**Molecular Characterization of Anaerobic Sulfur-Oxidizing Microbial
Communities in Up-flow Anaerobic Sludge Blanket Reactor Treating Municipal
Sewage**

Running title: Anaerobic sulfur-oxidizing microbial communities

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ABSTRACT

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

INTRODUCTION

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

(8,9,10). 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 1,178 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 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 consist of 3.2 mM bis-tris,

84 8 mM *p*-hydroxybenzoic acid and 50 mM boric acid at a flow rate of 1.0 ml/min.
85 Sodium sulfate was used as standard. The COD was analyzed using a HACH water
86 quality analyzer (HACH DR2500). Sulfide concentration was measured by iodometric
87 titration method based on standard methods published by the Japan Sewage Works
88 Association (12).

89 **Fig. 1**

90 **DNA extraction, polymerase chain reaction (PCR), cloning and sequencing**

91 Genomic DNA was isolated from approximately 500 mg of sludge sample
92 using a FastDNA® SPIN Kit for Soil (MP Biomedicals, LLC, USA) according to the
93 manufacturer's protocol. The DNA concentration was determined by using a
94 NanoDrop® Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). PCR
95 amplification of the 16S rRNA gene from extracted DNA was conducted using
96 Premix Ex Taq Kit (Takara Bio, Shiga, Japan). A set of bacteria-specific primers,
97 EUB338f and UNIV1500r, and a set of archaea-specific primers, ARC109f and
98 Univ1500r, were used for PCR amplification. Amplification of the 16S rRNA gene
99 was performed in a final volume of 20 µl containing 20 ng of extracted DNA under
100 the following conditions: initial denaturation step of 94°C for 2 min, followed by 35
101 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at
102 72°C for 1 min. The final cycle was followed by final extension at 72°C for 4 min.
103 The PCR products were purified according to the protocol provided in the QIAquick
104 PCR purification kit (Qiagen, Hilden, Germany) and then cloned using a TOPO PCR
105 cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's
106 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 1,000 replicate data sets was conducted using the ARB software for tree topology confidence estimation.

Terminal-restriction fragment length polymorphism (T-RFLP) 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 (Beckman Coulter, Fullerton, CA) as described previously by Hatamoto *et al.* (13). *In silico* T-RF prediction was performed using TRiFLe as described by Junier *et al.* (14) and the predicted T-RFs were correlated with the cloning results.

130

131 **RESULTS AND DISCUSSION**

132 **Reactor performance**

133 Over one year of operation, the COD, BOD, sulfate and sulfide concentration of
134 the process performance were recorded (Fig. 2). The average total COD concentration
135 of the UASB effluent was 162.4 mg/L with a removal rate of 48.1%. The total BOD
136 concentration of the UASB effluent was 67.4 mg/L with a 57.2% removal rate. This
137 reactor performance is common in UASB reactors treating domestic sewage under
138 ambient conditions (5,15). Before addition of sodium sulfate, the average UASB
139 influent sulfate concentration was 7 mg-S/L and the effluent sulfide concentration was
140 29 mg-S/L. Sodium sulfate was added to the influent sewage for the reproduction of
141 the anaerobic sulfur oxidation reaction because based on our previous analysis,
142 anaerobic sulfur oxidation had occurred when approximately 40-150 mg-S/L sodium
143 sulfate was added (16). After addition of sodium sulfate, the average UASB influent
144 sulfate concentration was 54 mg-S/L and the effluent sulfide concentration was 29
145 mg-S/L. The occurrence of the sulfur oxidation reaction in the UASB reactor can be
146 determined through the changes in the sulfate and sulfide concentrations during the
147 operation. Our results (Fig. 2) suggest that the sewage temperature affect the
148 concentrations of sulfate and sulfide in the UASB reactor. The level of oxidized sulfur
149 as indicated by the increase in sulfate concentration was inversely proportional to the
150 temperature after addition of sodium sulfate, where sulfide was oxidized to sulfate
151 during the cold season (average of 13.1°C) but was not oxidized during the hot season
152 (average of 23.3°C).

