Characterization of bacterial biofilm communities in tertiary treatment processes for wastewater reclamation and reuse

Tadashi Shoji, Shuichi Ochi and Masaaki Ozaki

ABSTRACT

The concern with wastewater reuse as a sustainable water resource in urban areas has been growing. For the reclamation and distribution of wastewater, biofilm development deserves careful attention from the point of view of its promotion (e.g. biofiltration) and inhibition (e.g. clogging and hygiene problems). As the first step to control biofilm development, bacterial biofilm communities in tertiary treatment processes were characterized by using molecular biological methods. The result of clone library analysis showed that Nitrospirae-related (nitrite-oxidizing bacteria) and Acidobacteria-related (probably oligotrophic bacteria) groups were dominant. The ratio of the Nitrospirae-related group to the Acidobacteria-related group was associated with ammonia load, whereas other operational conditions (process, media, temperature, salt) did not clearly affect the phylum-level community or the dominant sequence of nitrifying bacteria. The result of real-time PCR also indicated that high ammonia load promotes the proliferation of nitrite- and ammonia-oxidizing bacteria. Regarding water supply systems, some researchers also have suggested the dominance of Nitrospirae- and Acidobacteria-related groups in biofilm formed on water distribution pipes. In tertiary wastewater treatment, therefore, it is concluded that oligotrophic and autotrophic bacteria are the dominant groups in biofilm samples because assimilable organic carbon is too poor to proliferate various heterotrophic bacteria.

Key words | biofilm, heterotrophic bacteria, microbial community, nitrifying bacteria, tertiary treatment

INTRODUCTION

The concern with wastewater reclamation and reuse as a sustainable water resource in urban areas has been growing. In order to mitigate the risk and discomfort of reclaimed wastewater, several treatment technologies have been developed. For example, chemical or physical technologies such as ozonation, UV irradiation, and membrane filtration are useful to decrease organic carbon and health-related microorganisms. On the other hand, biological treatment is useful for nitrification and degradation of suspended solids. For this purpose, not suspended but fixed biomass should be preferred because washout of biomass can be averted under low-load conditions. In the case of tertiary treatment of wastewater, therefore, biofiltration using biofilm fixed on various media is one of the most useful processes. Although biofilm formation should be promoted for biological treatment, it may cause clogging problems in the physical filtration process (e.g. sandfiltration) and hygiene problems in the distribution system (Bishop 2007). In either case, the characteristics of biofilm deserve careful attention to optimize wastewater reclamation systems. For water supply systems, several studies have been made on bacterial biofilm communities formed on biological activated carbon (Kasuga et al. 2007) and distribution pipes (Regan et al. 2002; Martiny et al. 2005; Regan et al. 2003;
Martiny et al. 2005). However, little is known about bacterial biofilm in wastewater reclamation systems so far.

In this paper, therefore, bacterial biofilm communities in biofiltration or sandfiltration processes for tertiary wastewater treatment were examined by using molecular biological methods. Eleven biofilm samples formed on anthracite, chemo-treated or activated carbon, porous ceramics, and plastic media were gathered from 9 municipal wastewater treatment plants in Japan. Clone library analysis targeting the bacterial 16S rRNA gene was performed to identify and quantitatively characterize the microbial communities. Moreover, nitrifying bacteria (ammonia-oxidizing bacteria: AOB, and nitrite-oxidizing bacteria: NOB) were quantified by real-time PCR assay using specific primer and probe sets. The objectives of this study are to characterize the microbial communities of biofilms for tertiary wastewater treatment and to examine the effects of operational conditions on the communities.

**MATERIALS AND METHODS**

**Biofilm samples**

The biofilm samples were gathered from 9 municipal wastewater treatment plants in winter (January or February). Table 1 summarizes the characteristics of fixed media, influents, and operational conditions. The samples (about 100 mL) were brought to our laboratory with a cold insulator as soon as possible (mostly on the day of the sampling). The biofilm samples were peeled off by ultrasonication (38 kHz, 120 W for 5 min) in phosphate-buffered saline and concentrated by centrifugation (14,000 r.p.m. for 5 min).

