidizing bacteria was conducted in a continuous stirred tank reactor. The community composition was determined using a clone analysis targeting 16S rRNA and *aprA* at three different time points during a 26-week incubation. The community was dominated by autotrophic *Gammaproteobacteria*; however, heterotrophs were observed at all incubation times. *Betaproteobacteria* were the major group of *aprA* clones and were less abundant than the detected heterotrophs. At the end of the incubation period, a lithoautotroph was isolated from the continuous culture. Taxonomically, both the isolate and the dominant clones were positioned within the *Thiomicrospira* group, but they were grouped into different operational taxonomic units. The results of this study provide insight into the composition of environmental thiosulfate-oxidizing bacteria during continuous culture.

Key words: Autotrophic thiosulfate-oxidizing bacteria, continuous culture, isolation, and tidal flat.

In tidal flats, thiosulfate is the most abundant inorganic sulfur after sulfide. It is formed from the oxidation of sulfide during the sulfur cycle. Thiosulfate is detected in both oxidizing and reducing layer of sediment (Jorgensen, 1990). As a reduced sulfur compound, thiosulfate provides energy for a variety of lithoautotrophs specifically with eight electrons in the sulfur elements (Friedrich *et al*., 2001). Therefore, thiosulfate is an important source of energy for autotrophic sulfur oxidizers in tidal flats.

Thiosulfate-oxidizing bacteria can be detected by culture-independent and culture-dependent approaches. The former is used to analyze community composition based on 16S rRNA or functional gene markers involved in the oxidative pathway of sulfur compounds (Watanabe *et al*., 2013). The relative abundance of bacteria and thus the dominant group can be determined. The second approach is a conventional method for obtaining culturable strains that remained undetected with the culture-independent techniques (Vegas *et al*., 2013). The method is reliable in providing information on cell-level characteristics.

The initial step for bacterial isolation is selective enrichment to support the growth of targeted species. Serial batch culture is a common method to isolate various sulfur bacteria. However, this method has disadvantages with respect to maintaining a constant pH and chemical

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conditions. Thiosulfate-oxidizing bacteria often release acidic or alkaline compounds as products of thiosulfate oxidation. The pH of the medium can change drastically over a very short period of time (Teske *et al*., 2000). Delayed pH correction can reduce the viability of acid- or alkaline-sensitive sulfur bacteria. This is not a problem in a continuous culture in which the chemical conditions are kept constant.

Continuous culture is a common technique for isolation of autotrophic sulfur bacteria from the environment. However, little is known about the composition of communities during growth in continuous culture before isolation on agar plates. In this study, we performed enrichment cultures of autotrophic thiosulfate-oxidizing bacteria from tidal flat sediment samples using a continuous stirred tank reactor (CSTR). We examined the bacterial composition using the culture-independent method targeting 16S rRNA and *aprA*, which is widely distributed in autotrophic sulfur bacteria. We observed the community at several time points during the incubation and isolated thiosulfate-oxidizing bacteria at the end of the incubation period.

MATERIALS AND METHODS

Description of sampling site

The Midorikawa Tidal Flat is a part of the Midorikawa Estuary, which is located in western Kumamoto Prefecture, Kyushu Island, Japan. At the estuary, fresh water from the Midorikawa River meets seawater from the semi-closed Ariake Sea. The flat becomes submerged by seawater twice daily with a tidal range of 3–4 m (Azad *et al*., 2005). During low tide, the muddy flat becomes exposed and a number of foraging migratory birds and burrowing animals, such as mudskippers and crabs, can be observed.