This finding is supported by the sulfate and sulfide concentrations in the UASB reactor on days 91, 111, 167 and 335 (Fig. 3). Over the 91 days of operation, which was before the addition of sodium sulfate, the temperature was 23.2°C and sulfur oxidation did not occur. On day 111, which was 13 days after sodium sulfate addition, the temperature slightly decreased to 18.7°C but sulfur oxidation still did not occur, which indicates that the microbial community had not changed. Sulfur oxidation occurred after 167 days of operation where the temperature dropped gradually and sulfate concentration increased, which means the microbial community had changed. However, the sulfur oxidation stopped after day 335 when the temperature started to increase (23.4°C). When sulfur oxidation occurred, the ORP was always less than -300 mV, and the DO, nitrate and nitrite were not detected, which shows that anaerobic conditions were maintained. Additionally, lower sewage temperature seems to be important for the occurrence of the anaerobic sulfur oxidation reaction.

Fig. 2

Fig. 3

Phylogenetic analysis of bacterial and archaeal microbial communities

The microbial community present in the UASB reactor was successfully characterized through microbial 16S rRNA gene analyses. Samples from days 91, 111 and 167 were used for cloning analysis to monitor changes in microbial community structure between sulfur oxidation and non-sulfur oxidation conditions. The 16S rRNA cloning analysis results shown in Table 1 and 2 indicate that bacteria and archaea species were present in the reactor. Among the bacteria species, the major

bacterial groups in the UASB reactor were phylum *Proteobacteria* comprising an average of 39% of the total clones, followed by the phyla *Firmicutes* (18%), *Bacteroidetes* (16%) and *Caldiserica* (12%). The acetate-utilizing methanogen *Methanosaeta* spp. was the dominant group of the domain archaea at all times. The microbial structure was almost the same as that of other UASB reactors treating sewage except for phylum *Caldiserica* (17). However, at the genus level, genus *Smithella* of phylum *Proteobacteria* was the major bacterial group observed in the UASB reactor comprising an average of 19% of the total clones, followed by uncultured bacteria of phylum *Caldiserica* (12%). Within phylum *Proteobacteria*, which was the most abundant group of bacteria, various genera were present and most of the bacteria are sulfur-reducing bacteria (SRB) including the most dominant phylotype, genus *Smithella* (18).

Mori *et al.* (19,20) discovered a new bacteria strain that represented a new genus and novel species named *Caldisericum exile* within the phylum *Caldiserica*. This strain was previously known as uncultured phylum OP5 and obtained from anaerobic environments. Anaerobic growth of this bacterium was observed with the reduction of sulfur compounds such as thiosulfate, sulfite and elemental sulfur but without the presence of sulfate, fumarate, nitrate, nitrite and oxygen as electron acceptors. Furthermore, some of the OP5 clones were retrieved from relatively sulfur-rich environments. Based on the description of Mori *et al.*, the uncultured bacteria of phylum *Caldiserica* found in this study were not closely related and had low sequence similarity (the sequence similarity of the 16S rRNA gene was approximately 83%) to any other known bacteria in recognized phyla (shown in Fig. 4). Therefore, the uncultured bacteria of phylum *Caldiserica* present in this UASB reactor were probably involved in the sulfur cycle and may thus have participated in the sulfur

oxidation and/or sulfate reduction reactions in the UASB reactor.

Other dominant bacterial species were members of the genus *Smithella* but these phylotypes were phylogenetically related to *S. propionica* (the sequence similarity of the 16S rRNA gene was approximately 97%). *S. propionica* only grows in anaerobic conditions and is unable to use sulfate as an electron acceptor (21); it is also able to grow on propionate in syntrophic association with methanogens. *S. propionica* oxidizes propionate to acetate, carbon dioxide and hydrogen, particularly acetate. Under this condition where acetate is extensively produced, *Methanosaeta* spp., which have high affinity for acetate (22), will present a higher growth rate and higher yield, as observed in this study.