**Construction of clone libraries**

DNA was extracted by a Fast DNA SPIN Kit For Soil (Q-BIOgene, Irvine, CA), and purified by a MonoFas I (GL Sciences, Tokyo, Japan). PCR amplification of the 16S rRNA gene was performed with a PE9700 thermal cycler (Perkin Elmer, Waltham, MA). The primer sets used are 8f-1492r for bacteria and CTO189f-654r for AOB (nucleotide sequences are shown in Table 2). The thermal program for the 8f-1492r set was as follows: 94°C for 10 min, followed by 30 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 120 s, and a final incubation at 72°C for 10 min. The thermal program for the CTO189f-654r set was as follows: 95°C for 10 min, 92°C for 1 min, followed by 30 cycles at 92°C for 30 s, 57°C for 30 s, 72°C for 45 s, and a final incubation at 72°C for 5 min. The PCR products were ligated into the Blunting Kination Ligation Kit (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. Except for several samples, the cloning and sequencing works after ligation were carried out as subcontracting by Takara Bio. For the sample analyzed by ourselves, the sequencing works were performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) in an ABI 377 DNA sequencer (Applied Biosystems). The obtained sequences were identified using a BLAST program (DDBJ/EMBL/GenBank). Moreover, the phylogenetic trees were illustrated using MEGA3 (Kumar et al. 2004) with the option available on the ClustalW program through the Neighbour-Joining algorithm.

**Real-time PCR**

The quantification of the targeting genes was performed by real-time PCR with a LightCycler ST300 (Roche Diagnostics, Basel, Switzerland) and a QuantiTect Probe PCR Kit (Qiagen, Venlo, Netherlands) according to the manufacturer’s instructions. The primer and probe sets used were CTO189f-RT1r and TMP1 for AOB, and NSR1113f-1264r and NSR1143Taq for NOB (shown in Table 2). The thermal programs were as follows: 95°C for 15 min, followed by 50 cycles at 95°C for 30 s and 60°C for 60 s (AOB, described by Hermansson and Lindgren 2001), and 95°C for 15 min, followed by 50 cycles at 95°C for 30 s and 63°C for 60 s (NOB, described by Harms et al. 2003). Gene copies were calculated by comparison of the sequenced clones as the standard samples after calculation of their concentration and dilution from 10^3 to 10^7 orders of copies per μL.

