# Analysis of Microbial Communities Involved in Anaerobic Sulfur-Oxidation in UASB Reactor Treating Municipal Sewage

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## 1. INTRODUCTION

In sulfur cycle, sulfur-oxidizing microbes in particular, catalyze a central step in the global sulfur cycle (1). This sulfur oxidation by microbes is a reaction performed by a very heterogeneous group of organisms which share the ability to oxidize reduced sulfur compounds by the presence of electron acceptors (2). This sulfur oxidation reaction had occurred in our series of studies of the municipal sewage treatment without the electron acceptors such as oxygen, nitrate and oxidized-irons. In these studies, an up-flow anaerobic sludge blanket (UASB) reactor with sulfur-redox reaction under low-temperature conditions was used and the anaerobic sulfur oxidation occurred in the absence of electron acceptors. With these considerations in mind, we explore the most probable microbial community that drives the anaerobic sulfur oxidation reaction. Therefore, the aim of this study is to analyze and identify the changes in bacteria and archaea groups that contribute to the contrasting performance and microbial community characteristics in anaerobic sulfur oxidation conditions.

## 2. MATERIALS AND METHODS

### 2.1 Reactor operation and sample collection

The 1178 L UASB reactor with a height of 4.7 m was set up at the municipal sewage treatment plant in Nagaoka, Niigata, Japan. The system was fed with raw sewage that was added with sodium sulfate. The sodium sulfate concentration was adjusted from 40-150 mg-S·$L^{-1}$. The system was operated at ambient temperature and the hydraulic retention time was set to 8 h. The sludge samples were collected from the UASB reactor on day 91, before sodium sulfate was added and days 111, 167 and 335, after the addition of sodium sulfate. The collected samples were then kept in a container containing ice during delivery to the laboratory and stored at -20°C for further analysis.

### 2.2 16S rRNA gene clone library, T-RFLP and FISH analysis

Genomic DNA was isolated from the sludge sample and subjected to PCR amplification of 16S rRNA gene using Premix Ex Taq Kit (Takara Bio, Shiga, Japan). A set of bacteria-specific primer, EUB338f and UNIV1500r, and a set of archaea-specific primer, ARC109f and Univ1500r, was used for PCR amplification. The PCR products were purified and then cloned using TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The positive clones were selected and sent to Dragon Genomics Center (TAKARA BIO, Yokkaichi, Japan) for sequencing. The raw sequencing data of the 16S rRNA gene were analyzed for possible chimeras using the Bellerophon program (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl). The sequences with 97% similarity were then grouped into operational taxonomic units (OTUs) using FastGroup II program (http://biome.sdsu.edu/fastgroup/index.htm). Representative sequences were phylogenetically classified using the Classifier tool from the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) and the ARB program. For terminal restriction fragment length polymorphism (T-RFLP) analysis, PCR was performed by using fluorescently labeled primers 907r and ARC912r for bacteria and archaea, respectively. *Hha*I and *Taq*I were selected to digest the PCR products of bacteria and archaea 16S rRNA gene purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany), respectively and were run through capillary sequencer. For fluorescence in situ hybridization (FISH) analysis, Cy3 labeled probe specific to bacteria OP5 was used and the hybridized cells with the probes were observed using an epifluorescence microscope.

## 3. RESULTS AND DISCUSSION

### 3.1 Reactor performance

Time course of the reduced and oxidized sulfur concentration showed the oxidized sulfur were inversely proportional with the temperature after the addition of the sodium sulfate where sulfide was oxidized to sulfate during the cold season but did not oxidize during hot season. This can be further shown in the UASB profiles of sulfate and sulfide on days 91, 111, 167 and 335. The profiles were observed and showed that the sulfur oxidation stopped after 335 days when the temperature started to increase. Sulfur oxidation occurred after 167 days of operation where sulfide was oxidized to sulfate. However, sulfur oxidation did not occur during the 91 and 111 days of operation. When sulfur oxidation occurred, the oxidation-reduction potential (ORP) was observed to be always less than -300 mV, and the dissolved oxygen (DO), nitrate and nitrite were not detected which shows that the anaerobic conditions were maintained and lower sewage temperature was important for anaerobic sulfur oxidation occurrence.

### 3.2 Clone library analysis of bacterial and archaeal diversity

From the 16S rRNA cloning analysis results shown in Table 1, there are varieties of bacteria and archaea species present in the reactor. Among all the bacteria species, the major bacterial groups monitored in the UASB reactor were found to be genus *Caldisericum* and *Smithella*. Meanwhile, the acetate-utilizing methanogen, *Methanosaeta* spp. was the dominant group of the domain archaea. In the previous research carried out by Mori *et al.* (3,4), they discovered a new bacteria strain represents a