As mentioned above, most of the bacteria groups found in the UASB reactor were SRB and no signs of sulfur-oxidizing bacteria (SOB) were present. Previous reports indicated that members of genera such as *Acidianus* (23), *Bacillus* (24), *Beggiatoa* (25), *Paracoccus*, *Pseudomonas* and *Thiobacillus* (26) in aerobic conditions; *Allochromatium* (formerly known as *Chromatium*) (27), *Chlorobium*, *Rhodobacter*, *Rhodopseudomonas* and *Rhodovulum* (28) in anaerobic conditions; and the purple sulfur bacteria *Thiocapsa roseopersicina* (29) and *Allochromatium vinosum* (30) in addition to the purple non-sulfur bacteria *Rhodovulum sulfidophilum* (31), *Rhodocyclus genatinosus* and *Rhodopseudomonas acidophila* (32,33) which grows in the dark, are frequently observed SOB (34). However, none of these bacteria groups existed in the UASB reactor during the entire operating period. Therefore, it is likely that the SRB present in the UASB reactor had another function as oxidizing bacteria in sulfur oxidation reaction in an unknown manner.

One possibility of anaerobic sulfur oxidation is the reverse reaction of anaerobic oxidation of methane (AOM) with sulfate reduction reaction. Actually, AOM

consortia could catalyze the reverse direction of AOM with sulfate (35). A consortium consisting of anaerobic methanotrophic (ANME) archaea and SRB mediates the AOM with sulfate (36). In the UASB reactor, ANME-related archaea was not detected, however, methanogenic archaea and SRB were detected. Thus, the SRB present in the UASB reactor may play a key role in anaerobic sulfur oxidation reaction. Nevertheless, further research is needed to elucidate the phenomena.

Table 1

Table 2

Fig. 4

Fig. 5

Succession of microbial community

The T-RFLP profile presented in Fig. 5 confirms the trend detected in the clone libraries. The T-RFLP analysis of bacteria showed some changes before and after the addition of sulfate with characteristic fingerprints for bacterial communities obtained using both *Hae*III and *Hha*I restriction enzymes (REs). The archaeal community structure demonstrated stable microbial community until the end of the oxidation process (data not shown) where the predominant T-RF corresponds to *Methanosaeta* spp. *Hae*III and *Hha*I were used for fingerprinting of bacterial communities because each RE has its own limitation in deriving certain terminal restriction fragment sizes that can be measured within a measurable size range (37). As shown in Fig. 5, when

using *HaeIII*, T-RF sizes lower than 50 bp, where the simulated peak determined by TRiFLe for uncultured bacteria of phylum *Caldiserica* (~33 bp) is located, could not be measured, whereas when using *HhaI*, T-RF sizes higher than 500 bp, where the simulated peak for *Smithella* (~561 bp) is located, could not be measured.

The T-RFLP profiles showed consistent results with the cloning analysis where the major peaks observed corresponded to the genus *Smithella* (~185 bp) and uncultured bacteria of phylum *Caldiserica* (~197 bp) in the *HaeIII* and *HhaI* T-RF profiles, respectively. Several peaks showed changes in their profiles during the oxidation reaction. During the oxidation period, the height of some of the peaks in the *HaeIII* profile such as the peaks at 183 and 185 bp increased, and these peaks were identified as representing the genera *Desulfarculus* and *Smithella*, respectively. When the oxidation ended, some peaks such as those at 181 bp (representing genus *Desulfovibrio*) and 183 bp disappeared, and the height of some of the peaks such as the peaks at 71 bp (representing genus *Bacteroides*) and 185 bp decreased. In the *HhaI* profile, the height of the peaks at 68, 163, 165 and 197 bp increased during the oxidation period but then decreased when the oxidation ended. These peaks represented the genera *Desulfobulbus*, *Desulfotibacter* and *Desulfarculus*, and uncultured bacteria of phylum *Caldiserica*, respectively. The peaks that disappeared or showed reduced magnitude when the oxidation ended thus probably represented the bacteria groups contributing to the sulfur oxidation reaction.