Sediment sampling and enrichment in continuous culture

The top 2-cm of sediment was sampled and added to a sterile 50-mL polypropylene Falcon conical tube (BD Biosciences, Durham, NC, USA) during low tide in July 2014. The sampling tube was kept on ice during transport to the laboratory before inoculation. Thiosulfate mineral (TM) liquid medium was used as enrichment medium to support the growth of autotrophic thiosulfate-oxidizing bacteria. The TM medium was prepared as follows (per L): 1 g of NH_4Cl, 10 g of NaCl, 0.4 g of $MgSO_4.7H_2O$, 0.14 g of KH_2PO_4, 1 g of K_2HPO_4, 3 g of $Na_2S_2O_3.5H_2O$ and 1 g of $NaHCO_3$ (Nacalai Tesque, Kyoto, Japan) in distilled water. The first two compounds were combined with 1 mL of trace metal solution (Robertson and Kuenen, 1983) and autoclaved at 121 °C for 20 min. Subsequent components were added after filter sterilization with 1 mL of vitamin solution through a cellulose acetate membrane filter (0.2 μm).

Enrichment in a CSTR was initiated by inoculating 5 g of the sediment sample into 1.5 L of medium in a Middle-Scale BMS-P Fermenter (ABLE Biott, Tokyo, Japan). A schematic diagram of the CSTR system used in this study is presented in Figure 1. The culture was maintained at 28 °C and the pH was adjusted to 7.4 by an automatic pH regulator using 0.9 M $NaHCO_3$ and 0.5 M HCl as alkaline and acid solutions, respectively. Sterile medium was supplied constantly using a peristaltic pump to achieve a hydraulic retention time of 72 h. Dissolved oxygen was maintained at 70% to 100% of air saturation by aeration (0.5 L of membrane-filtered air per min) and stirring at 110 rpm. The continuous culture was maintained for 26 weeks prior to isolation on TM agar. To determine the community composition, DNA samples were obtained at weeks 2, 4, and 26 using PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA), and they were examined by a clone analysis.

Isolation of autotrophic thiosulfate-oxidizing bacteria

For isolation, bacteria were obtained from the 26-week-old continuous culture. The bacterial sample was subjected to serial dilution prior to spreading on TM agar plates. The medium was composed of the following (per L of distilled water): 1 g of NH_4Cl, 10 g of NaCl, 0.8 g of $MgSO_4{\times}7H_2O$, 2 g of KH_2PO_4, 5 g of $Na_2S_2O_3{\times}5H_2O$, 1 g of $NaHCO_3$, 1 mL of trace metal solution, 1 mL of vitamin solution and 1.5% agar (Nacalai Tesque). As a pH indicator, 2 mL of 0.5% phenol red was added and the final pH of the medium was adjusted to 7.1. The inoculated plates were incubated at 30 °C for 7 days under aerobic conditions. Colonies that induced a change in the color of the medium to yellow were picked up and purified on agar plates with the same composition.

PCR amplification

The 16S rRNA and *aprA* genes were amplified from DNA samples obtained from the CSTR using AmpliTaq Gold (Applied Biosystems, Carlsbad, CA, USA) in a T-Gradient Biometra Thermocycler (Biometra, Goettingen, Germany). The universal bacterial primer set of 27F (5t-AGAGTTTGATCCTGGCTCAG-3t) and 518R (5t-GTATTACCGCGGCTGCTGG-3t) was used to amplify the partial 16S rRNA gene. PCR was performed under the following conditions: initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min. Amplification of the partial *aprA* gene followed the procedures described in Meyer and Kuever (2007) using 60 °C as a single annealing temperature. To confirm that the amplicons were of the correct size, the PCR reactions were subjected to electrophoresis on 1.5% (w/v) agarose gels in 1× TAE buffer. Gels were stained with ethidium bromide and reaction products were visualized by illumination with ultraviolet light at 302 nm. PCR products that were confirmed to be of the correct size were purified using the UltraClean PCR Clean-Up Kit (MoBio, Carlsbad, CA, USA). PCR was also performed to examine the presence of other genes related to sulfur oxidation in isolated colonies. The procedures for both *soxB* and *sqr* gene amplifications were adopted from Petri *et al*. (2001) and Pham *et al*. (2008), respectively. Moreover, to confirm autotrophy in the isolates, PCR was used to detect the presence of *cbbL* gene with primer sets and cycle conditions as described in Spiridonova *et al*. (2004).

