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Succession of Internal Sulfur Cycles and Sulfur-Oxidizing Bacterial Communities in Microaerophilic Wastewater Biofilms

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The succession of sulfur-oxidizing bacterial (SOB) community structure and the complex internal sulfur cycle occurring in wastewater biofilms growing under microaerophilic conditions was analyzed by using a polyphasic approach that employed 16S rRNA gene-cloning analysis combined with fluorescence in situ hybridization, microelectrode measurements, and standard batch and reactor experiments. A complete sulfur cycle was established via S^0 accumulation within 80 days in the biofilms in replicate. This development was generally split into two phases, (i) a sulfur-accumulating phase and (ii) a sulfate-producing phase. In the first phase (until about 40 days), since the sulfide production rate (sulfate-reducing activity) exceeded the maximum sulfide-oxidizing capacity of SOB in the biofilms, H_2S was only partially oxidized to S^0 by mainly *Thiomicrospira denitirificans* with NO_3^- as an electron acceptor, leading to significant accumulation of S^0 in the biofilms. In the second phase, the SOB populations developed further and diversified with time. In particular, S^0 accumulation promoted the growth of a novel strain, strain SO07, which predominantly carried out the oxidation of S^0 to SO_4^{2-} under oxic conditions, and *Thiothrix* sp. strain CT3. In situ hybridization analysis revealed that the dense populations of *Thiothrix* (ca. 10^9 cells cm^{-3}) and strain SO07 (ca. 10^8 cells cm^{-3}) were found at the sulfur-rich surface (100 μm), while the population of *Thiomicrospira denitirificans* was distributed throughout the biofilms with a density of ca. 10^7 to 10^8 cells cm^{-3}. Microelectrode measurements revealed that active sulfide-oxidizing zones overlapped the spatial distributions of different phylogenetic SOB groups in the biofilms. As a consequence, the sulfide-oxidizing capacities of the biofilms became high enough to completely oxidize all H_2S produced by SRB to SO_4^{2-} in the second phase, indicating establishment of the complete sulfur cycle in the biofilms.

In wastewater biofilms, due to the relatively high organic input and low dissolved oxygen (DO) concentration, an internal sulfur cycle consisting of sulfate reduction and subsequent sulfide oxidation is an important process for carrying electrons from the deeper anoxic zone to the oxic surface zone. Consequently, the sulfur cycle could be responsible for mineralization of a substantial part of the organic matter and consumption of dissolved oxygen (19, 25, 34), which prevent the emission of odorous and toxic hydrogen sulfide gas from the biofilms.

The reductive side of the sulfur cycle (i.e., sulfate reduction) occurs only biologically. Thus, the population dynamics, biodiversity, and in situ ecophysiology of sulfate-reducing bacteria (SRB) in wastewater biofilms have been extensively investigated by combining a 16S rRNA gene approach and microelectrode measurements (11, 12, 25, 26, 27, 34). In contrast, the oxidative side of the sulfur cycle (i.e., sulfide oxidation) occurs both biologically and chemically. The chemical oxidation reaction is, however, rather slow at natural pHs and temperatures (e.g., in the range of minutes to several hours) (5, 9, 14) and proceeds via $S_2O_3^{2-}$ as a major intermediate, whereas the biological oxidation reaction occurs via S^0 (4, 14, 15). S^0 accumulation has often been observed in wastewater biofilms (26), indicating that biological oxidation is a dominant oxidation reaction of H_2S. However, only a few studies of the community structure and diversity of sulfur-oxidizing bacteria (SOB) responsible for H_2S oxidation and S^0 production have been done. Little is known about development of the sulfur cycle that occurs in wastewater biofilms.

One of the reasons for the limited information is probably the phylogenetic diversity of SOB. SOB are phylogenetically distributed in a wide range of *Proteobacteria* (30, 31). Therefore, complete detection and quantification of SOB with a 16S rRNA-targeting probe is almost impossible, and hence, a genus-specific probe for each SOB group is required. Another reason is the versatile metabolic ability of SOB to utilize various electron donors, such as H_2S, S^0, $S_2O_3^{2-}$, and SO_3^-, and various electron acceptors, such as O_2 and NO_3^- (30, 31), indicating that the sulfide oxidation reactions can take place under various conditions.

It is therefore necessary to multilaterally investigate sulfur oxidation by using several analytical techniques. In the present study, the succession of the SOB community structure and sulfur-oxidizing activity in developing wastewater biofilms was analyzed by combining molecular techniques based on the 16S rRNA gene and microelectrode measurements. In addition, the mass balance for SO_4^{2-}, S^0, and sulfide in biofilm reactors and S^0 accumulation in the biofilms were examined to evaluate the development of the internal sulfur cycle in the biofilms. After a complete sulfur cycle was developed, the spatial distri-

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butions of numerically important SOB, such as *Thiothrix*, novel strain SO07, and *Thiomicrospira denitrificans*, were determined by fluorescence in situ hybridization (FISH) and related to the vertical profiles of H_2S, O_2, pH, NO_3^-, and S^0 in the biofilms. The data sets resulting from these different approaches were integrated to obtain a clear overall picture of the development of the SOB community structure and complex sulfur cycle occurring in the biofilms.

MATERIALS AND METHODS

Biofilm samples. Microaerophilic mixed-population biofilms were grown in fully submerged rotating disk reactors (RDRs) on a primary settling tank effluent from a domestic wastewater treatment plant (Sapporo, Japan). Two RDRs (RDR1 and RDR2) were independently operated in the same manner but at different times (for RDR1, May to July 2002; for RDR2, July to September, 2002). The RDRs consisted of nine polymethylmethacrylate disks with a diameter of 18 cm. The reactor volume was 5.6 liters, and the dilution rate in the reactors was kept at 0.2 h^{-1}. Four removal slides (1 by 6 cm) were installed in each disk for sampling biofilms. The disk rotational speed was fixed at 14 rpm for both runs. The influent was supplemented with a KNO_3 solution to give a final NO_3^- concentration of ca. 186 μM. More details concerning the RDR operation have been described elsewhere (25). The average effluent NO_3^- concentrations were 121 ± 43 μM and 94 ± 69 μM (means ± standard deviations) for RDR1 and RDR2, corresponding to 35% and 49% reductions, respectively. The average DO concentrations in the bulk liquid were 30 ± 19 μM and 40 ± 30 μM for RDR1 and RDR2, respectively. These conditions indicated that the biofilms were grown under microaerophilic conditions in both runs.