In summary, these findings allowed us to identify the microbial community structures of the bacterial and archaeal groups present in the UASB reactor. The bacterial community presented high diversity, as determined by the 16S rRNA gene analysis, whereas the archaeal community had relatively low and simple diversity. It can therefore be concluded that (i) uncultured bacteria within the phylum *Caldiserica*

and genus *Smithella* probably play an important role in sulfur oxidation, (ii) some SRB probably have a dual function and play a role in sulfur oxidation, and/or (iii) missing peaks and peaks with the decreased height during the non-oxidation condition probably represent bacteria involved in sulfur oxidation in the UASB reactor. However, further analyses are necessary for a deeper understanding of microbial diversity and its contribution to the anaerobic sulfur oxidation reaction in the UASB reactor.

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REFERENCES

1. Sato, N., Okubo, T., Onodera, T., Lalit, K.A., Ohashi, A. and Harada, H.: Economic evaluation of sewage treatment processes in India, J. Environ. Manage., **84**, 447-460 (2007).
2. Uemura, S. and Harada, H.: Treatment of sewage by a UASB reactor under moderate to low temperature conditions, Bioresour. Technol., **72**, 275-282 (2000).
3. Yamaguchi, T., Bungo, Y., Takahashi, M., Sumino, H., Nagano, A., Araki, N., Imai, T., Yamazaki, S. and Harada, H.: Low strength wastewater treatment under low temperature conditions by a novel sulfur redox action process, Water Sci. Technol., **53**, 99–105 (2006).

4. **Chan, Y.J., Chong, M.F., Law, C.L. and Hassell, D.G.:** A review on anaerobic-aerobic treatment of industrial and municipal wastewater, *Chem. Eng. J.*, **155**, 1-18 (2009).
5. **Takahashi, M., Yamaguchi, T., Kuramoto, Y., Nagano, A., Shimozaki, S., Sumino, H., Araki, N., Yamazaki, S., Kawakami, S. and Harada, H.:** Performance of a pilot-scale sewage treatment: An up-flow anaerobic sludge blanket (UASB) and a down-flow hanging sponge (DHS) reactors combined system by sulfur-redox reaction process under low temperature conditions, *Bioresour. Technol.*, **102**, 753-757 (2011).
6. **Loy, A., Duller, S., Baranyi, C., Mußmann, M., Ott, J., Sharon, I., Béjà, O., Paslier, D.L., Dahl, C. and Wagner, M.:** Reverse dissimilatory sulfite reductase as phylogenetic marker for a subgroup of sulfur-oxidizing prokaryotes, *Environ. Microbiol.*, **11**, 289-299 (2009).
7. **Tang, K., Baskaran, V. and Nemat, M.:** Bacteria of the sulphur cycle: An overview of microbiology, biokinetics and their role in petroleum and mining industries, *Biochem. Eng. J.*, **44**, 73-94 (2009).
8. **Dunn, J.P., Stenger Jr., H.G. and Wachs, I.E.:** Oxidation of sulfur dioxide over supported vanadia catalysts: molecular structure - reactivity relationships and reaction kinetics, *Catal. Today*, **51**, 301-318 (1999).
9. **Kletzin, A.:** Oxidation of sulfur and inorganic sulfur compounds in *Acidianus ambivalens*, p. 184–201. In Dahl, C. & Friedrich, C.G., (ed.), *Microbial Sulfur Metabolism*, Springer Verlag, Berlin (2008).
10. **Shao, M.-F., Zhang, T. and Fang, H.H.-P.:** Sulfur-driven autotrophic denitrification: diversity, biochemistry, and engineering applications, *Appl. Microbiol. Biotechnol.*, **88**, 1027-1042 (2010).