Construction of clone library and nucleotide sequencing

The purified PCR products of 16S rRNA and *aprA* were ligated into pT7Blue T-vectors (Novagen, Madison, WI, USA) with DNA Ligation Kit Mighty Mix (TaKaRa, Otsu, Japan) and transformed into *Escherichia coli* DH5×á Competent Cells (TaKaRa) following the manufacturer's instructions. X-Gal Agar (Invivogen, San Diego, CA, USA) was used to isolate the transformed *E. coli*. The white colonies were picked up and cultivated in liquid LB medium. Extraction of inserted plasmids was subsequently carried out using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) following the manufacturer's instructions. The cloned plasmids were screened for correctly sized inserts by digestion at 37 °C for 60 min with *EcoR*×I and *Pst*×I restriction enzymes (TaKaRa). The digested plasmids were subjected to 1.0 % (w/v) agarose gel electrophoresis, stained with ethidium bromide, and visualized with a UV illuminator. Plasmids with inserts of the expected size were selected for sequencing, which was performed by TaKaRa using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequence analysis

Readable sequences of twenty clones from each sampling time and each gene were chosen for analysis. Sequences of 16S rRNA and *aprA* from the GenBank database were aligned using the web-based BLAST analysis tools (National Center for Biotechnology Information, Bethesda, MD, USA; www.ncbi.nlm.nih.gov/BLAST/) to determine their phylogenetic relationships and identify their closest relatives. The gene sequences for the isolates were aligned with their closest reference sequence using CLUSTAL×X 2.1 (Larkin *et al*., 2007). To determine the phylogenetic relationships between the sequences, a neighbor-joining tree was constructed using MEGA version 6 (Tamura *et al*., 2013). The bootstrap method was performed with 500 replicates to estimate the reliability of the inferred phylogenetic nodes.

RESULTS

Community composition in continuous culture

We determined the community composition using a clone analysis of 16S rRNA at different sampling times during the continuous culture. In the 2-week-old culture, the clones were dominated by autotrophic strains, which represented 80% of the total clones, while the remaining species were heterotrophic. This composition was not significantly different for longer incubation periods; autotrophs were dominant irrespective of incubation time (Figure 2). The autotrophs were identified as *Gammaproteobacteria*, and among them two genera were detected. One of these genera was the most abundant among all clones and shared

94.3-98.8% partial 16S rRNA sequence with *Thiomicrospira pelophila*, while the other was similar to *Thioalkalivibrio* sp. with identity of 90%.

We detected heterotrophic sulfur oxidizers even after a 26-week incubation. They were identified as *Alphaproteobacteria*, *Bacteroidetes*, and *Gammaproteobacteria*, and might be capable of thiosulfate oxidation. Sequence similarities between the obtained heterotrophic clones and reference strains were between 89% and 96%. Based on the similarity values, the detected autotrophs and heterotrophs were likely novel species.

According to *aprA* sequences, all clones obtained at each sampling time were related to uncultured bacteria with sequence similarities of 87-–98%. Most abundant belonged to the class *Betaproteobacteria* that shared 93–97% partial *aprA* sequence with *Thiobacillus denitrificans*. Meanwhile, the other clones were distributed in separated groups of *Betaproteobacteria* and *Gammaproteobacteria* (Figure 3). Over time, the diversity of *aprA* clones decreased (data not shown). At 2 weeks, ten operational taxonomic units were detected, while at 4 and 26 weeks, no more than three operational taxonomic units were found in each sample. Strains that harbor *aprA* were likely outcompeted by the dominant strains.