SORs. The potential sulfur (S^0) and sulfide (H_2S) oxidation rates (SORs) were determined in standard batch experiments by measuring the initial production of sulfate. Two 18-day-old and 65-day-old biofilms were taken from RDR1 and RDR2, homogenized, and inoculated into four serum vials (130 ml) containing 100 ml of synthetic media. A slightly modified medium for neutrophilic *Thiobacillus* (18) was used, which contained (per liter) 2.0 g of $NaHCO_3$, 1.0 g of KH_2PO_4, 1.0 g of K_2HPO_4, 1.0 g of NH_4Cl, 0.1 g of $CaCO_3$, 0.2 g of $MgSO_4$, and 1 ml of a trace element solution (18). The media also contained either S^0 (1.3 mM) or Na_2S (1.3 mM) as the sole electron donor, and the pH was adjusted to 7.0. The vials were incubated for 5 days at 20°C on a shaking table (at 100 rpm) in both oxic conditions and anoxic conditions with nitrate present. Therefore, a total of four different culture conditions were tested for each biofilm sample. At regular time intervals, subsamples were withdrawn, and the sulfate concentrations were measured by using an ion chromatograph equipped with an AS-9 column (Dionex, Japan). The sulfate concentrations during the initial 48-h incubation were used to calculate the SORs. Abiotic controls with no biomass added were incubated in parallel to determine the spontaneous chemical S^0 and H_2S oxidation rates, which were subsequently subtracted from the overall oxidation rates.

Finally, the duplicate cultures of the 18- and 65-day-old RDR1 biofilms were combined and harvested by centrifugation at 10,000 × *g* and then subjected to 16S rRNA gene-cloning analysis.

Measurement of reduced sulfur compounds. Elemental sulfur (S^0), acid-volatile sulfide (AVS) (H_2S, HS^-, S^{2-}, and FeS), and chromium-reducible sulfide (CRS) (FeS_2) in the reactor effluents and in the biofilms from RDR1 and RDR2 were determined by the method described by Fossing and Jørgensen (10) and modified by Nielsen et al. (23). Three biofilm samples were taken from each RDR at time intervals and were immediately subjected to measurement. The measurement procedure was described in detail by Okabe et al. (26). Four biofilm samples were taken from each RDR at day 60, and the vertical distributions of S^0 in each biofilm were determined by slicing the biofilms into 200-μm sections with a Microslicer (model DTK-1000; Dosaka EM Co., Ltd., Kyoto, Japan) without any pretreatment, as described previously (26). The biofilm sections (200 μm thick) were then subjected to the measurement procedure.

Microelectrode measurements. A Clark-type O_2 microelectrode (28), an H_2S microelectrode (29), and liquid ion-exchanging membrane microelectrodes for pH and NO_3^- (8) were prepared and calibrated as previously described. A biofilm was taken from RDR1 at day 14 and day 60 and incubated in the synthetic medium for about 3 h prior to measurement, which ensured steady-state concentration profiles. The medium contained the following; $NaNO_3$ (0 and 250 μM), $MgSO_4 \cdot 7H_2O$ (900 μM), NH_4Cl (600 μM), Na_2HPO_4 (570 μM), $MgCl_2 \cdot 6H_2O$ (84 μM), $CaCl_2$ (200 μM), EDTA · 2Na (270 μM), and sodium propionate (900 μM) as the sole carbon source. Propionate was used because propionate-utilizing *Desulfobulbus* spp. were the predominant SRB in this type of wastewater biofilms (12, 26). The medium without $NaNO_3$ was used to determine the potential in situ sulfate reduction rates in the biofilm. All measurements were performed in a water chamber containing 1.8 liters of synthetic medium at 20°C. The average liquid flow velocity (2 to 3 cm s^{-1}) above the biofilm was provided by a Pasteur pipette blowing a mixture of air and N_2 gas onto the water surface. Each measurement was performed three times at different positions by advancing the microelectrodes in steps of 50 μm through the biofilm. More details concerning the measurement procedure have been described elsewhere (12). In this study, microelectrode measurements for the RDR2 biofilm could not be performed due to time constraints.

Net specific consumption and production rates (in μmol cm^{-3} h^{-1}) were estimated from the measured microprofiles by using Fick's second law of diffusion. The details of this method have been described previously by Lorenzen et al. (21). The diffusive fluxes of NO_3^- and H_2S in the sulfide-oxidizing zone were calculated by using Fick's first law. We used molecular diffusion coefficients of 1.23×10^{-5} cm^2 s^{-1} for NO_3^- (3) and 1.39×10^{-5} cm^2 s^{-1} for H_2S (19) at 20°C.

16S rRNA gene-cloning and phylogenetic analysis. An aliquot of the homogenized RDR1 biofilm sample (0.2 ml) and 200-mg portions of centrifuged pellets of the preincubated 18- and 65-day-old RDR1 biofilm cultures were subjected to DNA extraction. DNA was extracted using a Fast DNA Spin kit (BIO 101) as described in the manufacturer's instructions. The nearly full-length 16S rRNA genes from mixed bacterial DNA were amplified by PCR using the universal primer set for *Bacteria*, 27f and 1492r (20). To minimize nonspecific annealing of the primers to nontarget DNA, a hot-start PCR program was used for all amplifications.

One microliter of the PCR-amplified bacterial 16S rRNA gene was directly ligated into the pGEM-T vector cloning system (Promega) and transformed into competent cells (high-efficiency *Escherichia coli* JM109; Promega) as described in the manufacturer's instructions. Plasmids were extracted and purified from clones with the Wizard Plus Minipreps DNA purification system (Promega) used in accordance with the manufacturer's instructions. The 16S rRNA gene inserts were sequenced by using an ABI model 310 genetic analyzer with a BigDye terminator Ready Reaction kit (Applied Biosystems).

All sequences were checked for chimeric artifacts by the CHECK_CHIMERA program in Ribosomal Database Project II (7) and were compared with the sequences available in public databases (GenBank and DDBJ) by the BLAST system (1). Sequence data were aligned with the CLUSTAL W package (36). Phylogenetic trees were constructed by the neighbor-joining method (33). Bootstrap resampling analysis for 100 replicates was performed to estimate the confidence of tree topologies.