- 316 11. **Overmann, J. and van Gernerden, H.:** Microbial interactions involving sulfur
317 bacteria: implications for the ecology and evolution of bacterial communities,
318 FEMS Microbiol. Rev., **24**, 591-599 (2000).
- 319 12. **Japan Sewage Works Association:** Japanese standard testing methods for
320 sewage, Japan Sewage Works Association, Tokyo, Japan (1997). (in Japanese)
- 321 13. **Hatamoto, M., Imachi, H., Yashiro, Y., Ohashi, A. and Harada, H.:** Diversity
322 of anaerobic microorganisms involved in long-chain fatty acid degradation in
323 methanogenic sludges as revealed by RNA-based stable isotope probing, Appl.
324 Environ. Microbiol., **73**, 4119-4127 (2007).
- 325 14. **Junier, P., Junier, T. and Witzel, K.-P.:** TRiFLe, a program for *in silico*
326 terminal restriction fragment length polymorphism analysis with user-defined
327 sequence sets, Appl. Environ. Microbiol., **74**, 6452-6456 (2008).
- 328 15. **Sumino, H., Takahashi, M., Yamaguchi, T., Abe, K., Araki, N., Yamazaki, S.,**
329 **Shimozaki, S., Nagano, A. and Nishio, N.:** Feasibility study of a pilot-scale
330 sewage treatment system combining an up-flow anaerobic sludge blanket
331 (UASB) and an aerated fixed bed (AFB) reactor at ambient temperature,
332 Bioresour. Technol., **98**, 177-182 (2007).
- 333 16. **Sumino, H., Yamaguchi, T., Tanikawa, D., Okazaki, Y., Araki, N.,**
334 **Kawakami, S., Yamazaki, S. and Harada, H.:** Sewage treatment by sulfur
335 redox cycle action in a system consisting of a UASB pre-reactor and a aerobic
336 post-reactor, Environ. Eng. Res., **40**, 431-440 (2003) (in Japanese).
- 337 17. **Takahashi, M., Ohya, A., Kawakami, S., Yoneyama, Y., Onodera, T.,**
338 **Syutsubo, K., Yamazaki, S., Araki, N., Ohashi, A., Harada, H. and**
339 **Yamaguchi, T.:** Evaluation of treatment characteristics and sludge properties in a
340 UASB reactor treating municipal sewage at ambient temperature, Int. J. Environ.

Res., **5**, 821-826 (2011).

18. **Garrity, G.M., Brenner, D.J., Krieg, N.R. and Staley, J.T. (eds.):** Bergey's Manual of Systematic Bacteriology, Volume Two: The Proteobacteria, Part C: The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. New York, New York: Springer (2005).

19. **Mori, K., Sunamura, M., Yanagawa, K., Ishibashi, J.-i., Miyoshi, Y., Iino, T., Suzuki, K.-i. and Urabe, T.:** First cultivation and ecological investigation of a bacterium affiliated with the candidate phylum OP5 in hot springs, Appl. Environ. Microbiol., **74**, 6223-6229 (2008).

20. **Mori, K., Yamaguchi, K., Sakiyama, Y., Urabe, T. and Suzuki, K.:** *Caldisericum exile* gen. nov., an anaerobic, thermophilic, filamentous bacterium of a novel bacterial phylum, *Caldiserica* phyl. nov., originally called the candidate phylum OP5, and description of *Caldiseriaceae* fam. nov., *Caldisericales* ord. nov. and *Caldisericia* classis nov., Int. J. Syst. Evol. Microbiol., **59**, 2894-2898 (2009).

21. **Liu, Y., Balkwill, D.L., Aldrich, H.C., Drake, G.R. and Boone, D.R.:** Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*, Int. J. Syst. Bacteriol., **49**, 545-556 (1999).