**RESULTS AND DISCUSSION**

**Characteristics of bacterial biofilm community**

As Figure 1 indicates, the Nitrospirae-related group was the most dominant (29% of total clones), especially in the high
<table>
<thead>
<tr>
<th>Name</th>
<th>Process</th>
<th>Media</th>
<th>Pretreatment</th>
<th>Temperature</th>
<th>NH₄⁻ N load</th>
<th>BOD</th>
<th>Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA¹</td>
<td>Pilot-scale</td>
<td>Activated carbon</td>
<td>Sand filtration</td>
<td>Low⁻⁴, about 10°C</td>
<td>Low⁻⁶, 0.1 mg/L</td>
<td>Inf: 10.7⁻⁶ mg/L, Eff: 9.4⁻⁶ mg/L</td>
<td>Inland (Data N.A.)</td>
</tr>
<tr>
<td>KC¹</td>
<td>Pilot-scale</td>
<td>Chemo-treated carbon</td>
<td>Sand filtration</td>
<td>Low⁻⁴, about 10°C</td>
<td>Low, 0.1 mg/L</td>
<td>Inf: 10.7⁻⁶ mg/L, Eff: 9.1⁻⁶ mg/L</td>
<td>Inland (Data N.A.)</td>
</tr>
<tr>
<td>FE</td>
<td>Upflow BF with</td>
<td>Anthracite</td>
<td>Coagulation, precipitation, ozonation</td>
<td>Middle, 17°C</td>
<td>High, 15.2 mg/L</td>
<td>Inf: 7.0⁻⁷ mg/L, Eff: 3.8⁻⁷ mg/L</td>
<td>Coast 310 mg/L</td>
</tr>
<tr>
<td>AR</td>
<td>Upflow BF with</td>
<td>Anthracite</td>
<td></td>
<td>Middle, 22°C</td>
<td>Low, 0.2 mg/L</td>
<td>Inf: 1 mg/L, Eff: 1 mg/L</td>
<td>Coast 260 mg/L</td>
</tr>
<tr>
<td>MK</td>
<td>Upflow BF with</td>
<td>Anthracite</td>
<td></td>
<td>Middle, 18°C</td>
<td>High, 15 mg/L</td>
<td>Inf: 5 mg/L, Eff: 2 mg/L</td>
<td>Inland 74 mg/L</td>
</tr>
<tr>
<td>OC</td>
<td>Upflow SF</td>
<td>Sand</td>
<td></td>
<td>Middle, 19°C</td>
<td>Low, 0.6 mg/L</td>
<td>Inf: 2 mg/L, Eff: 1 mg/L</td>
<td>Inland 50 mg/L</td>
</tr>
<tr>
<td>SS¹</td>
<td>Upflow SF</td>
<td>Sand</td>
<td></td>
<td>Middle, 18°C</td>
<td>High, 11 mg/L</td>
<td>Inf: 3 mg/L, Eff: data N.A.</td>
<td>Coast 220 mg/L</td>
</tr>
<tr>
<td>SB¹</td>
<td>Upflow BF with</td>
<td>Anthracite</td>
<td></td>
<td>Middle, 18°C</td>
<td>High, 11 mg/L</td>
<td>Inf: 3 mg/L, Eff: data N.A.</td>
<td>Coast 220 mg/L</td>
</tr>
<tr>
<td>NG</td>
<td>Upflow BF with</td>
<td>Porous ceramics</td>
<td></td>
<td>High, 25°C</td>
<td>High, 12.1 mg/L</td>
<td>Inf: 6.7 mg/L, Eff: 4.7 mg/L</td>
<td>Coast (Data N.A.)</td>
</tr>
<tr>
<td>IM</td>
<td>Upflow BF with</td>
<td>Anthracite</td>
<td></td>
<td>High, data N.A.</td>
<td>High, data N.A.</td>
<td>data N.A.</td>
<td>Coast (Data N.A.)</td>
</tr>
<tr>
<td>CN</td>
<td>Upflow BF with</td>
<td>Cylindrical plastic</td>
<td></td>
<td>High, 25°C</td>
<td>High, 15.1 mg/L</td>
<td>Inf: 3.1 mg/L, Eff: 1.5 mg/L</td>
<td>Coast 500 mg/L</td>
</tr>
</tbody>
</table>

¹: KA/KC and SS/SB were taken from same wastewater treatment plant.
²: BF, biofiltration; SF, sandfiltration.
³: Treatment between secondary settler and BF/SF process.
⁴: This could be nearly equal to air temperature because both the pilot-scale process and influent tank were set outside.
⁵: Not available. But qualitative estimation may be possible based on comments by the officials.
⁶: COD Cr.
⁷: COD Mn.
ammonia-loaded (about 10 mgN/L) groups, FE, MK, SB, SS, NG, IM, and CN. Although most of the ammonia was nitrified to nitrate in these biofiltration processes, the AOB-related clone was rarely detected. On the other hand, the second dominant group, the Acidobacteria-related one (15% of total clones), was more frequently detected from the low ammonia-loaded groups, KA, KC, AR, and OC. Next to them, α-Proteobacteria-, Bacteroidetes-, and β-Proteobacteria-related clones accounted for about 10% each. The dominance of Nitrospirae- and Acidobacteria-related clones has also been reported in a nonchlorinated water distribution pipe (Martiny et al. 2005). They reported that the most dominant clone detected from the old biofilm was Nitrospirae-related (29%), followed by the Acidobacteria-related one (14%). What needs to be emphasized is that the dominant groups are probably oligotrophic (Acidobacteria) according to Fierer et al. (2007) or autotrophic NOB (Nitrospirae). Moreover, there were about 5% of Verrucomicrobia- and Planctomycete-related groups in both their and our samples, which are rarely observed in activated sludge for secondary waste-water treatment. In tertiary wastewater treatment, therefore, it is concluded that the low load of organic carbon caused the dominance of oligotrophic or autotrophic bacteria, which was similar to the biofilm formed on water distribution pipes. On the other hand, it must be noted that the significant amount of nitrifying bacteria can be a source of organic carbon (Rittmann et al. 1994). Kindaichi et al. (2004) and Okabe et al. (2005) reported the dominance of nitrifying bacteria (the sum of AOB and NOB were 50–60%), in addition to the coexistence of heterotrophic bacteria, which utilize dead biomass and metabolites of nitrifying bacteria in autotrophic biofilm. Although the quantitative effect of organic carbon produced by nitrifying bacteria is too complicated to be examined here, it may be relatively considerable compared with the influent.