FISH. Biofilm samples taken on days 18 and 70 from RDR1 and RDR2 were fixed in a 4% paraformaldehyde solution and embedded in Tissue-Tek OCT compound. After freezing at −20°C, vertical 20-μm sections (cross sections) of the fixed biofilms were prepared (26). Dehydration and in situ hybridization were performed according to the procedure described by Amann (2). The following oligonucleotide probes were used: G123T (S-G-Thioth-697-a-A-18), specific for *Thiothrix* spp. (16); S-*-SO07-0655-a-A-19, specific for the SO07 bacterium belonging to the γ-*Proteobacteria* (13); and S-S-Tmsde-0257-a-A-18 (5′-AACCCG CTACCCGTCATT-3′), specific for *Thiomicrospira denitrificans*, designed by using the ARB program (http://www.arb-home.de) in this study. The previously published optimal hybridization conditions were used for each probe. The specificity of probe S-S-Tmsde-0257-a-A-18 was empirically evaluated using the following strains as reference strains: *Thiomicrospira denitrificans* DSM1251, *Thiobacillus denitrificans* JCM3870, *Atopobium parvulum* JCM10300, and *Sulfurimonas autotrophica* JCM11897. In addition, the specificity of this probe was also tested with the cells in the cultures used for the SOR determination as mentioned above. The hybridization stringency was adjusted by adding formamide to the hybridization buffer (40% for S-S-Tmsde-0257-a-A-18). All probes were synthesized and labeled with tetramethylrhodamine-5-isothiocyanate (TRITC) or with fluorescein isothiocyanate at the 5′ end by TaKaRa Shuzo Co., Ltd. (Shiga, Japan). After hybridization and a washing step, the slides were allowed to air dry and mounted in antifading solution (Slow Fade Light; Molecular Probe, Eugene, Oreg.). Fluorescent and phase-contrast images were recorded with an LSM 510 confocal scanning laser microscope (Carl Zeiss) equipped with an argon laser (488 nm) and an HeNe laser (543 nm). Image combining was performed with the standard software (LSM 510 software, version 2.01) provided by Zeiss.

Nucleotide sequence accession numbers. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the clones used for the phylogenetic analysis are AB181494 to AB181501.

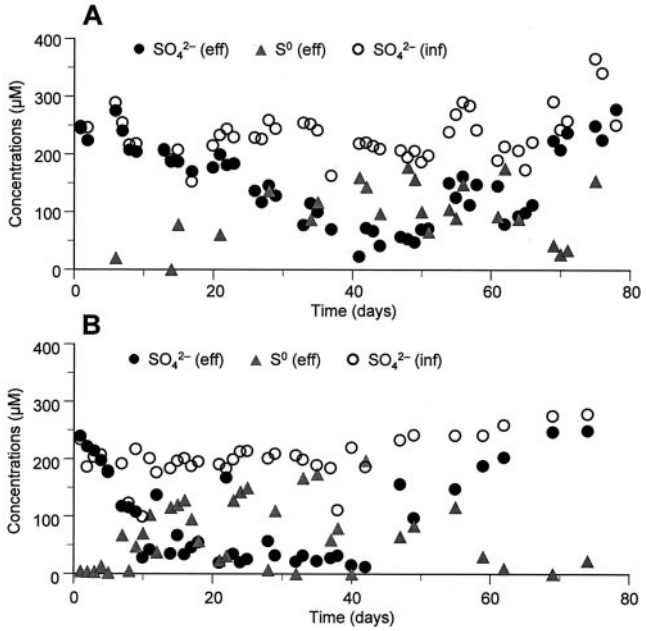


FIG. 1. Time courses for different sulfur compounds in the reactor influent (inf) and effluent (eff) during the experiments. (A) RDR1. (B) RDR2.

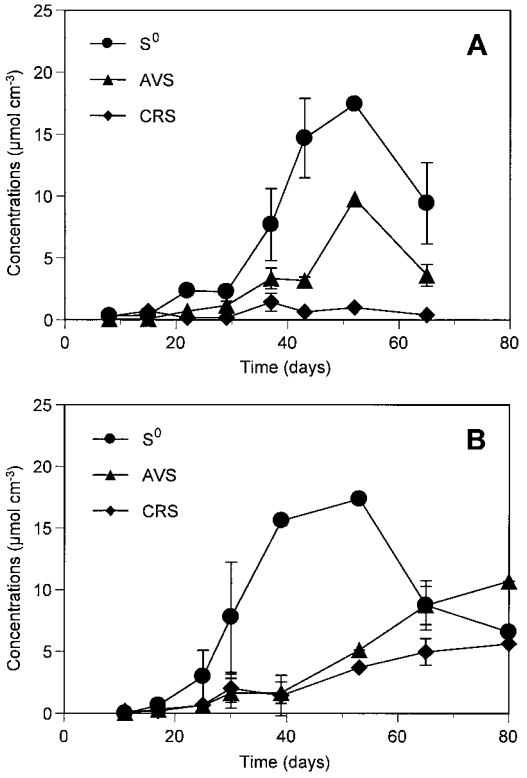


FIG. 2. Accumulation of S^0, AVS (H_2S, HS^-, S^{2-}, and FeS), and CRS (FeS_2) in the developing biofilms. (A) RDR1. (B) RDR2. The error bars indicate the standard deviations of the measurements for three biofilm specimens.

RESULTS

Reactor performance. Time courses for different sulfur compounds (sulfate [SO_4^{2-}] and elemental sulfur [S^0]) in RDR1 and RDR2 during the 80 days of operation are presented in Fig. 1. The SO_4^{2-} concentrations in the effluents started to decrease after the first few days and reached the lowest level (about 10 to 25 μM) at days 20 to 40 in both reactors. Although sulfate reduction occurred slightly earlier in RDR2 than in RDR1 due to a higher water temperature, the concentrations of SO_4^{2-} and S^0 in the effluent were well balanced, indicating that no other intermediate played an important role in the sulfur cycle. Finally, the effluent SO_4^{2-} concentration returned to the level in the influent (ca. 250 μM SO_4^{2-}) at day 70. On the other hand, the concentrations of S^0 in the effluents were almost equivalent to the amounts of SO_4^{2-} reduced. The change in the S^0 concentration was the inverse of the change in the SO_4^{2-} concentration. The concentrations of other reduced sulfur compounds, including H_2S, HS^-, and S^{2-}, and CRS (FeS_2) in the effluents of RDR1 and RDR2 were very low (less than 1.0 μM for AVS and CRS, respectively) during the experiments. After day 70, sulfur transformation in the reactors could not be seen from the mass balance of SO_4^{2-} and S^0 in both reactors because the internal sulfur cycle was completely developed. Although sulfate reduction and the subsequent sulfur oxidation occurred slightly earlier in RDR2 than in RDR1, the successional patterns of the sulfur cycle were essentially identical.

Reduced sulfur compounds in the biofilm. Time-dependent accumulations of reduced sulfur compounds, such as S^0, AVS (FeS, H_2S, HS^-, and S^{2-}), and CRS (FeS_2) in the biofilms are shown in Fig. 2. The biofilm thickness gradually increased from ca. 300 to 400 μm at days 8 to 15 to ca. 1,000 to 1,200 μm at days 35 to 42 and thereafter remained more or less constant in both reactors. The biofilm in RDR2 grew faster than that in RDR1, but the thicknesses at steady state were comparable. In addition, the biofilms obviously became denser and darker with time. The reduced sulfur compounds gradually accumulated in the biofilms from the beginning of development. Among the reduced sulfur compounds, S^0 was the most abundant sulfur pool in both biofilms. Significant accumulation of S^0 up to a concentration of 18 μmol cm^{-3} was observed during days 43 to 52, when the SO_4^{2-} concentration in the RDR1 effluent was lowest (Fig. 1). Then the concentration of S^0 decreased after day 52. This decrease in the S^0 concentration was probably due to an increase in biological S^0-oxidizing activity (Fig. 3A). The accumulation pattern for AVS, the second abundant sulfur pool, was similar to that for S^0. The CRS concentration was low throughout the experiment. Higher AVS and CRS concentrations were detected in the late biofilm developmental stage (at day 80) in RDR2 (Fig. 3B), which was probably due to the higher sulfate-reducing activity caused by the higher water temperature. The trend toward accumulation of reduced sulfur compounds in the RDR2 biofilm was, however, essentially the same as that in the RDR1 biofilm, suggesting that the two reactors had the same sulfur cycle succession.