22. **Smith, K.S. and Ingram-Smith, C.:** *Methanosaeta*, the forgotten methanogen?, Trends Microbiol., **15**, 150-155 (2007).

23. **Friedrich, C.G.:** Physiology and genetics of sulfur-oxidizing bacteria, Adv. Microb. Physiol., **39**, 235-289 (1998).

24. **Aragno, M.:** Aerobic, chemolithoautotrophic, thermophilic bacteria, p. 77-103. In Kristjansson, J.K. (ed.), Thermophilic Bacteria, CRC Press Inc., Boca Raton

- 366 U.S.A. (1992).
- 367 25. **Strohl, W.R.:** *Beggiatoales*, p 2089-2106. In Stanley, J.T., Bryant, M.P., Pfennig,
368 N. and Holt, J.G. (ed.), *Bergey's Manual of Systematic Bacteriology*, Volume
369 Three, Williams & Wilkins, Baltimore (1989).
- 370 26. **Friedrich, C.G. and Mitrenga, G.:** Oxidation of thiosulfate by *Paracoccus*
371 *denitrificans* and other hydrogen bacteria, *FEMS Microbiol. Lett.*, **10**, 209-212
372 (1981).
- 373 27. **Imhoff, J.F., Söling, J. and Petri, R.:** Phylogenetic relationship and taxonomic
374 reclassification of *Chromatium* species and related purple sulfur bacteria, *Int. J.*
375 *Syst. Bacteriol.*, **48**, 1029-1043 (1998).
- 376 28. **Brune, D.C.:** Sulfur oxidation by phototrophic bacteria, *Biochim. Biophys. Acta*,
377 **975**, 189-221 (1989).
- 378 29. **Kondratieva, E.N., Zhukov, V.G., Ivanovsky, R.N., Petushkova, Yu.P. and**
379 **Monosov, E.Z.:** The capacity of phototrophic sulfur bacterium *Thiocapsa*
380 *roseopersicina* for chemosynthesis, *Arch. Microbiol.*, **108**, 287-292 (1976).
- 381 30. **Kämpf, C. and Pfennig, N.:** Chemoautotrophic growth of *Thiocystis violacea*,
382 *Chromatium gracile* and *C. vinosum* in the dark at various O₂-concentrations, *J.*
383 *Basic Microbiol.*, **26**, 517-531 (1986).
- 384 31. **Hiraishi, A. and Umeda, Y.:** Intragenic structure of the genus *Rhodobacter*:
385 transfer of *Rhodobacter sulfidophilus* and related marine species to the genus
386 *Rhodovulum* gen. nov., *Int. J. Syst. Bacteriol.*, **44**, 15-23 (1994).
- 387 32. **Kondratieva, E.N.:** Chemolithotrophy of phototrophic bacteria, p. 283-287. In
388 Schlegel, H.G. and Bowien, B. (ed.), *Autotrophic bacteria*, Springer, Berlin, and
389 Science Tech Publishers, Madison, WI (1989).
- 390 33. **Siefert, E. and Pfennig, N.:** Chemoautotrophic growth of *Rhodopseudomonas*

species with hydrogen and chemotrophic utilization of methanol and formate,
Arch. Microbiol., **122**, 177-182 (1979).

34. **Friedrich, C.G., Rother, D., Bardischewsky, F., Quentmeier, A. and Fischer, J.:** Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism?, Appl. Environ. Microbiol., **67**, 2873-2882 (2001).

35. **Holler, T., Wegener, G., Niemann, H., Deusner, C., Ferdelman, T.G., Boetius, A., Brunner, B. and Widdel, F.:** Carbon and sulfur back flux during anaerobic microbial oxidation of methane and coupled sulfate reduction, Proc. Natl. Acad. Sci. USA., **108**, E1484-E1490 (2011).

