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Structural and Functional Dynamics of Sulfate-Reducing Populations in Bacterial Biofilms

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We describe the combined application of microsensors and molecular techniques to investigate the development of sulfate reduction and of sulfate-reducing bacterial populations in an aerobic bacterial biofilm. Microsensor measurements for oxygen showed that anaerobic zones developed in the biofilm within 1 week and that oxygen was depleted in the top 200 to 400 μm during all stages of biofilm development. Sulfate reduction was first detected after 6 weeks of growth, although favorable conditions for growth of sulfate-reducing bacteria (SRB) were present from the first week. In situ hybridization with a 16S rRNA probe for SRB revealed that sulfate reducers were present in high numbers (approximately 10^8 SRB/ml) in all stages of development, both in the oxic and anoxic zones of the biofilm. Denaturing gradient gel electrophoresis (DGGE) showed that the genetic diversity of the microbial community increased during the development of the biofilm. Hybridization analysis of the DGGE profiles with taxon-specific oligonucleotide probes showed that *Desulfobulbus* and *Desulfovibrio* were the main sulfate-reducing bacteria in all biofilm samples as well as in the bulk activated sludge. However, different *Desulfobulbus* and *Desulfovibrio* species were found in the 6th and 8th weeks of incubation, respectively, coinciding with the development of sulfate reduction. Our data indicate that not all SRB detected by molecular analysis were sulfidogenically active in the biofilm.

Although sulfate reduction is thought to be an anaerobic process, sulfate-reducing bacteria (SRB) are also important in aerobic environments if they can proliferate in anaerobic zones. For example, in marine sediments (16, 17) and in aerobic wastewater treatment systems (18, 20), sulfate reduction accounts for up to 50% of the mineralization of organic matter. Furthermore, sulfate reduction strongly stimulates microbially enhanced corrosion of metals (5, 7). Therefore, the detection of sulfate reducers and sulfate-reducing activity in sediments, wastewater treatment plants, and fouling biofilms is of great practical and scientific relevance. Conventional microbial techniques based on selective culturing are of limited usefulness for quantification and characterization of environmental populations, as it is now well recognized that most strains do not grow in vitro, either because cultivation media poorly resemble natural growth conditions or because different strains of microorganisms are interdependent (2, 49). Techniques based on the analysis of bacterial DNA and RNA may complement the conventional microbiological approach and nowadays are routinely used to determine the presence and distribution of individual bacterial species, including SRB, in complex communities such as those in bacterial biofilms (1, 33–35).

So far, these studies of microbial communities, i.e., bacterial biofilms, have mainly focused on the exploration of bacterial diversity and on the detection of individual bacterial taxa by molecular techniques. Studies relating community structure to community function are scarce, partially because of difficulties in monitoring microbial activities. Within biofilms, the convection of compounds is hindered, and consequently mass transfer to the cells often limits conversion rates. Because of this resistance to mass transfer, biofilms develop various microenvironments, which differ from the bulk liquid (6, 10, 18). This complicates the interpretation of community function analysis, because extrapolation of community behavior to that of individual cells is impossible without knowledge of their microenvironment.

The most direct way to study different microzonations in biofilms is with microsensors—needle-shaped devices with a tip measuring from <1 to 100 μm which are sensitive for a specific compound. Due to their small size, microsensor measurements cause minimal disturbance to the system. With microsensors, microenvironments can be examined, and microgradients can be measured. The measured gradients are a function of local transport and conversion rates. Thus, if the transport process (usually diffusion) is known, the spatial distribution of microbial activity can be derived from the substrate profiles (38). An important advantage of using microsensors is the capability to unravel closed cycles, such as sulfate reduction coupled with sulfide oxidation, within a biofilm or sediment (18). With these systems, measurements of net substrate consumption or product excretion lead to considerable underestimation of the actual processes within the biofilms. Internal metabolic cycles can be hidden, although they play a crucial role in the biofilm and will be reflected in the structure of the microbial community.

Ramsing et al. (33) were the first to use both microsensors and molecular techniques to study sulfate reduction in a trickling-filter biofilm, which was used for treating municipal wastewater. Recently, Schramm et al. (42) combined microsensor and molecular techniques to study nitrification in a trickling-filter biofilm. In both studies, a good correlation was found between microbial conversions (sulfate reduction and nitrification) and microbial population distributions within the biofilms.

The studies described above were performed with well-established and mature biofilms. In this work, we studied the transients of sulfate reduction, using microsensors, and fol-

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lowed the successional changes in the composition of microbial species, using molecular techniques, with a developing, multispecies bacterial biofilm. This topic is relevant, as biofilms are often subjected to changing conditions and sloughing is followed by recolonization. The aim of our study was to assess how closely species composition reflects activity in a biofilm with gradually changing microenvironments.

A biofilm developing in an aerobic wastewater treatment plant was used as a model for biofilms growing in aerobic sulfate-containing waters with high organic loads. Microsensors with high spatial resolution were used to measure oxygen and hydrogen sulfide profiles and to infer aerobic respiration and sulfate-reducing activities. Molecular techniques for studying SRB were as follows: denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S ribosomal DNA (rDNA) fragments to determine the complexity of the microbial community in the biofilm and to monitor its behavior over time, hybridization analysis of these DGGE profiles with group-specific oligonucleotide probes to identify SRB, and in situ hybridization with thin sections of the biofilm to quantify the SRB and determine their spatial distribution within the biofilm.

MATERIALS AND METHODS

Biofilm growth and sampling. Oxygen-impermeable plastic foil (Ril-O-Ten 80/100X; Otto Nielsen Emballage AS, Lyngby, Denmark) was submerged as a substratum for biofilm growth in the activated-sludge basin (first aerated stage) of the Seehausen municipal wastewater treatment plant in Bremen, Germany, during spring 1996. The temperature in the basin during the experiment varied between 17 and 20°C. In the bulk liquid, the concentrations of O_2 and SO_4^{2-} were 1.8 to 2.4 mg/liter (55 to 75 μM) and 75 to 115 mg/liter (800 to 1,200 μM), respectively. NO_3^- was absent. The biological oxygen demand was approximately 350 mg/liter (10 mM). Samples of the foil (ca. 6 by 12 cm each) on which the bacterial biofilm was growing were taken weekly over a period of 12 weeks. While submerged in wastewater, samples were transferred to the laboratory and used within 1 h for microelectrode measurements. Replicate samples were frozen at −20°C for molecular analysis.