Potential SORs of the biofilm. The potential S^0 and H_2S oxidation rates of the biofilms were determined at the early and late developmental stages of the sulfur cycle (Fig. 3). For the 18-day-old biofilm of RDR1, the aerobic and anaerobic H_2S oxidation rates were 3.7 ± 0.4 μmol cm^{-3} h^{-1} and 0.8 ±

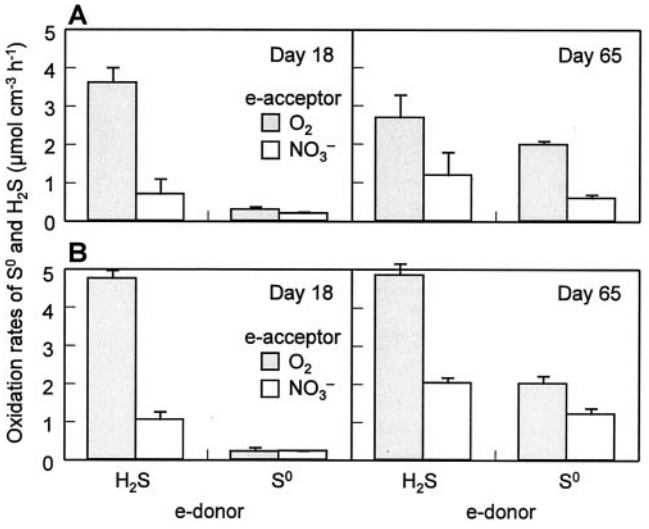


FIG. 3. Potential sulfur and sulfide oxidation rates of 18-day-old and 65-day-old biofilms taken from RDR1 (A) and RDR2 (B). The rates were determined in batch experiments with O_2 or NO_3^- as the sole electron acceptor (e-acceptor). The error bars indicate the standard deviations for duplicate experiments.

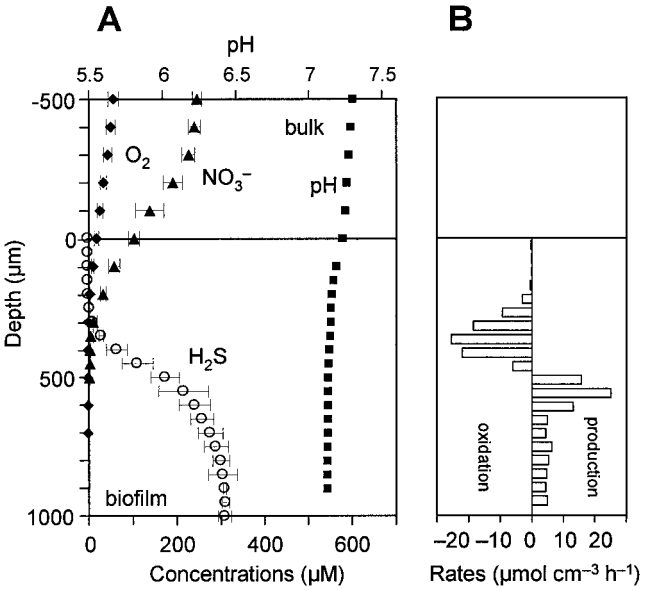


FIG. 4. (A) Steady-state concentration profiles for O_2, H_2S, NO_3^-, and pH in a 14-day-old wastewater biofilm taken from RDR1. The biofilm was incubated in DO-controlled (DO concentration, approximately 60 μM) synthetic medium with about 250 μM NO_3^-. The error bars indicate the standard deviations for three measurements at different positions. (B) Spatial distributions of the specific H_2S production and oxidation rates. The rates were calculated based on the corresponding microprofiles shown in panel A. The biofilm surface was at a depth of 0 μm.

0.4 μmol cm^{-3} h^{-1}, respectively, whereas the aerobic and anaerobic S^0 oxidation rates were less than 0.3 μmol cm^{-3} h^{-1}. As the biofilm grew, the aerobic and anaerobic S^0 oxidation rates increased by seven- and threefold, respectively, at day 65, whereas the aerobic and anaerobic H_2S oxidation rates remained relatively constant. The rates of S^0 and H_2S oxidation obtained for RDR1 at days 18 and 65 did not differ significantly from those determined for RDR2 (t test; $\alpha = 0.025$). Similar trends (i.e., increase in S^0 oxidation rates and no significant change in H_2S oxidation rates from day 18 to day 65) were also found in both biofilms (Fig. 3B). These results indicate that SOB populations responsible for aerobic and anaerobic H_2S oxidation were already developed to some extent in the early phase and that after this SOB that were capable of oxidizing S^0 to SO_4^{2-} developed in the late-phase biofilm.

Vertical profiles of O_2, H_2S, NO_3^-, pH, and S^0. Steady-state microprofiles of O_2, H_2S (total sulfide), pH, and NO_3^- in the 14-day-old biofilm taken from RDR1 were measured with microelectrodes (Fig. 4A). Oxygen and nitrate penetrated approximately 200 μm and 500 μm from the surface, respectively. An H_2S concentration of 310 μM was detected in the bottom of the biofilm, indicating that high sulfide-producing activity developed within 2 weeks. The H_2S profile barely overlapped the O_2 profile, but it overlapped the NO_3^- profile, indicating that H_2S was mainly oxidized anaerobically in the 200- to 500-μm zone with a maximum specific H_2S oxidation rate of 26 μmol cm^{-3} h^{-1} (Fig. 4B). A similar trend was also found in the 60-day-old biofilm taken from RDR1 (Fig. 5A). The H_2S concentration was slightly lower than the day 14 concentration, but H_2S oxidation occurred in the same zone (200 to 500 μm) when NO_3^- was present. When NO_3^- was removed, the H_2S concentration rapidly increased and reached 430 μM at a depth of 500 μm. The H_2S produced was completely oxidized at the biofilm surface with a maximum specific rate of 23 μmol cm^{-3} h^{-1}, showing that there was high potential sulfide-oxidizing activity (Fig. 5B).

