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Molecular characterization of anaerobic sulfur-oxidizing microbial communities in up-flow anaerobic sludge blanket reactor treating municipal sewage

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A novel wastewater treatment system consisting of an up-flow anaerobic sludge blanket (UASB) reactor and a down-flow hanging sponge (DHS) reactor with sulfur-redox reaction was developed for treatment of municipal sewage under low-temperature conditions. In the UASB reactor, a novel phenomenon of anaerobic sulfur oxidation occurred in the absence of oxygen, nitrite and nitrate as electron acceptors. The microorganisms involved in anaerobic sulfur oxidation have not been elucidated. Therefore, in this study, we studied the microbial communities existing in the UASB reactor that probably enhanced anaerobic sulfur oxidation. Sludge samples collected from the UASB reactor before and after sulfur oxidation were used for cloning and terminal restriction fragment length polymorphism (T-RFLP) analysis of the 16S rRNA genes of the bacterial and archaeal domains. The microbial community structures of bacteria and archaea indicated that the genus *Smithella* and uncultured bacteria within the phylum *Caldiserica* were the dominant bacteria groups. *Methanosaeta* spp. was the dominant group of the domain archaea. The T-RFLP analysis, which was consistent with the cloning results, also yielded characteristic fingerprints for bacterial communities, whereas the archaeal community structure yielded stable microbial community. From these results, it can be presumed that these major bacteria groups, genus *Smithella* and uncultured bacteria within the phylum *Caldiserica*, probably play an important role in sulfur oxidation in UASB reactors.

[**Key words:** Wastewater treatment; Up-flow anaerobic sludge blanket; Anaerobic sulfur oxidation; Microbial community; Molecular analysis]

Up-flow anaerobic sludge blanket (UASB) technology has been widely used for sewage treatment especially in warm regions because of its high treatment efficiency and cost efficacy (1). However, the efficiency of the anaerobic methanogenic process tends to decrease at low temperature because of the suspension methanogenic activity (2,3). Thus, an aerobic post-treatment system, which has also been reported to achieve high chemical oxygen demand (COD) removal at short hydraulic retention time (HRT) and has proven to be an effective method for various types of wastewater treatment (4), is necessary. In these series of studies for treatment of municipal sewage under low-temperature conditions, a wastewater treatment system consisting of a UASB and down-flow hanging sponge (DHS) reactor with a sulfur-redox reaction was developed. Previous studies showed that the sulfur-redox UASB-DHS system is applicable for low-strength wastewater treatment under low-temperature conditions such as below 10°C with over 90% biochemical oxygen demand (BOD) removal (3,5).

In these series of wastewater treatment studies, an interesting phenomenon occurred in the UASB reactor tank where anaerobic sulfur oxidation reaction was initiated without the presence of electron acceptors such as oxygen, nitrate, nitrite and oxidized-irons. It was presumed that this sulfur oxidation reaction was mediated biologically by the microorganisms that actuate the reaction. Sulfur-oxidizing microbes catalyze a central step in the global sulfur cycle (6). These microbes share the ability to oxidize reduced sulfur compounds such as sulfide, elemental sulfur, polythionates and thiosulfate to sulfate as a final oxidation product (7). Many studies have evaluated the characteristics of the sulfur-oxidizing microbial community (8–10). However, information regarding sulfur oxidation under anaerobic conditions and the actual diversity and distribution pattern of the microorganisms is still inadequate. In contrast to well-characterized laboratory-cultured microorganisms, the microorganisms in the reactor could have unknown behavior or contain novel or not-yet-cultured species (11). Therefore, the aim of this study was to analyze and identify the bacteria and archaea groups that contribute to the contrasting performance and microbial community characteristics during anaerobic sulfur oxidation by molecular analysis of the 16S rRNA gene.

MATERIALS AND METHODS

Reactor operation, sample collection and water quality analysis A 1178 L UASB reactor with a height of 4.7 m was set up at the municipal sewage treatment plant in Nagaoka, Niigata, Japan without an open settling compartment where no light enters (Fig. 1). The system was fed with raw sewage that was supplemented

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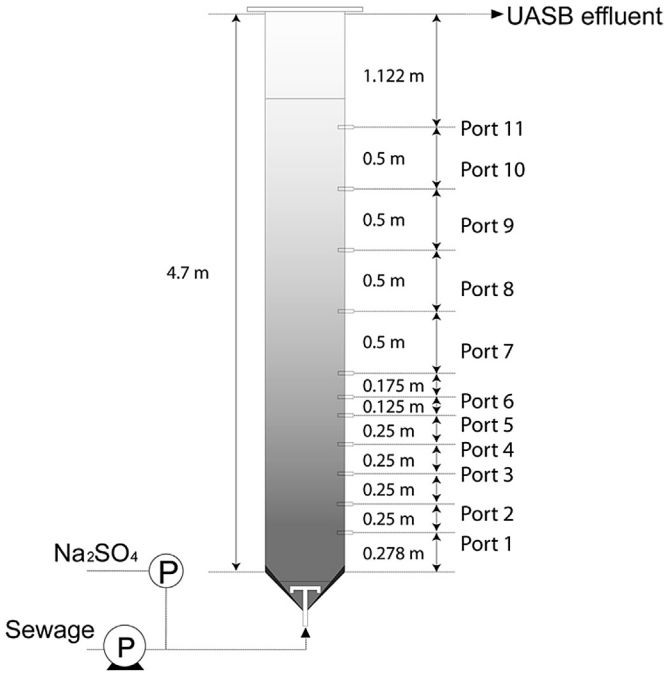

