Structure and function of nitrifying biofilms as determined by molecular techniques and the use of microelectrodes

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Abstract The phylogenetic diversity of a nitrifying bacterial community of two types of nitrifying biofilms, a domestic wastewater biofilm and an autotrophic nitrifying biofilm grown on rotating disk reactors (RDR), was characterized by 16S ribosomal DNA (rDNA)-cloning analysis. Thereafter, successional development of the bacterial community within both biofilms was visualized in situ by fluorescent in situ hybridization (FISH) with a set of fluorescently labeled 16S rRNA-targeted DNA probes. In situ hybridization revealed that *Nitrosomonas ureae* was the numerically dominant species of the ammonia-oxidizing population in the domestic wastewater biofilm and that a population shift from *N. urea* to *N. europaea* and *N. eutropha* occurred when the culture medium was switched to the synthetic media from the domestic wastewater. After reaching the steady-state condition, microprofiles of NH4+, NO2-, NO3-, and O2 in the biofilms were measured by use of microsensors, and the spatial distributions of in situ nitrifying activities were determined. The relationship between the spatial organization of nitrifying bacterial populations and the in situ activity of these populations within the biofilms was discussed. Microelectrode measurements revealed that the active ammonia-oxidizing zone was vertically separated from the active nitrite-oxidizing zone. This vertical separation became more evident with increase of the substrate C/N ratio, leading to deterioration of nitrification efficiency. The combined use of these techniques made it possible to relate in situ nitrifying activity directly to the occurrence of nitrifying bacterial populations.

Keywords Fluorescent in situ hybridization (FISH); microelectrodes; nitrifying biofilm; 16S rDNA-cloning analysis

Introduction Microbial nitrification processes for nitrogen removal are becoming more important due to strict regulations on nitrogen discharge. The process of nitrification is carried out by two phylogenetically unrelated groups of lithoautotrophic bacteria, the ammonia-oxidizing bacteria and nitrite-oxidizing bacteria. Since the diverse nitrifying bacterial populations are expected to be present in wastewater biofilms, different species of NH4+- and NO2- oxidizing bacteria exhibit different in situ growth kinetics, substrate affinities, and sensitivities to various environmental factors (e.g. pH, temperature, O2 concentration and substrate concentrations). In fact, there is increasing evidence of dominance by species or genera other than *Nitrosomonas europaea* and *Nitrobacter* spp., which have been commonly isolated and well investigated as representative ammonia- and nitrite-oxidizing bacteria, respectively, in practical environments. Application of molecular techniques (16S rRNA gene cloning analysis, denaturing gradient gel electrophoresis (DGGE), and fluorescent in situ hybridization (FISH)) revealed a high genetic diversity within the ammonia oxidizing bacteria of the β-subclass of Proteobacteria in lake water, soils, and wastewater treatment processes (Kowalchuk et al., 1998; Kowalchuk et al., 1999; Purkhold et al., 2000). Thus, a better understanding of microbial diversity, microbiology, and ecology of nitrifying bacteria in wastewater treatment processes is essential for improving process performance and
control. However, investigation of the diversity and microbial ecology of nitrifying bacteria in wastewater biofilm and activated sludge systems by conventional culture-based techniques has been hampered by their slow growth rates and by the biases inherent in all culture-based techniques. It is now widely recognized that only a small fraction, possibly below 10%, of the naturally occurring microorganisms, even in mesotrophic activated sludge systems, has been isolated and characterized so far. During the last few years, molecular techniques have been combined successfully with microelectrode measurements with high spatial and temporal resolution, which are the most reliable way of studying microbial nitrification in nitrifying biofilms (Schramm et al., 1996; Okabe et al., 1999) and in microbial flocs (Schramm et al., 1998).

Therefore, the 16S ribosomal DNA clone library was constructed from two types of biofilms grown on rotating disk reactors (RDR) with domestic wastewater and a synthetic nutrient medium to identify the ammonia-oxidizing bacteria in this paper. Based on the phylogenetic analysis, successional development of nitrifying bacterial community structure during the biofilm growth phase was monitored by fluorescent in situ hybridization (FISH) with a set of fluorescently labeled 16S rRNA-targeted DNA probes. Furthermore, the in situ nitrifying activity was measured by using microelectrodes and directly related to the spatial distribution of nitrifying bacteria. The data sets resulting from these different approaches were carefully integrated to perceive valid overall pictures of nitrifying bacteria community and their activity development in the biofilms.