The vertical distributions of elemental sulfur (S^0) that accumulated in the 60-day-old biofilms taken from RDR1 and RDR2 are shown in Fig. 5C. No significant accumulation of S^0 was observed in both biofilms at day 14 (Fig. 2). The concentration of S^0 was highest (ca. 22 μmol cm^{-3}) in the surface 200 μm and decreased with depth in the RDR1 biofilm. The large standard deviation at the surface (0 to 200 μm) was probably due to the heterogeneous structure of the biofilm surface. In the RDR2 biofilm, a high concentration of S^0 (ca. 23 to 25 μmol cm^{-3}) was also detected at the surface (0 to 400 μm), and S^0 became undetectable at depths below 600 μm.

Phylogenetic analysis. Three 16S rRNA gene clone libraries of bacteria were constructed from the RDR1 biofilm sample and the preincubated biofilm cultures to investigate the diversity of the SOB community structure. A total of 102 clones were analyzed for the biofilm clone library. Only one clone was affiliated with chemolithoautotrophic *Thiothrix* sp. strain CT3, with 99% sequence similarity (Table 1). None of the other phylotypes related to known SOB was retrieved directly from the biofilm without preincubation. Therefore, the biofilm samples taken from RDR1 at day 18 and day 65 were preincubated for 5 days with different combinations of electron donors (H_2S and S^0) and acceptors (O_2 and NO_3^-), and then the 16S rRNA gene was analyzed to detect the SOB community. In total, 171 clones were analyzed. Among the clones analyzed, four clone types were closely related to known SOB species: *Thiobacillus denitrificans* (β-*Proteobacteria*), *Pseudomonas* spp. (γ-*Proteobacteria*), *Thiomicrospira denitrificans* (ε-*Proteobacteria*),

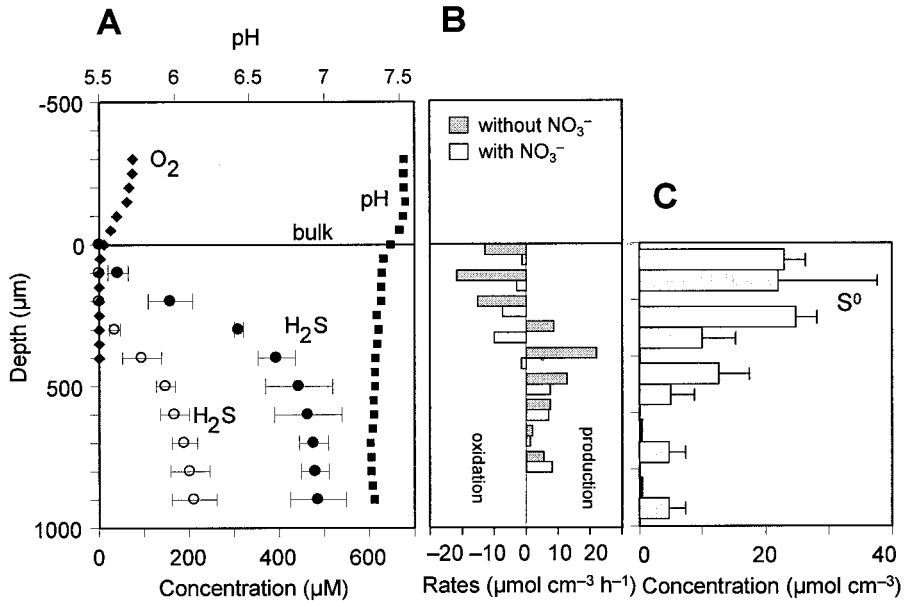


FIG. 5. (A) Steady-state concentration profiles for O_2, H_2S, NO_3^-, and pH in a 60-day-old wastewater biofilm taken from RDR1. The biofilm was incubated in DO-controlled (DO concentration, approximately 80 μM) synthetic medium with no addition of NO_3^- (solid circles) and with 90 μM NO_3^- (open circles). The error bars indicate the standard deviations for three measurements at different positions. (B) Spatial distributions of the specific H_2S production and oxidation rates when NO_3^- was absent and present. The rates were calculated based on the corresponding microprofiles shown in panel A. (C) Vertical distributions of S^0 in the biofilms obtained from RDR1 (gray bars) and RDR2 (open bars). The error bars indicate the standard deviations of the measurements for four biofilm specimens. The biofilm surface was at a depth of 0 μm.

and a novel sulfur-oxidizing bacterium, SO07 (γ-*Proteobacteria*), distantly related to *Halothiobacillus* spp. (Table 1 and Fig. 6). In particular, clones related to *Thiomicrospira denitrificans* (represented by HSN-59) were most frequently detected (detection frequency, 33%) from anaerobic incubation of an 18-day-old biofilm supplemented with NO_3^- and H_2S. In addition, clones closely related to SO07 (represented by SO-07) were also obtained with a high detection frequency (36%) from aerobic incubation of a 65-day-old biofilm with S^0 as the electron donor. Clones closely related to *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* (represented by SN-84 and SN-94, respectively) were also retrieved from anaerobic incubation of a 65-day-old biofilm with S^0 as the electron donor.

In situ identification of SOB in the biofilm. The surfaces of the RDR1 and RDR2 biofilms were whitish and were covered with a number of filamentous bacteria. Based on the 16S rRNA gene clone analyses, FISH targeting for *Thiothrix*, SO07, and *Thiomicrospira denitrificans* was conducted to investigate their in situ abundance and distributions in the biofilms obtained at days 18 and 70 from RDR1 and RDR2, respectively. Microscopic observation of a thin vertical section of the 70-day-old RDR1 biofilm revealed that probe G123T-hybridized filamentous *Thiothrix* was dominant at the surface of the biofilm (Fig. 7A and B). The population density of the probe G123T-hybridized *Thiothrix* was estimated to be 2×10^9 cells cm^{-3} (from a conversion of filament length to cell number; cell

TABLE 1. Phylogenetic analysis of 16S rRNA gene fragments derived from the biofilm taken from RDR1 and cultures incubated with combinations of an electron donor (S^0 or H_2S) and an electron acceptor (O_2 or NO_3^-)

Sample origin	Clone name	Closest relative (accession no.)	Taxonomic group	Frequency (%)[a]	% Similarity[b]
18-day-old biofilm incubated with[c]:					
$H_2S + O_2$	HSO-32	*Pseudomonas putida* (D86000)	γ-*Proteobacteria*	16 (7/44)	99 (1,470)
$H_2S + NO_3^-$	HSN-59	*Thiomicrospira denitrificans* (L40808)	ε-*Proteobacteria*	33 (11/33)	99 (1,437)
$H_2S + NO_3^-$	HSN-95	*Pseudomonas* sp. (AJ297355)	γ-*Proteobacteria*	6 (2/33)	98 (1,405)
$H_2S + NO_3^-$	HSN-82	*Thiobacillus denitrificans* (AJ243144)	β-*Proteobacteria*	3 (1/33)	95 (1,371)
65-day-old biofilm incubated with[c]:					
$S^0 + O_2$	SO-07	Novel sulfur-oxidizing bacterium SO07 (AB118236)	γ-*Proteobacteria*	36 (17/47)	98 (1,412)
$S^0 + NO_3^-$	SN-84	*Thiobacillus denitrificans* (AJ243144)	β-*Proteobacteria*	6 (3/47)	98 (1,458)
$S^0 + NO_3^-$	SN-94	*Thiomicrospira denitrificans* (L40808)	ε-*Proteobacteria*	2 (1/47)	96 (1,447)
65-day-old biofilm	Biofilm-30	*Thiothrix* sp. strain CT3 (AF148516)	γ-*Proteobacteria*	1 (1/102)	99 (1,422)

[a] The numbers in parentheses are number of clones obtained/number of clones analyzed in the library.
[b] The numbers in parentheses are the sequence lengths compared (in base pairs).
[c] The biofilm was taken from RDR1 and then preincubated for 5 days.