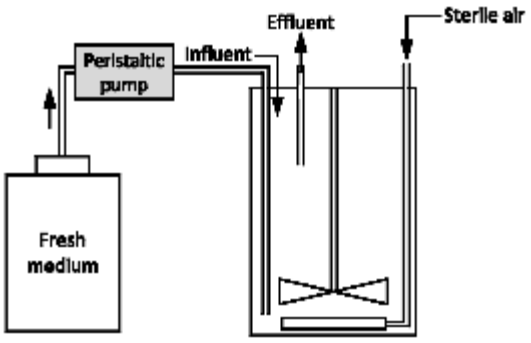

Fig. 1. Schematic diagram of the continuous reactor for the enrichment of autotrophic thiosulfate-oxidizing bacteria

Isolation of autotrophic thiosulfate oxidizers from continuous culture

We sampled the culture after 26 weeks to isolate autotrophic sulfur-oxidizing bacteria on agar plates with TM medium. Within the first day of incubation, phenol red in the agar plates turned to pink, indicating the production of alkaline compounds by the growing colonies. Hydroxides are commonly produced by heterotrophic sulfur bacteria during the oxidation of thiosulfate to tetrathionate (Mason and Kelly, 1988). Small yellow spots were observed over the next few days, and after 5–7 days, the color of the medium became completely yellow. This indicated that sulfuric acid was released during the oxidation of thiosulfate by autotrophic and acid-producing heterotrophic sulfur-oxidizing bacteria (Teske *et al*., 2000).

As evidenced by the existence of the *cbbL* gene, nine acid-producing isolates were obtained. The isolates formed an intense yellow color in the agar plate and their viability was very low in acidic conditions. It was difficult to achieve successful subculture on agar plates when grown in high-density conditions for more than 3 days. Sufficient distance between colonies on the plate improved viability. It is possible that the released acid was destructive to the cells.

The partial 16S rRNA genes of the isolates were sequenced and analyzed. Their sequences were identical and thus we concluded that the nine isolates belonged to a single strain. Hence, we choose one isolate for additional

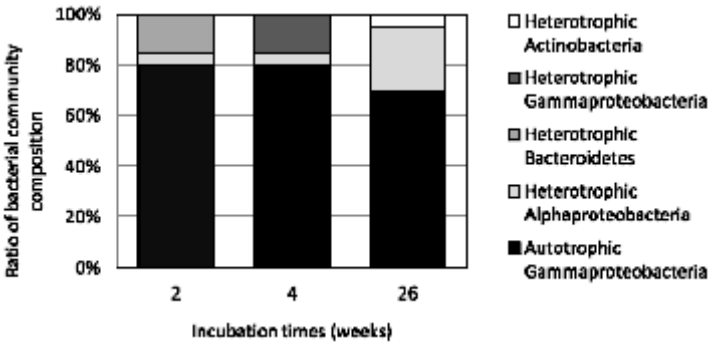

Fig. 2. Comparison of bacterial communities for different incubation times at the continuous culture

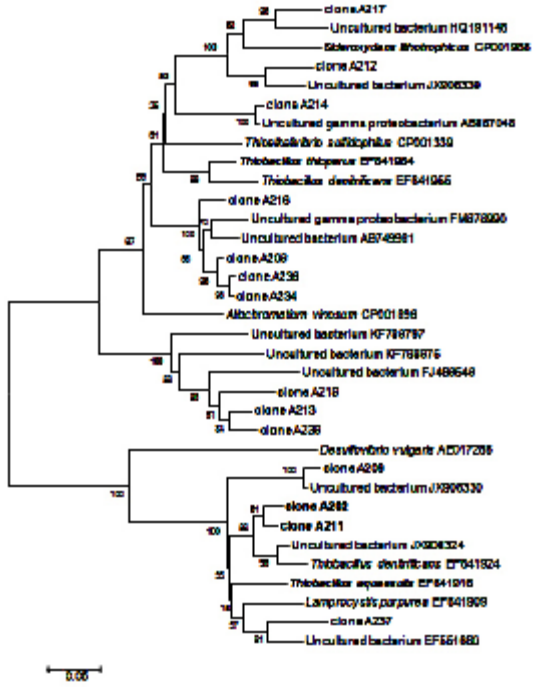

Fig. 3. Phylogenetic tree of partial AprA sequences from autotrophic-thiosulfate oxidizers in the continuous culture and reference strains. The representatives of dominant clones are typed in bold. The tree was generated using the neighbor-joining method. Bootstrapping values are indicated near the nodes. The scale bar corresponds to 5% estimated sequence divergence.