**Experimental materials and methods**

**Biofilm samples**

Two types of biofilms, a domestic wastewater biofilm and an autotrophic nitrifying biofilm, were studied. Both biofilms were cultured in partially submerged rotating disk reactors (RDR) consisting of 5 poly-methyl-methacrylate disks. Eight removable slides (1 × 6 cm) were installed in each disk for sampling biofilms. The autotrophic nitrifying biofilms were first cultured with the primary settling tank effluent from the Shoseigawa municipal wastewater treatment plant, Sapporo, Japan for a few days and then were cultured with synthetic nutrient medium. The nutrient medium was composed of the following (mM): NH₄Cl, (3.6); NaHCO₃, (17.8); K₂HPO₄, (0.03); MgSO₄·7H₂O, (0.41); NaCl, (1.25); pH = 7.0 ± 0.2. The reactor volume was 1370 cm³, and total biofilm area was 2,830 cm². Temperature was maintained at 20°C. Disk rotational speed was fixed at 14 rpm. Dilution rate in the reactors was kept at 0.2 h⁻¹.

**Nucleic acid extraction and PCR amplification**

Approximately, 1 g of each wet biofilm sample was mixed with 1 ml of AE buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA) in clean 15-ml tubes. After ultrasonic treatment (10 min) and lysis (5 mg of lysozyme per ml for 40 min at 37°C and 2 mg of protease K per ml for 30 at 55°C), bacterial DNA was extracted from biofilm samples by a combined freeze-thaw (three cycles of freezing in liquid nitrogen and heating at 37°C), 1% (w/vol) sodium dodecyl sulfate (SDS) treatment, and hot phenol-chloroform-isoamyl alcohol treatment (Teske et al., 1996). The 16S rRNA genes (rDNA) from mixed bacterial DNA were amplified by PCR with the primer set of CTO189f and CTO654r as described by Kowalchuk et al. (1997). For general bacteria, almost-full length bacterial 16S rDNA fragments were amplified using the primer set of GM3f (Escherichia coli 16S rDNA positions 8 to 24) and GM4r (E. coli positions 1492 to 1507) as described by Muyzer et al. (1995). To minimize nonspecific annealing of the primers to nontarget DNA, a hot-start and touch-down PCR program was used for all amplification (Muyzer et al., 1997). The PCR products were evaluated on a 1% (w/v) agarose gel.
Cloning of 16S rDNA

One microlitre of the amplified bacterial 16S rDNA fragments (465 bp including variable V3 region) was directly ligated into the pGEM-T vector cloning system (Promega) and transformed into competent cells (high-efficiency *E. coli* JM109 [Promega]) as described in the manufacturer’s instruction.

Sequencing and phylogenetic analysis

Plasmids were extracted and purified from clones with the Wizard Plus Minipreps DNA purification system (Promega) in accordance with the manufacturer’s instructions. To avoid redundant sequencing, PCR-amplified DNA fragments of all clones were analyzed by RFLP (Restriction fragment length polymorphism) after digestion with restriction enzymes of *cfoI* or *haeIII* as described in the manufacturer’s instruction. The PCR fragments digested were loaded on a 2.0% (w/v) agarose gel. Similar fragment migration patterns were defined as identical recombinants, and one representative of each group of recombinants was selected for comparative sequence analysis. Partial sequencing (ca. 465 bp) of the 16S rDNA inserts was performed with an automatic sequencer (DNA Sequencer 5500, HITACHI). All sequences were checked for chimeric artifacts by the CHECH_CHIMERA program in the Ribosomal Database Project (RDP) (Maidak *et al*., 1997) and compared with similar sequences of the reference organisms by BLAST search (Altschul *et al*., 1990). Phylogenetic trees were constructed by the neighbour-joining method (Saito and Nei, 1987) with Tree Explore. Bootstrap resampling analysis for 100 replicates was performed.