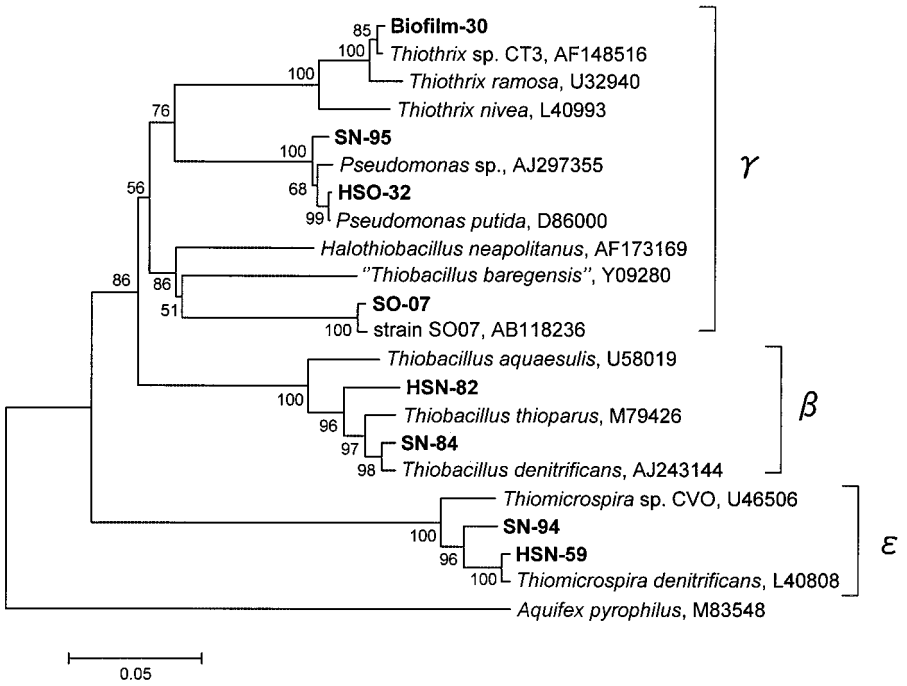


FIG. 6. Phylogenetic distance tree showing the affiliations of 16S rRNA gene clone sequences related to SOB shown in Table 1. The nearly full-length sequences of 16S rRNA genes (>1,380 bp) were retrieved from the RDR1 biofilm (Biofilm-) and RDR1 biofilms precultured with H_2S-O_2 (HSO-), H_2S-NO_3^- (HSN-), S^0-O_2 (SO-), and S^0-NO_3^- (SN-) as combinations of sole electron donor and sole electron acceptor. The bar represents 5% estimated divergence, and the numbers at the nodes are bootstrap values (100 replicates) with more than 50% bootstrap support. *Aquifex pyrophilius* served as an outgroup.

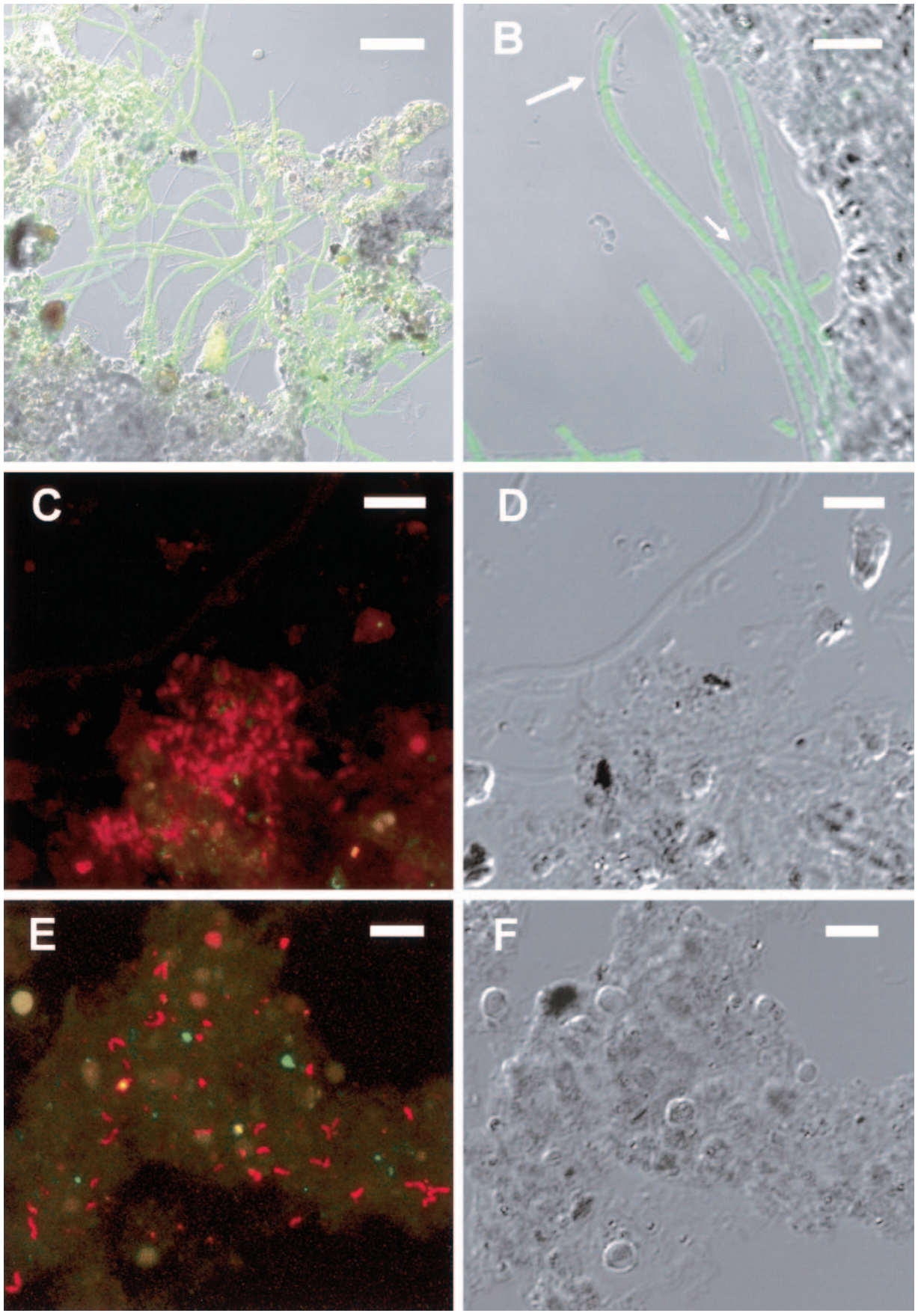
length, 1.5 μm) at the surface. The cells were sheathed filamentous cells (Fig. 7B), like *Thiothrix* sp. strain CT3 cells (32), and thus were distinguishable from type 021N *Thiothrix* species (lacking a sheath). In situ hybridization with the SO07-specific probe revealed that a number of rod-shaped SO07 cells (approximately 10^8 cells cm^{-3}) were mainly present along with some filamentous bacteria at the surface (100 μm) of the biofilm (Fig. 7C and D). Spiral-shaped *Thiomicrospira denitrificans* cells that were 0.5 to 1.0 μm thick were distributed throughout the biofilm at a density of approximately 10^7 to 10^8 cells cm^{-3} (Fig. 7E and 7F). It should be noted that the population was larger (ca. 1×10^8 to 5×10^8 cells cm^{-3}) near the oxic-anoxic interface. In the 18-day-old biofilm, only very few cells were detected with the probes targeting *Thiothrix* and SO07, whereas the population of *Thiomicrospira denitrificans* was comparable to that in the 70-day-old biofilm. The developmental pattern, spatial distribution, and abundance of these three SOB in the RDR2 biofilm were comparable to those in the RDR1 biofilm.