analysis, MKAI-19. Taxonomically, the isolate was closely related (99.1% identity) to *Hydrogenovibrio marinus*, a sulfur bacterium capable of hydrogen oxidation isolated from seawater in Japan (Nishihara *et al*., 1991). In addition, it also shared 97.8% and 95.5% partial 16S rRNA sequence to *Thiomicrospira kuenenii* and *Tms. crunogena*, respectively.

The phylogenetic positions of MKAI-19 and the representative dominant clone, which were affiliated with *Thiomicrospira*, and related strains are illustrated in Figure 4. Both were in the *Thiomicrospira* group, but they were located on separated branches with 88.1% sequence similarity. PCR detection of sulfur oxidation genes revealed the absence of *aprA* and the existence of both *soxB* and *sqr* in MKAI-19. This indicated that the isolate uses the Sox enzyme system in thiosulfate oxidation and is capable of sulfide oxidation using sulfide:quinone reductase instead of the Apr enzyme system.

DISCUSSION

Community composition in the continuous culture

The clone analysis showed that there was no change in the bacterial community composition for longer incubation periods in the continuous culture. It indicated that the prolonged incubation period did not significantly affect the community composition. The thiosulfate mineral medium specifically supports the growth of autotrophic

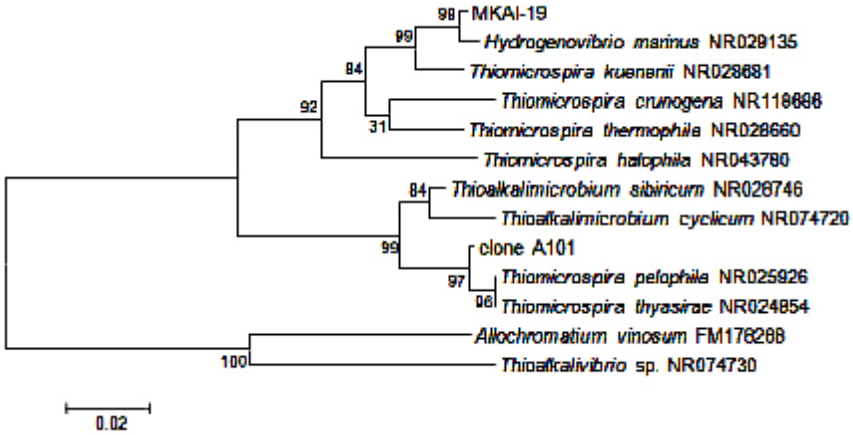

Fig. 4. Phylogenetic tree of partial 16S rRNA sequences presents the relationships between the isolated MKAI-19 and a representative of dominant clones from the continuous culture. The tree was generated using the neighbor-joining method. Bootstrapping values are indicated near the nodes. The scale bar corresponds to 2% estimated sequence divergence.

thiosulfate oxidizers, but heterotrophic thiosulfate oxidizers, such as *Paracoccus* and *Marinobacter*, remained. They were not eliminated by an extended incubation period. It is likely that they obtained sufficient organic carbon to support growth from excreted waste products of the autotrophs and products of lysed cells. According to 16S rRNA clone library, *Gammaproteobacteria* were the dominant autotroph. In contrast, the majority of *aprA* clones was related to uncultured *Betaproteobacteria* and was not related to the 16S rRNA clones. This suggested that autotrophic *Betaproteobacteria* existed in the continuous culture, but were much less abundant than *Gammaproteobacteria*, and even less abundant than the detected heterotrophs.