Biofilm fixation and slicing. Biofilm samples for in situ hybridization analysis were fixed by incubation in paraformaldehyde (4% [wt/vol] in phosphate-buffered saline) at 4°C for 1 h and washed subsequently in phosphate-buffered saline. After fixation, samples were embedded in embedding medium (OCT compound; Sakura Finetek USA, Torrance, Calif.) and frozen above evaporating liquid nitrogen. Thin sectioning of the biofilm was performed with a cryomicrotome (Microm model HM 505 E) at −18°C. Vertical slices about 10 μm thick were collected on gelatin-coated microscopic slides. The slides were air dried and dehydrated in a series of increasing concentrations of ethanol (50, 80, and 96% [vol/vol]).

Biofilm thickness measurement. Biofilm thickness was determined by positioning a thin glass needle mounted on a micromanipulator on the surface of the biofilm. The needle was moved down until it touched the substratum, which was detected by bending the needle, viewed through a dissection microscope. Biofilm thickness was inferred from micromanipulator readings.

Microsensor measurements. Microsensor measurements were performed at room temperature (20 to 23°C) in a flow cell with aeration and circulation of artificial wastewater containing the following ingredients: 50 mM KH_2PO_4, 400 μM K_2HPO_4, 760 μM $(NH_4)_2SO_4$, 41 μM $MgSO_4 \cdot 7H_2O$, and 200 μM Na-acetate at pH 7.5 (the last ingredient was added as an additional carbon source to the bound carbon inside the biofilm to ensure that oxygen remained the limiting substrate). The oxygen concentration in the flow cell was kept the same as that in the activated-sludge basin, i.e., approximately 70 μM O_2, by bubbling with air and nitrogen. The oxygen concentration in the flow cell was continuously monitored with an oxygen microsensor. Microsensors, mounted on micromanipulators, were positioned on the surface of the biofilm, which was viewed through a dissection microscope. Profiles were recorded by penetrating the biofilm with the microsensor in increments of 50 or 100 μm and using a micromanipulator.

Microsensors were used to measure oxygen (37), hydrogen sulfide (19), and pH (38). The oxygen and pH electrodes were calibrated as described before (37, 38). The H_2S microsensor was calibrated by measuring the signal in dilution series of a standard solution (sulfide dissolved in artificial wastewater with a constant pH, flushed with nitrogen to avoid oxidation of the sulfide) (18). The concentration of total dissolved sulfide (H_2S plus HS^- plus S^{2-}) in the dilution series was determined by a spectrophotometric method (8). The total dissolved sulfide measured in the biofilm was calculated by using the slope and intercept of the calibration curves. The sulfide electrode had a linear response to H_2S concentrations of up to 1,000 μM. The detection limit of the microsensors varied between 1 and 3 μM total sulfide. No pH correction was necessary, since the sensor was calibrated at the same pH as the sample medium and the buffer prevented the development of pH gradients inside the biofilm. The diffusive fluxes were calculated by using Fick's first law, $J = -D \times (\delta c/\delta x)$, where J is flux (in micromoles per square meter per second), D is diffusion coefficient (in square meters per second), and $\delta c/\delta x$ is concentration gradient (in micromoles per cubic meter). Diffusion coefficients inside the biofilm were assumed to equal the molecular diffusion coefficients (7). Values of 2.12×10^{-5} cm^2/s for oxygen (3) and 1.39×10^{-5} cm^2/s for total sulfide (18) at 20°C were used.

Total iron determination. Ten milliliters of 0.5 M HCl and 0.5 g of dithionite were added to the biofilm sample to extract Fe oxides, FeS, and $FeCO_3$ (46). The total iron concentration in this extract was determined spectrophotometrically at 562 nm after reaction of the extract with Ferrozine solution (1% [wt/vol] hydroxylammoniumchloride and 10% [wt/vol] Ferrozine in 50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid buffer, pH 7.0) (46). The detection limit was 1 μM total iron.

Nitrate determination. The nitrate concentration in the bulk liquid of the activated-sludge basin was measured spectroscopically with Spectroquant model 14773 (E. Merck, Darmstadt, Germany). The detection limit was 16 μM (1 mg/liter) NO_3^-.

Net sulfate reduction measurement with radiotracers. Net sulfate reduction (sulfate reduction minus sulfide oxidation) was determined by measuring the accumulation of reduced sulfur compounds in the 7-week-old biofilm. Carrier-free $^{35}SO_4^{2-}$ (2.5 MBq; Amersham) was added to the medium in the microsensor setup after a blank sample of the biofilm was taken for a reference. During the 30-h incubation period, samples were taken from the biofilm (3 to 4 cm^2 each) and from the medium (4 ml each) at intervals of at least 2.5 h to determine total radioactivity and sulfate concentration. The entire sample (foil plus adhering biofilm) was preserved in a 20% (wt/vol) Zn-acetate solution. The fraction of reduced $^{35}SO_4^{2-}$ per square centimeter of plastic foil (i.e., radioactive H_2S, FeS, S^0, and FeS_2) was determined with the foil and biofilm by the hot acidic Cr-II procedure as described by Fossing and Jørgensen (13). The net sulfate reduction was expressed as an areal rate, i.e., moles of reduced ^{35}S per square meter per second for comparison with microsensor flux measurements.

Nucleic acid extraction and PCR amplification. DNA was extracted from the biofilm samples by a combined freeze-thaw (freezing three times in liquid nitrogen and thawing at 37°C) and hot phenol-chloroform-isoamyl alcohol treatment (45). The ribosomal DNA was enzymatically amplified as described by Muyzer et al. (29) with either the universal primer 907R and the bacterial primer GM5F with a GC clamp or the universal primer 907R and the SRB385 primer (1) with a GC clamp, targeting SRB of the delta subdivision as well as some other bacteria (Table 1). A hot-start, touch-down PCR program was used for all amplifications to minimize nonspecific amplification (29). The PCR mixture (100 μl) contained 50 pmol of each primer, 25 nmol of each of the four deoxynucleoside triphosphates, 300 μg of bovine serum albumin, 10 μl of 10× PCR buffer (HT Biotechnology Ltd.), and 10 to 20 ng of template DNA.