Table 2 | Primers and probes used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>8f</td>
<td>AGAGTTGATCM^2TGGCTCAG</td>
<td>Lane (1991)</td>
</tr>
<tr>
<td>1492r</td>
<td>GGTACCTTGTTACGAGTTT</td>
<td>Gordon &amp; Giovannonni (1996)</td>
</tr>
<tr>
<td>CTO189f A/B</td>
<td>GGAGR^2AAAGCAGGGGATCG</td>
<td>Kowalchuk et al. (1997)</td>
</tr>
<tr>
<td>CTO189f C</td>
<td>GGAGAAAAGTAGGGGATCG</td>
<td>Kowalchuk et al. (1997)</td>
</tr>
<tr>
<td>CTO654r</td>
<td>CTAGC^2TTGATGTTCAAAACGC</td>
<td>Kowalchuk et al. (1997)</td>
</tr>
<tr>
<td>RT1r</td>
<td>GTCCTCTACGACACCTAAGCTCAG</td>
<td>Hermansson &amp; Lindgren (2001)</td>
</tr>
<tr>
<td>TMP1</td>
<td>^3(FAM)-CAACTAGCTAATCAG^12CATCRGCCGTC-(TAMRA)</td>
<td>Hermansson &amp; Lindgren (2001)</td>
</tr>
<tr>
<td>NSR1113f</td>
<td>CCGCCAGGCTTGTGCGCCG</td>
<td>Dionisi et al. (2002)</td>
</tr>
<tr>
<td>NSR1264r</td>
<td>GTCGCCAGCGGGTTGTAGCG</td>
<td>Dionisi et al. (2002)</td>
</tr>
<tr>
<td>NSR1143Taq</td>
<td>^3(FAM)-AGCACTCTGAAAGGACTGCCAG-(TAMRA)</td>
<td>Harms et al. (2003)</td>
</tr>
</tbody>
</table>

* 1: CTO189f is a 2:1 mixture of 189fA/B and 189fC.  
* 2: M indicates a degenerate nucleotide of A and C; R indicates that of A and G; Y indicates that of C and T.  
* 3: FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.

Figure 1 | Microbial community composition of the bacterial biofilm determined by PCR-cloning analysis (N = 59–92) and BLAST search. Although Nitrosomonadaceae is one of the members of α-proteobacteria, they are classified as a different group. Since some sequences could not be identified, the sum of the proportion (percentage of total clones) is less than 100%.
Quantification of nitrifying bacteria in the biofilm

Since nitrification is one of the most important roles of biofiltration processes, nitrifying bacteria need to be examined in detail. Figure 2 illustrates the copy numbers of *Nitrosomonas*-related AOB and *Nitrospirae*-related NOB. As well as the clone library analysis, the results of quantification can be classified into two groups; FE, MK, SB, SS, NG, IM, and CN are categorized as the high ammonia-load group, whereas KA, KC, AR, and OC are categorized as the low ammonia-load group. Moreover, the quantity of NOB ($10^3$ to $10^5$ copies/ng nucleic acid) was about 1 order of magnitude higher than that of AOB in each sample. Regarding the quantity of NOB, Dionisi *et al.* (2005) reported that $10^3$ to $10^4$ copies/ng-nucleic acid was detected from activated sludge fed with municipal wastewater by using the same primer set. Therefore, the biofilm samples for tertiary treatment contain the same level of NOB as activated sludge for secondary treatment in the case of low ammonia load, or 1 order of magnitude higher in the case of high ammonia load. Assuming that the mass of nucleic acid reflects the copy number of total bacterial DNA, this result also suggests the dominance of autotrophic bacteria in the biofilm samples. Regarding the difference between AOB and NOB, it has also been reported that NOB outnumber AOB in various environments, such as wastewater treatment plants (Dionisi *et al.* 2002), phosphate-removing biofilms (Gieseke *et al.* 2001), nitrifying fluidized bed reactors (Schramm *et al.* 1998), and water treatment lagoons (Mitsuhashi *et al.* 1983). However, there seems to be no established theory to explain the difference so far.