DISCUSSION

Development of a complete sulfur cycle in the biofilm. The succession of the SOB populations and their in situ activity in the wastewater biofilms was analyzed by a polyphasic approach that employed analyses of the biofilm reactor performance, potential SOR measurement, the 16S rRNA gene clone library, FISH, and microelectrode measurement. Two biofilm systems were independently operated, and consistent results were obtained. This clearly indicated that the succession of the internal sulfur cycle and responsible SOB populations in the complex wastewater biofilms was reproducible. The results obtained from all these experiments demonstrated that a complete internal sulfur cycle was established via S^0 accumulation within approximately 80 days in the biofilm system. The formation of S^0 in H_2S oxidation is mainly dependent on the rate of sulfide production by SRB, the availability of electron acceptors (i.e., O_2 and NO_3^-), and the size and type of the SOB populations in the biofilm. In general, the development of the complete sulfur cycle in this biofilm can be split into two phases, (i) a sulfur-accumulating phase and (ii) a sulfate-producing phase.

Sulfur-accumulating phase. In the first phase (until about day 40), partial oxidation of H_2S to S^0 occurred as a result of the rapid development of sulfate-reducing activity (Fig. 4A and B), resulting in the accumulation of S^0 in the biofilm (Fig. 2 and 5C). During this period, although sulfate-reducing activity had developed (Fig. 4A), the SOB communities were not sufficiently developed yet, and available electron acceptors, such as O_2 and NO_3^- for SOB, were somewhat limited in the biofilm (Fig. 4A). Thus, the sulfide production rate (SRB activity) exceeded the maximum sulfide-oxidizing capacity of the SOB present in the biofilm. In order to be able to oxidize increasing amounts of H_2S produced by SRB, the SOB have to convert H_2S to S^0 ($HS^- \rightarrow S^0 + H^+ + 2e^-$) instead of SO_4^{2-} ($HS^- + 4H_2O \rightarrow SO_4^{2-} + 9H^+ + 8e^-$), thereby keeping the electron flux constant (39). This prevents the accumulation of sulfide in the biofilm.

The 16S rRNA gene-cloning analysis of the 18-day-old bio-


A
B
C
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E
F

film sample revealed that *Thiomicrospira denitrificans*, *Thiobacillus denitrificans*, and *Pseudomonas* spp. (6, 35) first appeared in the biofilm during the first phase (Table 1). In addition, the FISH analysis revealed that a high concentration (ca. 10^7 to 10^8 cells cm^{-3}) of *Thiomicrospira denitrificans* was distributed throughout the biofilm, especially near the oxic-anoxic interface in the 18-day-old biofilm (ca. 1×10^8 to 5×10^8 cells cm^{-3}). This is because *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* prefer extremely low oxygen concentrations (optimum growth occurs at ca. 1% of air saturation) (17, 37, 38). Microelectrode measurements showed that the H_2S profiles did not really overlap the O_2 profile when NO_3^- was present, indicating that H_2S was oxidized mainly anaerobically during the first phase (Fig. 4A). The anaerobic H_2S oxidation zone (200 to 500 μm from the surface) shown in Fig. 4B overlapped the main location of *Thiomicrospira denitrificans* in the biofilm. Based on these results, it could be speculated that *Thiomicrospira denitrificans* was one of the main contributors to the partial anaerobic oxidation of H_2S to S^0, which led to the accumulation of S^0 in the biofilm during the first phase.

The contribution of these nitrate-reducing SOB to sulfide oxidation in the biofilm systems was roughly estimated. When the oxidation of H_2S was carried out with NO_3^- as the electron acceptor and S^0 as the end product, it could be described by the following stoichiometric equation: $5HS^- + 2NO_3^- + 7H^+ \rightarrow 5S^0 + N_2 + 6H_2O$ (equation 1). Based on this equation, the oxidation of 1 mol of HS^- requires 0.4 mol of NO_3^- and produces 1 mol of S^0 (HS^-:NO_3^-:S^0, 1:0.4:1; HS^-/NO_3^-, 2.5). The average concentrations of SO_4^{2-} reduced, NO_3^- reduced, and S^0 produced in RDR1 were 142 μM SO_4^{2-}, 67 μM NO_3^-, and 125 μM S^0, respectively, during days 40 to 55, which gives a ratio of HS^- to NO_3^- to S^0 of 1:0.47:0.88. A similar ratio (HS^-:NO_3^-:S^0, 1:0.53:0.86) was found in RDR2 during days 30 to 45. These ratios agree reasonably well with the theoretical ratio. The slightly higher values for NO_3^- could be explained by the occurrence of heterotrophic denitrification and aerobic oxidation of H_2S. Furthermore, the ratio of H_2S flux to NO_3^- flux determined from the concentration gradients of H_2S and NO_3^- (i.e., in situ H_2S oxidation and NO_3^- consumption rates) in the H_2S-oxidizing zone (ca. 200 to 400 μm), where both profiles overlapped, was calculated to be 2.7 (Fig. 4A). This ratio was also close to the theoretical value, 2.5, as described in equation 1. It should be noted that the sulfur (S^0) production was maximal at this ratio.