DGGE analysis of 16S rDNA fragments. DGGE was performed with the D-Gene system (Bio-Rad) and the following ingredients and conditions: 1× TAE (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.3), 1-mm-thick gels, and a denaturant gradient containing 35 to 65% urea-formamide at 60°C, with 100 V constantly for 17 h version (29, 30). DGGE gels were photographed on a UV transillumination table (302 nm) with a Polaroid camera. Photos were scanned with the software program Fotolook version 2.05 (Agfa), and inverse images were prepared with Photoshop version 4.0 (Adobe).

Blotting and hybridization analysis of DGGE gels. DGGE gels were blotted onto nylon membranes (Hybond+; Amersham) as described by Muyzer et al. (29). Hybridization analysis was performed with probes specific for different groups of sulfate reducers (Table 1). Probe 660 is specific for *Desulfobulbus* species; probe 687 targets *Desulfovibrio* species as well as some members of the genera *Geobacter*, *Desulfomonas*, *Desulfuromonas*, *Desulfomicrobium*, *Bilophila*, and *Pelobacter*; and probe 804 targets *Desulfobacter*, *Desulfobacterium*, *Desulfosarcina*, *Desulfococcus*, and *Desulfobotulus* species (11). The probes were end labeled with radioactive [γ-^{32}P]ATP (New England Biolabs). The hybridization buffer used was described by Martinez-Picado and Blanch (25) and contained the following: 10× Denhardt solution, 4× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate at pH 7.0), 0.1% (wt/vol) sodium dodecyl sulfate, 2 mM EDTA, and 50 μg of salmon sperm DNA per ml. Membrane blots were prehybridized for 3 to 4 h at 40°C before the radioactively labeled probe was added. Hybridization was performed for about 17 h at 40°C. Thereafter, the membranes were washed twice at 40°C for 30 min with 2× SSC–0.1% (wt/vol) sodium dodecyl sulfate. To eliminate nonspecific binding, the membranes were washed two more times for 15 min at the appropriate dissociation temperature (56°C for probe 660, 48°C for probe 687, and 52°C for probe 804), determined by the method described by Raskin et al. (36). The hybridized membranes were dried and exposed for 1 to 2 days to a PhosphoImager screen. These screens were further analyzed with the PhosphoImager and the program ImageQuant (Molecular Dynamics, Inc.).

In situ hybridization. The protocol described by Manz et al. (22) was used for fluorescence in situ hybridization (FISH) of the biofilm slices, with probe NON338 as a negative control and probe SRB385 for the detection of sulfate reducers (Table 1). Probes NSO1225, NIT3, and NSR1156 were used to estimate the amount of nitrifying bacteria in the biofilm. The probes were synthesized and labeled with the fluorochrome CY3 (Interactiva GmbH, Ulm, Germany). The

TABLE 1. Oligonucleotides used in this study

Oligo-nucleotide	Sequence	Position[a]	Formamide concn (%)[b]	NaCl concn (mM)[c]	Specificity
GM5F[d]	5′-CCTACGGGAGGCAGCAG-3′	341–357	Not used for FISH	Not used for FISH	Bacteria (30)
907R	5′-CCGTCAATTCCTTTGAGTTT-3′	907–928	Not used for FISH	Not used for FISH	All organisms (universal probe) (30)
SRB385[d]	5′-CGGCGTCGCTGCGTCAGG-3′	385–402	35	80	SRB of the delta proteobacteria (1) plus several gram-positive bacteria (e.g., *Clostridium*) (32)
NON338	5′-ACTCCTACGGGAGGCAGC-3′	338–355	0	900	None (negative control probe) (22)
Probe 660	5′-GAATTCCACTTTCCCCTCTG-3′	660–679	Not used for FISH	Not used for FISH	*Desulfobulbus* (11)
Probe 687	5′-TACGGATTTCACTCCT-3′	687–702	Not used for FISH	Not used for FISH	*Desulfovibrio* (11) plus some members of the genera *Geobacter*, *Desulfomonas*, *Desulfuromonas*, *Desulfomicrobium*, *Bilophila*, and *Pelobacter*
Probe 804	5′-CAACGTTTACTGCGTGGA-3′	804–821	Not used for FISH	Not used for FISH	*Desulfobacterium*, *Desulfobacter*, *Desulfococcus*, *Desulfosarcina*, *Desulfobotulus* (11)
NSO1225	5′-CGCGATTGTATTACGTGTGA-3′	1225–1244	35	80	Ammonium-oxidizing beta proteobacteria (28)
NIT3[e]	5′-CCTGTGCTCCATGCTCCG-3′	1035–1048	40	56	*Nitrobacter* spp. (50)
NSR1156	5′-CCCGTTCTCCTGGGCAGT-3′	1156–1173	30	112	Freshwater *Nitrospira* spp. (41)

[a] Position in the 16S rRNA of *Escherichia coli* (4).
[b] Formamide concentration in hybridization buffer.
[c] Sodium chloride concentration in washing buffer.
[d] When used for DGGE, this primer has the following GC clamp at its 5′ end: 5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′.
[e] Used with unlabeled competitor oligonucleotide cNIT3 (5′-CCTGTGCTCCAGGCTCCG-3′) (50).

hybridization buffer contained 0.9 M NaCl, the percentage (vol/vol) of formamide shown in Table 1, 20 mM Tris-HCl (pH 7.4), and 0.01% (wt/vol) sodium dodecyl sulfate. The probe concentration was 5 ng/μl. Hybridization was performed for 1 to 2 h at 46°C. The biofilm was washed at 48°C for 15 min in a washing buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, the NaCl concentration indicated in Table 1, and 0.01% (wt/vol) sodium dodecyl sulfate.