36. **Knittel, K., Lösekann, T., Boetius, A., Kort, R. and Amann, R.:** Diversity and Distribution of Methanotrophic Archaea at Cold Seeps, Appl. Environ. Microbiol., **71**, 467-479 (2005).

37. **Engebretson, J.J. and Moyer, C.L.:** Fidelity of select restriction endonucleases in determining microbial diversity by terminal-restriction fragment length polymorphism, Appl. Environ. Microbiol., **69**, 4823-4829 (2003).

FIGURE LEGENDS

Fig. 1. Schematic diagram of the UASB reactor.

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

Fig. 3. UASB profiles of sulfide and sulfate concentrations along the reactor height.

Fig. 4. Phylogenetic relationships of bacterial 16S rRNA gene sequences constructed using neighbor-joining method. The sequences determined in this study are shown in bold.

Fig. 5. T-RFLP profile of bacterial 16S rRNA gene from UASB sludge on different

414 sampling days. A, Before sodium sulfate addition (Day 91); B, After sodium
415 sulfate addition (Day 111); C, Sulfur oxidation occurrence (Day 167); D,
416 Sulfur oxidation end (Day 335).

Fig. 1.

Fig. 2

Fig. 3

Fig. 4

Fig. 5

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

Phylogenetic affiliations	No of clones (% of total clones)		
	Day 91	Day 111	Day 167
Bacteria			
<i>Acidobacteria</i>	6 (6.5)	3 (3.2)	6 (7.1)
<i>Actinobacteria</i>			2 (2.4)
<i>Bacteroidetes</i>	15 (16.3)	23 (24.7)	5 (5.8)
<i>Caldiserica</i>	4 (4.3)	14 (15.1)	13 (15.3)
<i>Chlorobi</i>		1 (1.1)	1 (1.2)
<i>Chloroflexi</i>	2 (2.2)	1 (1.1)	4 (4.7)
<i>Firmicutes</i>	16 (17.4)	8 (8.6)	25 (29.4)
<i>Nitrospira</i>	2 (2.2)		
<i>Planctomycetes</i>	2 (2.2)	1 (1.1)	1 (1.2)
<i>Proteobacteria</i>			
<i>Desulforhabdus</i>	1 (1.1)	2 (2.2)	5 (5.9)
<i>Smithella</i>	22 (23.9)	17 (18.2)	11 (12.9)
<i>Syntrophorhabdus</i>	1 (1.1)	3 (3.2)	4 (4.7)
<i>Other Proteobacteria</i>	17 (18.5)	16 (17.2)	7 (8.2)
<i>Spirochaetes</i>	2 (2.2)	1 (1.1)	
<i>Synergistetes</i>		1 (1.1)	1 (1.2)
<i>Verrucomicrobia</i>	1 (1.1)	2 (2.1)	
<i>WS3</i>	1 (1.1)		
Total	92 (100)	93 (100)	85 (100)

Table 2 Phylogenetic affiliation and numbers of archaeal 16S rRNA gene clones in the UASB sludge.

Phylogenetic affiliations	No of clones (% of total clones)		
	Day 91	Day 111	Day 167
Archaea			
<i>Crenarchaeota</i>			
<i>Thermofilum</i>			2 (4.5)
<i>Euryarchaeota</i>			
<i>Halopelagius</i>			1 (2.3)
<i>Methanobacterium</i>	11 (25.0)	5 (10.6)	3 (6.8)
<i>Methanomethylovorans</i>	1 (2.3)	1 (2.1)	2 (4.5)
<i>Methanoregula</i>	2 (4.5)	11 (23.4)	
<i>Methanosaeta</i>	26 (59.1)	25 (53.2)	36 (81.8)
<i>Methanospirillum</i>	3 (6.8)	5 (10.6)	
<i>Thermogymnomonas</i>	1 (2.3)		
Total	44 (100)	47 (100)	44 (100)