FIG. 1. Schematic diagram of the UASB reactor.

with 150 mg-S/L sodium sulfate. The system was operated at ambient temperature and the HRT was set to 8 h. The sludge samples were collected from each port of the UASB reactor on day 91, before sodium sulfate was added, and on 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 microbial analysis. The temperature, pH, oxidation-reduction potential (ORP) and dissolved oxygen (DO) were measured on site by portable devices. Sulfate was analyzed using high-performance liquid chromatography (HPLC) (Shimadzu LC 20-ADsp) equipped with a Shin-pack IC-A3 column after filtration using 0.2 μm membrane filter. The mobile phase used consists of 3.2 mM bis-tris, 8 mM *p*-hydroxybenzoic acid and 50 mM boric acid at a flow rate of 1.0 ml/min. Sodium sulfate was used as standard. The COD was analyzed using a HACH water quality analyzer (HACH DR2500). Sulfide concentration was measured by iodometric titration method based on standard methods published by the Japan Sewage Works Association (12).

DNA extraction, polymerase chain reaction, cloning and sequencing Genomic DNA was isolated from approximately 500 mg of sludge sample using a FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, USA) according to the manufacturer's protocol. The DNA concentration was determined by using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene from extracted DNA was conducted using Premix Ex Taq Kit (Takara Bio, Shiga, Japan). A set of bacteria-specific primers, EUB338f and UNIV1500r, and a set of archaea-specific primers, ARC109f and Univ1500r, were used for PCR amplification. Amplification of the 16S rRNA gene was performed in a final volume of 20 μl containing 20 ng of extracted DNA under the following conditions: initial denaturation step of 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The final cycle was followed by final extension at 72°C for 4 min. The PCR products were purified according to the protocol provided in the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and then cloned using a 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 chimerism using the Bellerophon program (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl). Sequences with 97% similarity were then grouped into operational taxonomic units (OTUs) using the FastGroup II program (http://biome.sdsu.edu/fastgroup/index.htm) and classified using the Classifier tool from the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/).

Phylogenetic analysis Phylogenetic analyses were conducted using the 16S rRNA gene sequence. The 16S rRNA gene sequences were aligned with an ARB data set using ARB software and the resulting alignment was re-corrected manually considering primary and secondary structures. A phylogenetic tree was constructed using the neighbor-joining method and a bootstrap analysis of 1000 replicate data sets was conducted using the ARB software for tree topology confidence estimation.

Terminal restriction fragment length polymorphism analysis PCR was performed by using fluorescently labeled primers 907r and ARC912r for bacteria and archaea, respectively. *Hae*III and *Hha*I were selected to digest the PCR products of bacteria, whereas *Taq*I was selected to digest the PCR products of archaea 16S rRNA gene purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The digested PCR products were then run through a CEG-2000XL capillary sequencer

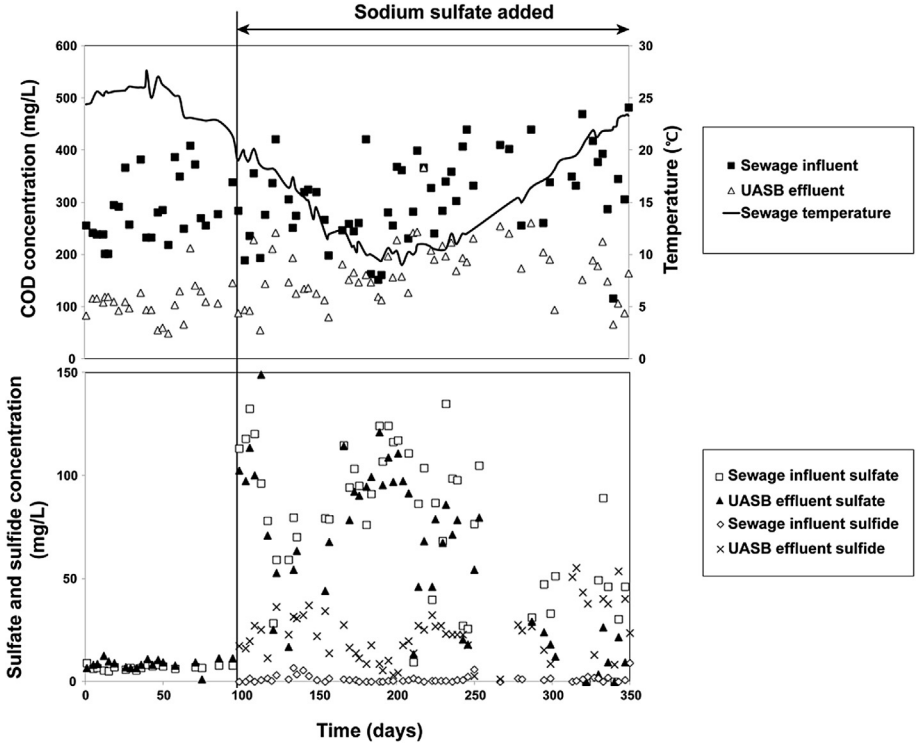

FIG. 2. COD, sulfate and sulfide concentrations in the UASB reactor.