The specimens were examined with an Axioplan epifluorescence microscope (Carl Zeiss, Oberkochen, Germany). CY3-stained cells were counted in 20 to 22 areas of 125 by 625 μm^2, both at the bottom and at the top of the biofilm. The density of the cells in the biofilm was too high to allow enumeration of the total number stained with 4′,6-diamidino-2-phenylindole (DAPI). Therefore, the number of stained bacteria was expressed per unit of volume of biofilm.

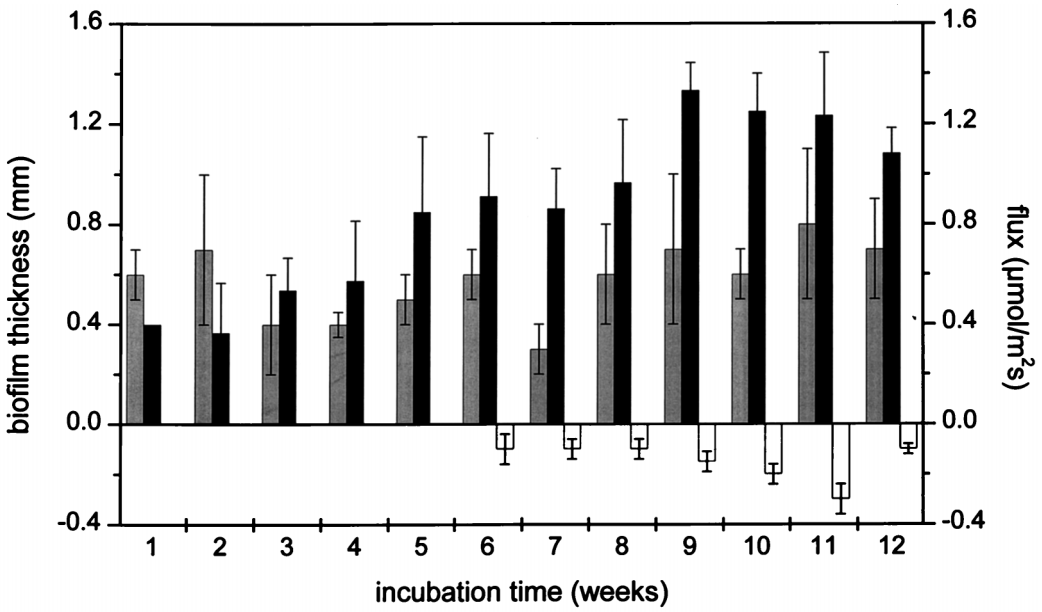

FIG. 1. Development of biofilm thickness (black bars) and fluxes of oxygen into the biofilm (gray bars) and sulfide into the oxic zone (white bars) calculated from the microprofiles.

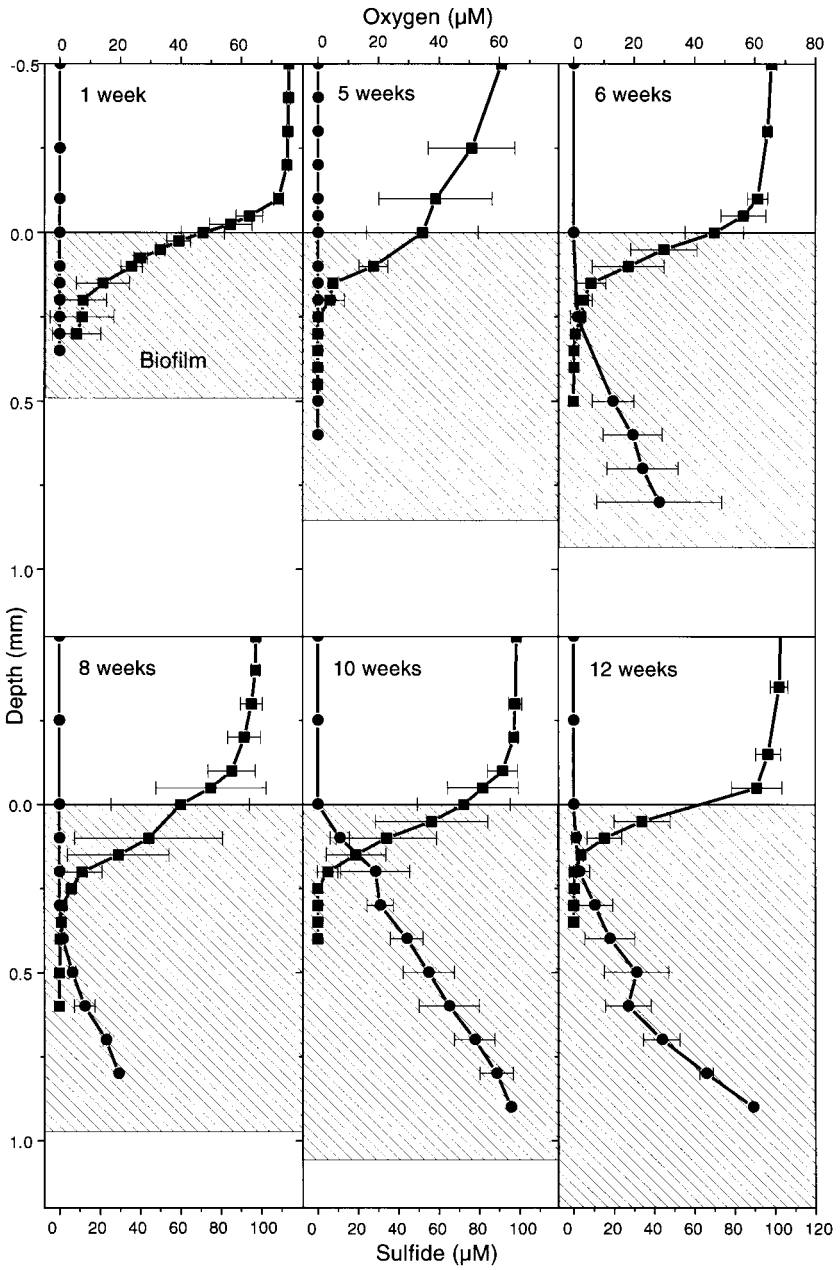

FIG. 2. Oxygen (■) and total sulfide (●) microprofiles in 1-, 5-, 6-, 8-, 10- and 12-week-old biofilms. Biofilm thickness is indicated by the shaded areas. The biofilm surface is at a depth of 0 mm, the water phase is indicated by negative values, and the biofilm is indicated by positive values.