Sulfate-producing phase. The biofilm S^0 oxidation rates determined by the batch experiments significantly increased from day 18 to day 65 in both biofilms (Fig. 3). This result suggested that the SOB populations particularly responsible for S^0 oxidation developed in the second phase. The 16S rRNA gene-cloning and FISH analyses revealed that the SOB populations developed further and diversified with time; in particular, novel strain SO07 and *Thiothrix* sp. became numerically important members of the SOB in the biofilms (Table 1). High concentrations of novel strain SO07 (ca. 10^8 cells cm^{-3}) and *Thiothrix* sp. (ca. 2×10^9 cells cm^{-3}) were present mainly in the oxic surface zone (0 to 200 μm), where S^0 mainly accumulated (Fig. 7A to D). This result suggested that the novel SOB strain SO07 could be responsible for oxidation of S^0 to SO_4^{2-} under oxic conditions. Further isolation and characterization of strain SO07 revealed that strain SO07 was an aerobic chemolithoautotrophic sulfur-oxidizing bacterium capable of oxidizing S^0 and H_2S to SO_4^{2-} (13). More details concerning the ecophysiological characteristics of strain SO07 have been reported previously (13). Consequently, the overall sulfide-oxidizing capacity of the biofilm was high enough to completely oxidize or efficiently utilize all of the H_2S produced by SRB and the accumulated S^0 to SO_4^{2-} among the SOB community members. This is probably a reason for the decrease in the S^0 concentrations in the reactor effluents and in the biofilms after around day 40 (Fig. 1 and 2).

In the final stage of development of the sulfur cycle, the probe G123T-hybridized filamentous organism *Thiothrix* sp. became the most abundant SOB in the 70-day-old biofilms (Fig. 7A and B). The sheathed filaments and 16S rRNA gene sequence analysis suggested that the G123T-hybridized *Thiothrix* was most likely a member of *Thiothrix* sp. strain CT3. The versatile physiological ability of *Thiothrix* sp. strain CT3 to utilize various reduced sulfur compounds with either O_2 or NO_3^- as the electron acceptor and the heterotrophic growth on acetate (32) might provide a competitive advantage in a wastewater biofilm growing under microaerophilic conditions in which acetate was often detected (25).

Recently, specific thiosulfate oxidation rates of pure cultures of *Thiothrix* isolated from activated sludge (32) and *Thiothrix*-dominated activated sludge (24) have been determined to be 1.9×10^{-11} and 0.9×10^{-11} mmol $S_2O_3^{2-}$ $cell^{-1}$ h^{-1}, respectively. By multiplying these oxidation rates by the population density of *Thiothrix* determined in the biofilms (ca. 2×10^9 cells cm^{-3}), the potential thiosulfate oxidation rate of *Thiothrix* in the biofilms was calculated to be 18 to 38 μmol $S_2O_3^{2-}$ cm^{-3} h^{-1}. This oxidation rate is 2 orders of magnitude higher than the average sulfate reduction rate (0.26 μmol cm^{-3} h^{-1}) of the biofilm reactors. The thiosulfate oxidation rate may be slightly different from the sulfide oxidation rate, and the availability of electron acceptors in the biofilms was uncertain. Nevertheless, this calculation suggests that the population of *Thiothrix* in the surface biofilms was large enough to completely oxidize all of the H_2S produced to SO_4^{2-}, which was clearly demonstrated by microelectrode measurements when NO_3^- was absent (Fig. 5A). Therefore, it could be speculated that the probe G123T-hybridized *Thiothrix* must have contributed significantly to H_2S oxidation in the late stage of development. Although an abun-

FIG. 7. In situ detection of sulfur-oxidizing bacteria in a thin vertical section (thickness, 20 μm) of a 70-day-old wastewater biofilm taken from RDR1. (A and B) FISH with fluorescein isothiocyanate-labeled probe G123T (specific for *Thiothrix* sp.) at the surface of the biofilm (0 to 100 μm): confocal scanning laser microscope projection image (A) and differential interference contrast (DIC) image (B). (C) FISH with TRITC-labeled probe for strain SO07 in the surface 100 μm of the biofilm. (D) DIC image of the field shown in the panel C. (E) FISH with TRITC-labeled probe for *Thiomicrospira denitrificans* at a depth of approximately 400 μm in the biofilm. (F) DIC image of the field shown in panel E. The yellowish signals were autofluorescence. Bars = 20 μm (A), 10 μm (B), and 5 μm (C to F).

dant *Thiothrix* population was detected by FISH analysis, no clone related to *Thiothrix* was retrieved from the biofilm cultures incubated with S^0 and H_2S. The incubation in liquid synthetic media with vigorous shaking probably inhibited the growth of filamentous *Thiothrix*.

As the biofilm grew, the active sulfide production zone moved upward to the oxic-anoxic interface and intensified with time due to the increasing SRB activity (12). Sulfate reduction and sulfide oxidation were therefore found to occur in close proximity at the oxic-anoxic interface in the biofilm (Fig. 5A), as in other wastewater biofilms (12, 19, 22, 26). This probably enabled SOB to efficiently utilize electrons that H_2S carried, and thus the turnover rate of H_2S was very high. The average turnover rates of H_2S in the H_2S-oxidizing zone in the biofilm were 36 s and 18 s when NO_3^- was present and absent, respectively, which were determined from the microprofiles in Fig. 5A. Such high H_2S turnover rates could be attributed to biological oxidation because the time scale of the spontaneous chemical reaction of H_2S and O_2 could be in the range of minutes to several hours (9, 22). This indicates that the chemical oxidation of H_2S does not play an important role in wastewater biofilms growing under microaerophilic conditions.

In summary, the data sets resulting from these different approaches were integrated to obtain a clear understanding of how the SOB community structure and the complex internal sulfur cycle are established in a wastewater biofilm. Although the water quality of wastewater fluctuated with time and the wastewater biofilms were generally heterogeneous, the succession of the internal sulfur cycle and the responsible SOB populations in the complex wastewater biofilms was reproducible. However, the contributions of each phylogenetic SOB group to the overall sulfide oxidation in the biofilm were not really clear in this study. Therefore, further molecular ecological studies using, for example, microautoradiography combined with fluorescence in situ hybridization with $^{35}SO_4^{2-}$ or $H^{14}CO_3^-$ are required to analyze which phylogenetic groups of SOB preferentially utilize which types of reduced sulfur compounds (H_2S, S^0, and so on) under in situ conditions. This obviously would provide more detailed information on a complex sulfur cycle that occurs in microaerophilic biofilms.

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