Fixation and cryosectioning of biofilm samples

Biofilm samples were taken at regular time intervals during the periods of the biofilm development. The biofilm samples were fixed with freshly prepared paraformaldehyde solution (4% in phosphate buffered saline (PBS), pH = 7.2) for 4 to 8 h at 4°C and embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) overnight to infiltrate the OCT compound into the biofilm. After rapid freezing at −21°C, 10 to 20 µm thick vertical slices were cut with a cryostat (Reichert-Jung Cryocut 1800, Leica) and placed on a gelatin-coated slide (Cell-line, USA, 0.1% gelatin and 0.01% chromium potassium sulfate). After air drying overnight, the slices were dehydrated by successive passage through 50, 80, and 98% ethanol washes (for 3 min each), air dried, and stored at room temperature.

Oligonucleotide probes

The following oligonucleotide probes were used: Nso190 (Mobarry *et al*., 1996), NEU (Wagner *et al*., 1995), Nsm156 (Mobarry *et al*., 1996), NmV (Pommerening-Rosser *et al*., 1996), Nsv443 (Mobarry *et al*., 1996), NIT2 (Wagner *et al*., 1996), NIT3 (Wagner *et al*., 1996), Ntpa454 (Hovanec *et al*., 1998), Ntpa685 (Hovanec *et al*., 1998), and Ntpa1026 (Juretschko *et al*., 1998). Probes were labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine-5-isothiocyanate (TRITC). Unlabelled competitor CNIT3 (Wagner *et al*., 1996) and CTE (Schleifer *et al*., 1992) probes were added to an equimolar amount of NIT3 and NEU probes, respectively.

In Situ hybridization

The previously published optimal hybridization conditions were used for each probe. All in situ hybridizations were performed according to the procedure described by Amann (1995) in hybridization buffer (0.9 M NaCl, 20 mM Tris hydrochloride (pH = 7.2), 0.01% sodium dodecyl sulfate (SDS), x% formamide) at 46°C for 2–3 hours. The final probe
concentration was approximately 5 ng µl⁻¹. Subsequently, a stringent wash step was performed at 48°C for 20 min in 50 ml of pre-warmed washing solution (× mM NaCl, 20 mM Tris hydrochloride (pH = 7.2), 0.01% SDS). The stringency of the washing step (at 48°C) was adjusted by lowering the sodium chloride concentration to achieve the appropriate specificity. The slides were then rinsed briefly with ddH₂O and allowed to air dry. Simultaneous hybridization with probes requiring different stringency was performed by a successive hybridization procedure: hybridization with the probe requiring higher stringency was performed first, and then hybridization with the probe requiring lower stringency was performed (Wagner et al., 1995). Slides were mounted in SlowFade™-light antifade kit (Molecular Probes, Eugene, OR).

Microelectrode preparation and measurements
For determination of concentration profiles in the biofilms, cathode type oxygen micro-electrodes with a tip diameter of about 10 µm were prepared and calibrated as described previously by Revsbech and Jorgensen (1986). Liquid ion-exchanging membrane (LIX) microsensors for NH₄⁺, NO₂⁻, and NO₃⁻ were prepared according to de Beer et al. (1997). The LIX microsensors were calibrated in dilution series (10⁻³–10⁻⁶ M) of NH₄⁺, NO₂⁻, and NO₃⁻ in the medium used for the measurements. All measurements were performed as described previously (de Beer and Heuvel, 1988) in a flow cell reactor at 20°C, with an average liquid velocity of 2–3 cm s⁻¹ by blowing air on the liquid surface. The composition of the medium used for microprofile measurements was described previously by de Beer et al. (1993). The biofilm samples taken from the reactor were acclimated in the medium a few hours before the measurement, to ensure that steady state profiles were obtained.

Estimation of consumption and production rate profiles
Net specific consumption and production rates (R; µmol cm⁻³ h⁻¹) of NH₄⁺, NO₂⁻, and NO₃⁻ were estimated from the measured microprofiles by using the Fick’s second law of diffusion as previously described by Lorenzen et al. (1998). Molecular diffusion coefficients of 1.38 × 10⁻⁵ cm²s⁻¹ for NH₄⁺, 1.23 × 10⁻⁵ cm²s⁻¹ for NO₂⁻, and 1.23 × 10⁻⁵ cm²s⁻¹ for NO₃⁻ at 20°C were used for the calculations (Andrussow, 1969).