RESULTS

Biofilm growth and development process. Within 1 week, a patchy biofilm ca. 400 μm thick developed on the plastic foil in the activated-sludge basin (Fig. 1). The thickness of the biofilm increased until the eighth week, after which it remained more or less constant at 1,000 to 1,200 μm.

Oxygen and sulfide profiles measured at different stages of biofilm development are shown in Fig. 2. The graphs show mean values of four to six profiles measured at different positions in the biofilm. pH values measured in the biofilm were within 0.05 U of the pH in the bulk liquid (data not shown) because of the buffering capacity of the medium used for measuring the profiles. Within 1 week, the biofilm developed anaerobic zones, which grew thicker during the following weeks. Oxygen was depleted within the top 200 to 400 μm in all stages of biofilm development (except in some profiles of the 1-week-old biofilm). Although the anaerobic environment in the biofilm would have allowed sulfate reduction, as there was enough substrate and sulfate available, no sulfide production was measured until the sixth week.

In the sixth week, a total sulfide concentration of 40 μM was detected, which increased to approximately 100 μM in the 10- and 12-week-old biofilms (Fig. 2). No sulfide diffused out of the biofilm, and oxygen and sulfide profiles overlapped, indicating that all sulfide produced by sulfate reduction was oxidized in the aerobic part of the biofilm.

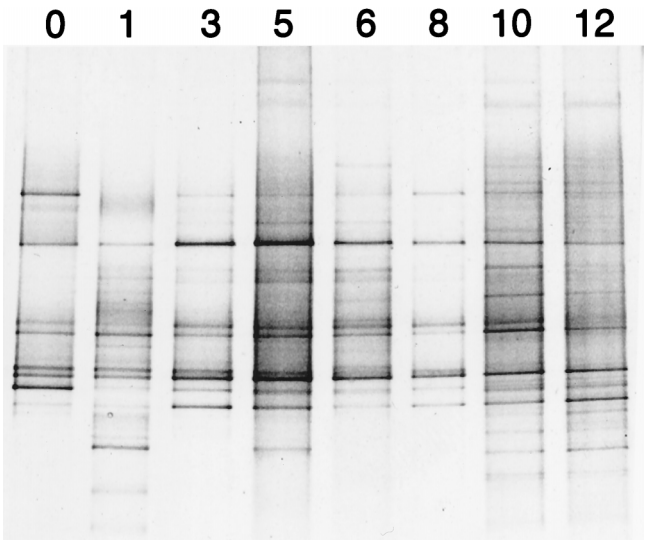

FIG. 3. DGGE analysis of 16S rDNA fragments obtained after PCR amplification with primer pair GM5F-907R and genomic DNA from bacterial biofilm samples taken at different time points. From left to right: sample taken directly from the activated sludge ($t = 0$ weeks) and samples taken from 1-, 3-, 5-, 6-, 8-, 10-, and 12-week-old biofilms (as indicated above the lanes).

Iron, which could have bound the sulfide, was not detected either in biofilm samples (measured at weeks 5 and 11) or in the activated sludge (data not shown).

Sulfate reduction rates measured by a radiotracer method with $^{35}SO_4^{2-}$ showed much lower values (approximately 0.017 $\mu mol/m^2 \cdot s^{-1}$) than the rates calculated with microsensor data (Fig. 1). Also, the radioactive tracer technique showed that no reduced S compounds accumulated inside the biofilm or bulk water.

Community development. To determine how the structure of the community developed with time and to identify the presence of SRB, DGGE of PCR-amplified DNA fragments and hybridization analysis of these DGGE profiles with group-specific probes were performed. Figure 3 shows the DGGE pattern of 16S rRNA gene fragments obtained after PCR amplification with a primer pair which amplifies members of the domain *Bacteria*. Figure 4 shows a graph of the total number of bands as well as new bands appearing relative to the activated sludge and the total number of bands remaining from the activated sludge. Nearly all bands obtained from the activated sludge remained in the biofilm samples. However, new bands also appeared during the maturation of the biofilm.

Detection of SRB in DGGE profiles. DGGE gels with DNA fragments amplified with primers for members of the domain *Bacteria* were blotted and hybridized with probes specific for different groups of SRB (probes 660, 687, and 804). No hybridization was observed, probably due to the small ratio of each SRB strain to the total number of bacteria in the biofilm.

To increase the sensitivity of SRB detection in our samples, we used the SRB385 sequence with a GC clamp as a forward primer, which targets sulfate reducers as well as some other delta proteobacteria and gram-positive bacteria, and the 907R sequence as the reverse primer. In this way, we excluded the amplification of 16S rDNA fragments of most of the *Bacteria* and enriched PCR products from SRB. These DGGE profiles were subsequently blotted and hybridized with radioactively labeled oligonucleotide probes specific for different groups of SRB.

In these SRB-enriched DGGE gels, clear hybridization was obtained with probes 660 and 687 (Fig. 5B and 6B) but not with 804 (data not shown). This result indicates the presence of *Desulfobulbus* (probe 660) and possibly *Desulfovibrio* (probe 687, which also targets members of the genera *Geobacter*, *Desulfomonas*, *Desulfuromonas*, *Desulfomicrobium*, *Bilophila*, and *Pelobacter*) and the absence of all species targeted by probe 804 (*Desulfobacter*, *Desulfobacterium*, *Desulfosarcina*, *Desulfococcus*, and *Desulfobotulus*) in all stages of biofilm development and in activated sludge. Furthermore, hybridization analysis of the DGGE profiles with probe 660 showed the initial presence of two *Desulfobulbus* strains and the appearance of another strain after week 6 (compare lanes 2 to 5, Fig. 5B, with lanes 6 to 9), coinciding with the first detected sulfide production by microsensors. The result for probe 687 was similar; initially two *Desulfovibrio* strains were present, and a third appeared during week 8 (compare lanes 2 to 6, Fig. 6B, with lanes 7 to 9).