In the culture, the *aprA* gene was considered less abundant than the other functional gene, *soxB*. The *soxB* is widely distributed in *Thiomicrospira*, *Thioalkalivibrio*, and *Thiobacillus* (Meyer *et al*., 2007; Muyzer *et al*., 2011). In contrast, *aprA* was not found in genus *Thiomicrospira* as reported by (Meyer and Kuever, 2007). The gene is commonly found in *Thiobacillus*, however *Betaproteobacteria* were existed in the culture at very low abundance. The isolated MKAI-19, which was close to the genus *Thiomicrospira*, lacked *aprA* and harbored *soxB*. Moreover, the diversity of *aprA* clones in the culture decreased as a function of time. This showed that the bacteria harboring *aprA*, especially *Thiobacillus*, were not well supported in the culture conditions. The Sox enzyme system catalyzes the oxidation of several reduced inorganic sulfur compounds, including thiosulfate, while Apr, associated with the rDsr, is responsible for the oxidation of H_2S (Hugler *et al*., 2010; Loy *et al*., 2009). As reported by Beller *et al*. (2006), the metabolically versatile *Thiobacillus* use *soxB* for thiosulfate oxidation, but in this study their growth was outcompeted in the continuous culture by *Thiomicrospira*. Presumably, in the competition for thiosulfate oxidation, *Thiomicrospira* was more specialized than *Thiobacillus*.

Isolation of the dominant genus in the continuous culture

The isolated strain MKAI-19 and the dominant clones belonged to the group *Thiomicrospira*, but they were not located in the same branch at the phylogenetic tree. The dominant clones were existed in high abundance; however, they were unculturable on the agar plate. The environmental conditions for bacteria in the liquid culture were dissimilar to those on the agar plate. In liquid culture, the interchange of metabolically important compounds among various strains in the community was allowed. Therefore, the growth of dominant strains in the culture may have been supported by the existing heterotrophic thiosulfate oxidizers.

The autotrophic isolate grew very well on the agar plate by oxidizing thiosulfate to sulfate. Existence of *soxB* and *sqr* indicated that MKAI-19 had a role in oxidation of thiosulfate and sulfide in the sulfur cycle. Additionally, we compared the 16S rRNA sequence of the isolate and the dominant clones with the clone library of Midorikawa Tidal Flat samples from the previous study in our laboratory. The clones that were similar to both of them were not detected in the clone library. As a lithoautotroph, the isolate had high affinity to sulfur compounds owing to their dependence on inorganic sulfur compounds as energy sources. Despite relatively low population sizes, MKAI-19 may exhibit high activity and therefore be important in the sulfur cycle of the tidal flat.

CONLUSIONS

In the continuous culture, extended period of incubation did not influence the community composition of thiosulfate-oxidizing bacteria. Autotrophic *Gammaproteobacteria* were dominating the community while thiosulfate-oxidizing heterotrophs were detected along the incubation period. The culture conditions supported growth of the dominant strains; however, they had low viability on agar plate. It showed their dependence on other strains in the community.

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REFERENCES

1. Azad, M.A.K., Ohira, S., Oda, M., Toda, K. On-site measurements of hydrogen sulfide and

sulfur dioxide emissions from tidal flat sediments of Ariake Sea, *Japan. Atmos. Environ.,* 2005; : 6077-87.