Results and discussion
General bacteria
Thirty-two clones were selected at random for the autotrophic nitrifying biofilm cultured at the substrate C/N (g/g) = 0 (Figure 1). Twelve different clones were identified and sequenced. Seven 16S rDNA clone sequences were affiliated with the a subclass of Proteobacteria (58%). Four and one clone sequences were affiliated with the β subclass of Proteobacteria (33%) and the phylum Cytophaga-Flavobacterium-Bacteroides (8%), respectively. Three 16S rDNA clone sequences (EGM-01, –28, –23) were closely related to Nitrosomonas europaea. Two clone sequences (EGM-08 and –22) were related to the 16S rDNA of a Sphingomonas sp. of the α subclass of Proteobacteria. Microbial community structure of the biofilm cultured at the substrate C/N=1 was significantly different from that in the biofilm cultured at the substrate C/N = 0. A major group represented in the clone library of the biofilm at C/N = 1 was the β subclass of Proteobacteria (62%) (data not shown). Within the β subclass of Proteobacteria, three clone sequences were closely related to Sphaerotilus spp. Only one clone sequence was closely related to ammonia-oxidizing bacteria, i.e. Nitrosomonas europaea, which was identical with the clone retrieved from the biofilm at C/N = 0 (99% similarity). Three clone sequences were affiliated with the γ subclass of Proteobacteria (23%). No clone sequence was found in the α subclass of
Proteobacteria. No sequences affiliated with the genus *Nitrobacter* were retrieved from any biofilms in this study.

**Nitrifying bacteria**

The phylogenetic microbial diversity of ammonia-oxidizing bacteria in a domestic wastewater and autotrophic nitrifying biofilms were determined by 16S rDNA-cloning and compared (Figure 2). 16S rDNA clone libraries were constructed by PCR with a β-subdivision ammonia-oxidizing bacteria-specific primer set (CTO189f and CTO654r), and partial sequencing (465 bp) including variable V3 region of the clonal 16S rDNAs was conducted for phylogenetic analysis. Among the clones analyzed, 10 and 7 different clone sequences were found in the DWWB and ANB libraries, respectively. 16S rDNA sequence analysis revealed that about 62% of the total domestic wastewater biofilm (DWWB) clones sequenced were closely related to members of *Nitrosomonas ureae* with more than 97% sequence similarity. These clones were closely related to each other. We also detected three clones closely related to *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis*, respectively. One clone (DWWB-22) was affiliated with a deeply branched group of *Nitrosovibrio* and *Nitrosococcus*. In the ANB clone library, the most dominant sequence was affiliated with *Nitrosomonas eutropha* with more than 95%
sequence similarity. Six clone sequences were closely related to *Nitrosomonas europaea*. This result might indicate that although the strains affiliated with *N. ureae* were numerically dominant NH$_4^+$-oxidizers in the domestic wastewater biofilm, *N. eutropha* and *N. europaea* who have higher growth rates became dominant populations in the autotrophic nitrifying biofilm after switching to the synthetic nutrient medium. The results of 16S rDNA-cloning analysis, however, do not allow quantitative conclusions about the abundance of the ammonia-oxidizing bacteria due to the biases inherent in molecular techniques.

To confirm and visualize such population dynamics of NH$_4^+$-oxidizing bacteria in the autotrophic nitrifying biofilm, fluorescent in situ hybridization (FISH) with a set of 16S rRNA-targeted oligonucleotide probes (i.e. Nso190, Nsm156 and NEU) were, therefore, performed (data not shown). In situ hybridization of vertical biofilm thin sections clearly indicated that the numbers of probe NEU-stained NH$_4^+$-oxidizing bacteria (i.e. *Nitrosomonas marina*-lineage, *Nitrosomonas europaea*-lineage, *Nitrosomonas eutropha*, and *Nitrosospira halophila*) were very low in the young autotrophic nitrifying biofilm, and other *Nitrosomonas*-lineages which hybridized with probe Nsm156 but did not hybridize with NEU were numerically dominant populations. As the biofilm grew, probe NEU-stained NH$_4^+$-oxidizing bacteria became the dominant populations in the autotrophic nitrifying biofilm. This population shift might be attributed to the inhibitory effect of NO$_2^-$.
which accumulated up to approximately 1.5 mM during the biofilm growth and higher growth rates.