The DGGE patterns obtained with primers targeting sulfate reducers (Fig. 5 and 6) show less variation with time than those obtained with bacterial primers (Fig. 3). This trend can be seen more clearly in Fig. 7, which contains a graph of the number of total, new, and remaining DGGE bands. The number of bands representing SRB varies only between 14 and 21, whereas Fig. 4 shows a variation of 14 to 37 bands from all bacteria during biofilm development.

In situ hybridization of sulfate-reducing and nitrifying bacteria. In situ hybridization with the SRB385 probe of thin sections of biofilms taken at weeks 2 and 11 stained *Vibrio*-shaped bacteria and short fat rods. Both morphotypes, resembling *Desulfovibrio* and *Desulfobulbus* cells, were present over the whole thickness of the biofilm. In the 2-week-old biofilm, the number of SRB was (64 ± 23 [mean $\pm$ standard deviation]) $\times 10^6$ SRB/ml on the surface and $114 \pm 33 \times 10^6$ SRB/ml at the bottom of the biofilm. In the 11-week-old biofilm, the SRB varied from $201 \pm 78 \times 10^6$ SRB/ml to $(358 \pm 127) \times 10^6$ SRB/ml on the surface and at the bottom of the biofilm, respectively.

In situ hybridization with probes specific for nitrifying bacteria revealed the presence of ammonia-oxidizing bacteria (30×10^6 to 90×10^6 cells per ml) and nitrite-oxidizing bacteria, *Nitrobacter* species (0.6×10^6 to 1×10^6 cells per ml) and

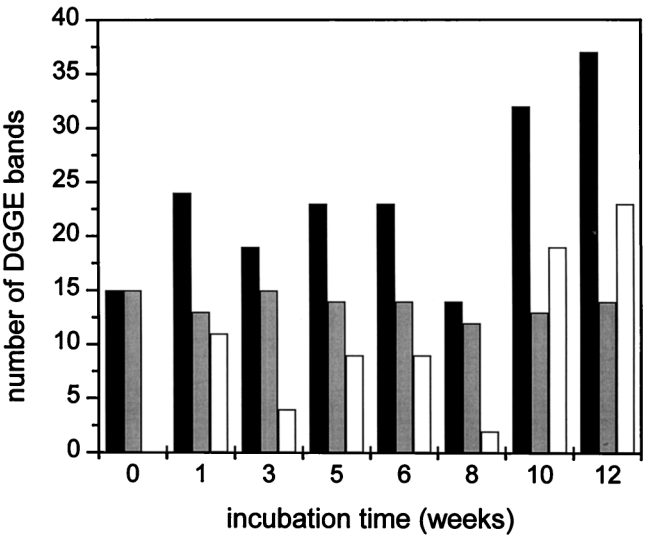

FIG. 4. Graphical representation of the number of bands in the denaturing gradient gel shown in Fig. 3. Total number of DGGE bands, black bars; total bands remaining from week 0 (activated sludge), gray bars; new bands after week 0, white bars.

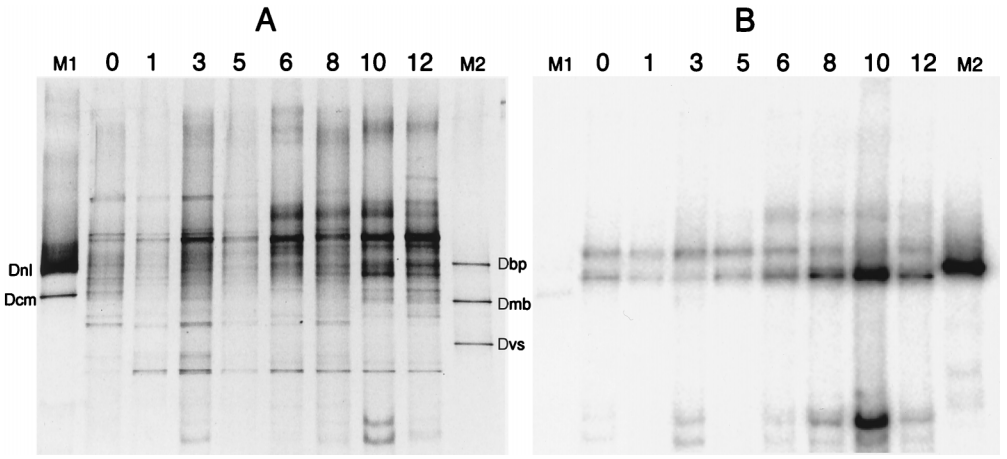

FIG. 5. (A) DGGE analysis of PCR-amplified 16S rDNA fragments obtained with primers SRB385 and 907R and bacterial genomic DNA from biofilm samples taken at different time points. From left to right: marker 1 (M1), *Desulfonema limicola* (Dnl) and *Desulfococcus multivorans* (Dcm); sample taken from the activated sludge ($t = 0$ weeks); samples from 1-, 3-, 5-, 6-, 8-, 10-, and 12-week-old biofilms (as indicated above the lanes); marker 2 (M2), *Desulfobulbus propionicus* (Dbp, positive control for probe 660), *Desulfomicrobium baculatum* and *Desulfovibrio salexigens* (Dmb and Dvs, positive controls for probe 687). (B) Hybridization results of the same DGGE pattern hybridized with probe 660, which is specific for *Desulfobulbus* species. The bands in the lower parts of the gels are single-stranded DNA and should be disregarded.

Nitrospira species (50×10^6 to 100×10^6 cells per ml), respectively, in the 2- and 6-week-old biofilms.

DISCUSSION

Process and biofilm development. The thickness and community characteristics of the biofilm developed gradually during 12 weeks without large-scale sloughing. The biofilm had a heterogeneous structure during the whole experimental period, as was reported in other studies (10, 26), explaining the variations in the amounts of oxygen and sulfide (Fig. 1). Heterogeneity may increase surface area exchange, therefore allowing more efficient transport of substrates and products. In determining the fluxes, we assumed that diffusion was the only transport mechanism; thus, the fluxes might be underestimated. However, the fluxes measured in this study are close to those calculated by Kühl and Jørgensen (18) for a trickling-filter biofilm.