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new genus and novel species named *Caldisericum exile* within the phylum *Caldiserica*, previously known as uncultured phylum OP5, obtained from anaerobic environments. The anaerobic growth of this bacterium is observed with the reduction of sulfur compounds such as thiosulfate, sulfite and elemental sulfur but with no presence of sulfate, fumarate, nitrate, nitrite and oxygen as electron acceptors. Furthermore, some of the OP5 clones were retrieved from relatively sulfur-rich environments. The description given in the study showed that this species is not closely related and with low percentage of sequence similarity to any other known bacteria in recognized phyla. Therefore, from this findings, it indicated that this genus *Caldisericum* present in this UASB reactor probably related to a sulfur cycle which may be the main player in the sulfur oxidation reaction process occurred in the UASB reactor which is consistent with the results reported by Mori *et al*. Other dominated bacterial species was affiliated to the genus *Smithella* but phylogenetically distantly related to *S. propionica* (sequence similarity of 16S rRNA gene is around 97%). *S. propionica* is strictly grown in an anaerobic condition and are unable to use sulfate as an electron acceptor (5). They are able to grow on propionate in syntrophic association with methanogens. They oxidized propionate to acetate, carbon dioxide and hydrogen, which acetate is produced more. With this conditions where acetate is widely produced, *Methanosaeta* spp. which have much higher affinity for acetate (6) will have much faster growth rate and higher yield such as the results shown in this study.

### 3.3 T-RFLP and FISH analysis

The T-RFLP profile presented in Figure 2 also confirms the trend detected in the clone libraries. The T-RFLP analysis of bacteria showed some changes before and after the addition of sulfate where the results yielded characteristic fingerprints for bacterial communities. Meanwhile, the archaeal community structure yielded stable microbial community till the end of the oxidation process. The availability of T-RFLP data through the operational period allowed us to monitor changes in community structure over time. Furthermore, *Caldisericum* cells were detected from UASB retained sludge by FISH analysis, which confirmed the actively presence of this bacteria in the UASB reactor which may indicates its contribution in this sulfur oxidation reaction process.

## 4. CONCLUSIONS

Through all these findings, the microbial community structures of the bacterial and archaeal groups present in the UASB reactor were identified. Thus, it can be concluded that genus *Caldisericum* and probably *Smithella* also may have contributed and played an important role in sulfur oxidation reaction in the UASB reactor.

**Table 1**. Phylogenetic affiliation and numbers of bacterial and archaeal 16S rRNA gene clones in the UASB sludge.

| Phylogenetic affiliations | No of clones (% of total clones) | | |
|---|---|---|---|
| | Day 91 | Day 111 | Day 167 |
| Bacteria | | | |
| *Acidobacteria* | | | |
| *Holophaga* | 1 (1.1) | 2 (2.2) | 5 (5.9) |
| *Bacteroidetes* | | | |
| *Prolixibacter* | 4 (4.3) | 9 (9.7) | 1 (1.2) |
| *Caldiserica* | | | |
| *Caldisericum* | 4 (4.3) | 14 (15.0) | 13 (15.3) |
| *Firmicutes* | | | |
| *Acidaminobacter* | 4 (4.3) | 2 (2.2) | |
| *Proteobacteria* | | | |
| *Desulforhabdus* | 1 (1.1) | 2 (2.2) | 5 (5.9) |
| *Smithella* | 22 (23.9) | 17 (18.2) | 11 (12.9) |
| *Syntrophorhabdus* | 1 (1.1) | 3 (3.2) | 4 (4.7) |
| Others | 55 (59.8) | 44 (47.3) | 46 (54.1) |
| Total | 92 (100) | 93 (100) | 85 (100) |

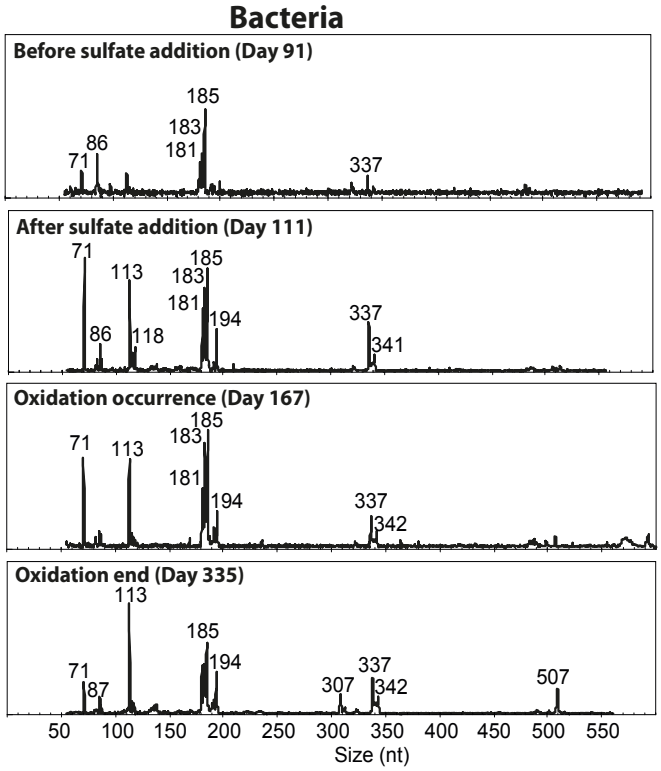


**Figure 2**. T-RFLP profile of bacterial 16S rRNA gene from different time periods of UASB reactor.

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