2. Beller, H.R., Chain, P.S.G., Letain, T.E., Chakicherla, A., Larimer, F.W., Richardson, P.M., Coleman, M.A., Wood, A.P., Kelly, D.P. The genome sequence of the obligately chemolithoautotrophic, facultatively anaerobic bacterium *Thiobacillus denitrificans*. *J. Bacteriol.,* 2006; **188**(4): 1473-88.
3. Friedrich, C.G., Rother, D., Bardischewsky, F., Quentmeier, A., Fischer, J. Oxidation of reduced inorganic sulfur compounds by bacteria: Emergence of a common mechanism?. *Appl. Environ. Microbiol.,* 2001; **67**(7): 2873-82.
4. Hugler, M., Gartner, A., Imhoff, J.F. Functional genes as markers for sulfur cycling and CO_2 fixation in microbial communities of hydrothermal vents of the Logatchev field. *FEMS Microbiol. Ecol.,* 2010; **73**(3): 526-37.
5. Jorgensen, B.B. A thiosulfate shunt in the sulfur cycle of marine sediments. *Science.,* 1990; **249**: 152-4.
6. Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G. Clustal W and Clustal X version 2.0. *Bioinformatics.,* 2007; **23**: 2947-8.
7. Loy, A., Duller, S., Baranyi, C., Mussmann, M., Ott, J., Sharon, I., Beja, O., Le Paslier, D., Dahl, C., Wagner, M. Reverse dissimilatory sulfite reductase as phylogenetic marker for a subgroup of sulfur-oxidizing prokaryotes. *Environ. Microbiol.,* 2009; **11**(2): 289-99.
8. Mason, J., Kelly, D.P. Thiosulfate oxidation by obligately heterotrophic bacteria. *Microb. Ecol.,* 1988; **15**(2): 123-34.
9. Meyer, B., Imhoff, J.F., Kuever, J. Molecular analysis of the distribution and phylogeny of the *soxB* gene among sulfur-oxidizing bacteria - Evolution of the Sox sulfur oxidation enzyme system. *Environ. Microbiol.,* 2007; **9**(12): 2957-77.
10. Meyer, B., Kuever, J. Molecular analysis of the diversity of sulfate-reducing and sulfur-oxidizing prokaryotes in the environment, using *aprA* as functional marker gene. *Appl. Environ. Microbiol.,* 2007; **73**(23): 7664-79.
11. Nishihara, H., Igarashi, Y., Kodama, T. *Hydrogenovibrio marinus* gen. nov., sp. nov., a marine obligately chemolithoautotrophic hydrogen-oxidizing bacterium. *Int. J. Syst. Bacteriol.,* 1991; **41**(1): 130-3.
12. Petri, R., Podgorsek, L., Imhoff, J.F. Phylogeny and distribution of the *soxB* gene among thiosulfate-oxidizing bacteria. *FEMS Microbiol. Lett.,* 2001; **197**: 171-8.
13. Pham, V.H., Yong, J.J., Park, S.J., Yoon, D.N., Chung, W.H., Rhee, S.K. Molecular analysis of the diversity of the sulfide:quinone reductase (*sqr*) gene in sediment environments. *Microbiology.,* 2008; **154**(10): 3112-21.
14. Robertson, L.A., Kuenen, J.G. *Thiosphaera pantotropha* gen. nov. sp. nov., a facultatively anaerobic, facultatively autotrophic sulphur bacterium. *J. Gen. Microbiol.,* 1983; **129**: 2847-55.
15. Spiridonova, E.M., Berg, I.A., Kolganova, T.V., Ivanovsky, R.N., Kuznetsov, B.B., Tourova, T.P. An oligonucleotide primer system for amplification of the ribulose-1,5-bisphosphate carboxylase/oxygenase genes of bacteria of various taxonomic groups. *Microbiology.,* 2004; **73**(3): 316-25.
16. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.,* 2011; **28**(10): 2731-9.
17. Teske, A., Brinkhoff, T., Muyzer, G., Moser, D.P., Rethmeier, J., Jannasch, H.W. Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. *Appl. Environ. Microbiol.,* 2000; **66**(8): 3125-33.
18. Vegas, C., Gonzalez, A., Mateo, E., Mas, A., Poblet, M., Torija, M.J. Evaluation of representativity of the acetic acid bacteria species identified by culture-dependent method during a traditional wine vinegar production. *Food Res. Int.,* 2013; **51**(1): 404-11.
19. Watanabe, T., Kojima, H., Takano, Y., Fukui, M. Diversity of sulfur-cycle prokaryotes in freshwater lake sediments investigated using *aprA* as the functional marker gene. *Syst. Appl. Microbiol.,* 2013; **36**(6): 436-43.