In contrast, *Nitrosomonas* spp. which hybridized with probe Nsm156 but did not hybridize with NEU were the numerically dominant species in the domestic wastewater biofilm. According to the results of 16S rDNA-cloning analysis, NH$_4^+$-oxidizing bacteria which hybridized with probe Nsm156 but did not hybridize with NEU could be a member of *Nitrosomonas ureae*. The FISH result reflected the results of 16S rDNA-cloning analysis. The NO$_2^-$-oxidizing bacteria belonging to the genus *Nitrobacter* could not be detected in any biofilm sample studied in this biofilm; instead, *Nitrospira* were found to be the main NO$_2^-$-oxidizing bacteria in both types of biofilms.

**Microelectrode measurements**

We also measured the microprofiles of NH$_4^+$, NO$_2^-$, and NO$_3^-$ and could, therefore, directly correlate the in situ activity of NH$_4^+$ and NO$_2^-$ oxidation with the spatial distributions of NH$_4^+$- and NO$_2^-$-oxidizing bacteria within biofilms. An example of microsensor measurements is presented in Figure 3. Oxygen penetrated approximately 150 µm into the autotrophic nitrifying biofilm (Figure 3A). The NH$_4^+$ and NO$_2^-$ profiles showed that NH$_4^+$ and NO$_2^-$ were both consumed and converted to NO$_3^-$ in the upper 100 µm. Thus, the active NH$_4^+$ oxidation zone completely overlapped with the active NO$_2^-$ oxidation zone (Figure 3B). This result was in good agreement with the spatial organization of NH$_4^+$- and NO$_2^-$-oxidizing bacteria (data not shown), showing that both NH$_4^+$- and NO$_2^-$-oxidizing bacterial clusters were densely present in the upper 100 µm. We obtained the average specific NH$_4^+$ and NO$_2^-$ oxidation rates of 32.6 ± 17.5 µmol NH$_4^+$ cm$^{-3}$ h$^{-1}$ and 24.1 ± 15.3 µmol NO$_2^-$ cm$^{-3}$ h$^{-1}$, respectively, which are comparable with the values reported in previous microsensor studies of nitrifying biofilms and aggregates (de Beer et al., 1993; de Beer et al., 1997; Schramm et al., 1998). When biofilms were cultured with domestic wastewater and synthetic media containing organic carbon, the active NH$_4^+$ oxidation zone was vertically separated from the active NO$_2^-$ oxidation zone. That is, the active NH$_4^+$ oxidation zone was located in the outer part of a biofilm, whereas the active NO$_2^-$ oxidation zone was located just below the NH$_4^+$ oxidation zone. Accordingly, NH$_4^+$-oxidizing
bacteria were present throughout the biofilms, whereas NO$_2^-$-oxidizing bacteria was mainly found in the lower part of the oxic biofilm strata, and the location of NO$_2^-$-oxidizing bacteria overlapped with the active NO$_2^-$ oxidation, as determined with FISH. We will discuss more about the relationship between the in situ activity of NH$_4^+$ and NO$_2^-$ oxidation and the spatial distributions of NH$_4^+$- and NO$_2^-$-oxidizing bacteria within biofilms grown in different media.

Conclusions
We have combined molecular techniques (i.e. 16S ribosomal DNA (rDNA)-cloning analysis and fluorescent in situ hybridization (FISH) with a set of fluorescently labeled 16S rRNA-targeted DNA probes and microsensor measurements for NH$_4^+$, NO$_2^-$, NO$_3^-$, and O$_2$. The combined use of these techniques made it possible to relate in situ nitrifying activity directly to the occurrence of nitrifying bacterial populations. In summary, the combination of molecular techniques and microsensor measurements is a very powerful research tool and provides new insights into microbial nitrification occurring in wastewater biofilm processes.

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