The microprofiles (Fig. 2) showed that no sulfide diffused out of the biofilm. Since iron was absent, iron sulfide precipitation can be excluded. The low value for the sulfate reduction rate, determined with radiotracers, compared to the rates calculated from microsensor data, can be explained only by cycling between reduced and oxidized ^{35}S compounds. Most of the $^{35}S^{2-}$ formed is rapidly oxidized to $^{35}SO_4^{2-}$ or other oxidized ^{35}S compounds, which are not detectable by our radiotracer method (H_2S, S^0, FeS_2, and FeS). Examination of the specific activity of the reduced sulfur pool during the course of the experiment showed that the pool rapidly became equilibrated (within 3 h) with the initially labeled sulfate. This result indicates that a small reduced sulfur pool (sulfide) with a high turnover rate is present in the biofilm.

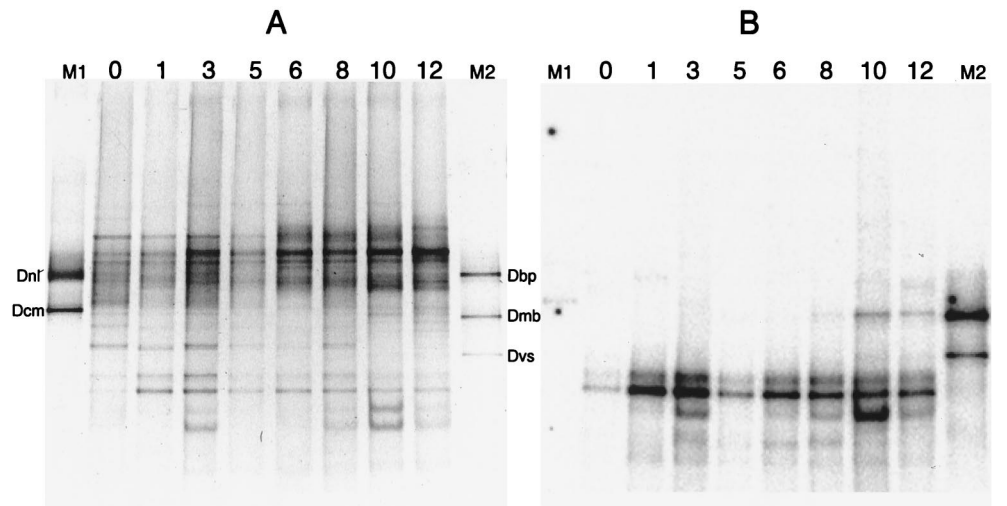

FIG. 6. (A) DGGE analysis of PCR-amplified 16S rDNA fragments obtained with primer set SRB385 and 907R. From left to right: marker 1 (M1), *Desulfonema limicola* (Dnl) and *Desulfococcus multivorans* (Dcm); sample taken from the activated sludge ($t = 0$ weeks); samples from 1-, 3-, 5-, 6-, 8-, 10-, and 12-week-old biofilms (as indicated above the lanes); marker 2 (M2), *Desulfobulbus propionicus* (Dbp, positive control for probe 660), *Desulfomicrobium baculatum*, and *Desulfovibrio salexigens* (Dmb and Dvs, positive controls for probe 687). (B) Hybridization results of the same DGGE pattern hybridized with probe 687, which targets *Desulfovibrio* species. The bands in the lower parts of the gels are single-stranded DNA and should be disregarded.

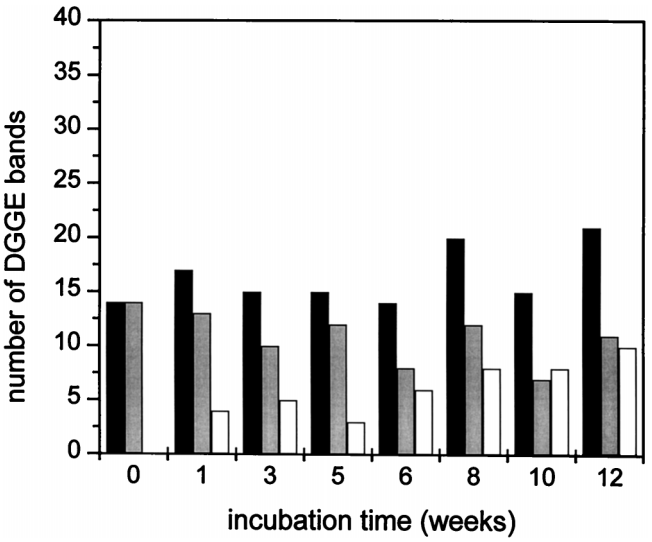

FIG. 7. Graphical representation of the number of bands in the denaturing gel shown in Fig. 5A and 6A. Total number of DGGE bands, black bars; total bands remaining from week 0 (activated sludge), gray bars; new bands, white bars.

We concluded that all sulfide, produced by sulfate reduction in the anoxic zone, was oxidized in the oxic layer in the biofilm. Thus, at the point of the steepest gradient, the sulfide flux in the zone adjacent to the aerobic zone can be used as a conservative measure of SRB activity. Diffusive fluxes of oxygen into the biofilm and sulfide from the anaerobic zone into the aerobic zone, calculated from the microprofiles, are given in Fig. 1. Sulfide fluxes ranged from 15% in week 6 to 35% in week 11 of the oxygen fluxes (Fig. 1). Since oxidation of 1 mol of sulfide to sulfate requires 2 mol of oxygen, a large part (up to 70%) of the oxygen diffusing into the biofilm was used for the reoxidation of sulfide. Thus, sulfate reduction may be as important as aerobic mineralization in the biofilm. Since the biomass yield by sulfate reduction is much lower than by aerobic mineralization (21), rates of biomass production in wastewater treatment plants based on biofilms may be affected significantly. The turnover of the sulfide pool in the biofilm, calculated by dividing the pool size by the fluxes (both determined from microsensor profiles), lasted only minutes. Consequently, sulfate reduction rates in biofilms can be reliably measured in situ only by microsensors.

The development of anaerobic zones in the biofilm created an optimal environment for sulfate reducers. However, sulfide production could not be measured during the first 6 weeks of biofilm development. In theory, sulfide could have been oxidized anaerobically, e.g., by Fe^{3+} or NO_3^- as an electron acceptor. However, neither Fe^{3+} nor NO_3^- was present in the bulk liquid of the activated-sludge basin. In situ hybridization showed the presence of ammonia and nitrite oxidizers in the 2- and 6-week-old biofilms; consequently, some nitrate could have been formed by nitrification inside the biofilm. However, even if sulfide reduction was coupled with anaerobic sulfide oxidation in the initial stages of biofilm development, some sulfide should have been detected by the sensitive sulfide microsensor. We therefore conclude that no sulfate reduction occurred in the biofilm during the first 6 weeks. Similar lag phases in developing biofilms have been detected for sulfate-reducing (48) and other slow-growing bacteria, such as nitrifiers (47) and methanogens (14). Slow adaptation of the sulfate-reducing population to the biofilm microenvironment might explain the lag phase in sulfate reduction that was found in our study.

Community development. Comparative DGGE analysis of PCR products obtained with primers for the 16S rDNA of all bacteria showed that the species composition of the biofilm changed within 1 week from that of the activated sludge (Fig. 3). The original strains remained, but new bands appeared each week (Fig. 4), leading to a more complex bacterial community. Exceptions were week 8 and, to a lesser degree, week 3, when fewer bands were detected. These exceptions might have been caused by a lower amount of DNA being loaded on the gel. Our results show that biofilms can harbor a much wider range of microbial species than bulk liquid. The increase in bacterial diversity, shown by the increasing number of DGGE bands, was the result of the development of different microhabitats inside the biofilm. Due to resistance to mass transfer and conversions, microzonations develop within the biofilm, providing a broader range of niches for bacteria with different physiological characteristics.

DGGE analysis of PCR products obtained with primers specific for sulfate reducers, i.e., primer pair SRB385F/907R, showed that the community of delta proteobacteria, including the sulfate reducers, was more stable during biofilm development than the total community (compare Fig. 4 and 7). *Desulfobulbus* and *Desulfovibrio* species were present from the beginning. Additional populations of *Desulfobulbus* and of *Desulfovibrio* appeared, respectively, after 6 and 8 weeks of incubation. We speculate that the additional strains from weeks 6 and 8 are responsible for the sulfide production in the biofilm.

The question of the metabolic activity of the species identified as SRB, present in the first 5 weeks, is unresolved. Our FISH data indicate that a significant portion of the SRB are present and proliferate in the oxic part of the biofilm. The ability of *Desulfobulbus* and *Desulfovibrio* species to respire oxygen (12, 24) could explain their presence in activated sludge and the initial biofilm. As the activated-sludge basin is aerated, other nonrespiring sulfate reducers may be unable to survive in this aerobic environment. Also, the finding of Teske et al. (45), that *Desulfobulbus* and *Desulfovibrio* species are the main SRB in the aerobic layers of a stratified fjord, underlines their ability to survive (and maybe thrive) in the presence of oxygen. Alternatively, SRB could use a different metabolic pathway. Some *Desulfobulbus* and *Desulfovibrio* species can utilize nitrate instead of sulfate as a terminal electron acceptor (27, 43, 52). There is conflicting evidence about whether or not nitrate is used in the presence of sulfate. It has been reported that nitrate can suppress sulfate reduction (43) and, conversely, that sulfate inhibits the use of nitrate as a terminal electron acceptor (9). However, no nitrate was found in the bulk liquid of the activated-sludge basin. Another metabolic role of SRB could be the fermentation of organics, such as fumarate or malate (51).

With in situ hybridization, we found 10^7 to 10^8 SRB/ml of biofilm volume. Considering the total cell density of 10^{10} to 10^{11} cells per ml of biofilm reported by others (7, 31), we estimate that the relative percentage of bacteria that are SRB is less than 1%. This, however, contradicts our finding that a large part of the oxygen diffusing into the biofilm is used for oxidation of the formed sulfide and that sulfate reduction, therefore, plays an important role in the biofilm. An explanation for this discrepancy is that either the SRB are extremely active or our FISH technique underestimates the number of SRB. From the H_2S fluxes and the number of SRB (determined by FISH), the specific sulfate reduction rate (moles of SO_4^{2-} $cell^{-1}$ day^{-1}) was calculated to be 70×10^{-15} mol of SO_4^{2-} $cell^{-1}$ day^{-1} (at week 11). This value is in the upper

range of the specific sulfate reduction rates reported by Jørgensen (15).

By using the molecular techniques described in this study, we were able to follow community development and to detect different groups of SRB in complex biofilms with many species. This would be difficult to achieve with conventional cultivation techniques. However, molecular techniques also have biases and limitations. First, PCR amplification is not quantitative, as preferential amplification can occur (39, 44). Therefore, band intensities cannot be extrapolated to indicate the abundance of a particular bacterial population. Second, the number of DGGE bands is dependent on the amount of DNA that is applied to the gel as well as on the number of different DNA fragments. Third, some of the oligonucleotides, i.e., SRB385 and probe 687, which were used in this study, are not as specific as originally described. However, we used only primer SRB385 in our PCRs to exclude most non-SRB and to enrich the SRB populations. Probe SRB385 was also used for in situ hybridization analysis. For this application, the specificity of the probe is increased, as the gram-positive cells, targeted by SRB385, are not accessible to the oligonucleotide when paraformaldehyde is used as the fixative (40). These biases demonstrate the importance of combining different molecular methods and comparing them with activity measurements.

Relating community analysis obtained by molecular techniques to processes occurring within microbial consortia is of great practical relevance. However, the molecular techniques currently available, and used in this study, seem insufficient to accurately predict the behavior of a gradually changing but complex microbial community. More specific techniques, perhaps involving the detection or expression of specific genes, are needed so that molecular techniques can be used as reliable diagnostic tools. Further analysis of the biofilm samples, nitrate microsensor measurements, and in situ hybridization with more specific probes (23) might provide a more detailed picture of the abundance, distribution, and functioning of the SRB groups.

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