Characterization of nitrifying bacteria in the biofilm

Figure 3 illustrates the phylogenetic trees of AOB (based on 16S rRNA gene sequences amplified by the CTO primer set targeting *Nitrosomonas*-related AOB) and NOB (based on 16S rRNA gene sequences identified as *Nitrospirae*-related NOB). The CTO primer-based analysis was performed for AR, CN, FE, IM, NG, SF, and SB only, due to experimental problems. The AOB-like sequences were classified into four clusters, I, II, III, and IV. Cluster I mainly consists of SB (7 of 14 clones), SF (10 of 17 clones), and IM (8 of 18 clones), but it contains no sequence of pure culture. Cluster II mainly consists of CN (18 of 18 clones), FE (14 of 34 clones), and NG (9 of 23 clones), but it also contains no sequence of pure culture. Cluster III contains many sequences of pure cultures such as *N. oligotropha*, and contains of NG (9 of 23 clones), IM (5 of 18 clones), and a few clones from every sample except for CN. Cluster IV contains *N. communis* as a sequence of pure culture, and mainly consists of AR (5 of 11 clones) and IM (5 of 18 clones). Although the relationship between phylogenetic position and function is still controversial, Limpiyakorn *et al.* (2004) suggested that *N. communis*-like organisms (cluster IV) were more tolerant to the absence of oxygen or prefer a fluctuated-oxygen condition. In the present study, however, this assumption may not be applicable because both AR and IM samples were collected from aerated biofiltration processes. Moreover, it has been reported that *N. europaea*-like sequences are observed in wastewater treatment systems treating rich ammonia (Logemann *et al.* 1998; Pynaert *et al.* 2003). The absence of this kind of sequence in tertiary treatment indirectly approve of the assumption. On the other hand, the *Nitrospirae*-related sequences shown in Figure 3(b) were classified into two clusters, the *N. defulvii*-like group and the *N. moscoviensis*-like one. The most noteworthy point is that most of the high ammonia-load group (181 of 226 clones, 80%) belongs to the former cluster, whereas most of the low ammonia-load group (33 of 43 clones, 77%) belongs to the latter cluster. It should be added that very little nitrite was observed in both the influent and effluent of the treatment processes. These results imply that not nitrite but the ammonia load is one of the most important factors to proliferate specific NOB in tertiary wastewater treatment.
Figure 3  | Phylogenetic trees of (a) Nitrosomonas-related AOB (about 460 base pair sequences) and (b) Nitrospirae-related NOB (about 640 base pair sequences) based on the 16S rRNA gene. The figures after the sample names mean detected number/total clone number. Sample names in bold font indicate multiple-detected clone.
Effect of operational condition on the microbial community

It was pointed out in the previous paragraphs that the ammonia load affects the microbial community, such as the ratio of the Nitrospirae-related group to the Acidobacteria-related one, and the dominant cluster of the Nitrospirae-related group. However, the effect of other factors (process, media, temperature, and salt) is relatively smaller than that of the ammonia load. Comparing KA with KC or SB with SS, for example, the same influent caused quite similar clone libraries (Figure 2) and similar dominant sequences of AOB (Figure 3a), despite the difference of media (KA, activated carbon; KC, chemo-treated carbon) or process (SB, biofiltration with aeration; SS, sandfiltration without aeration). In order to examine other possible affecting factors, the direction of this study will be to compare biofilm samples in various water environments.

CONCLUSIONS

The bacterial biofilm communities in tertiary wastewater treatment were examined. The result of clone library analysis showed that Nitrospirae-related (nitrite-oxidizing bacteria) and Acidobacteria-related (probably oligotrophic bacteria) groups were dominant. Their ratio was associated with ammonia load, whereas other operational conditions (process, media, temperature, salt) did not clearly affect the phylum-level community or the dominant sequence of nitrifying bacteria. The result of real-time PCR also indicated that the ammonia load promotes the proliferation of nitrite- and ammonia-oxidizing bacteria. In tertiary wastewater treatment, therefore, it is concluded that oligotrophic and autotrophic bacteria are the dominant groups in biofilm samples because assimilable organic carbon is too poor to proliferate various heterotrophic bacteria.

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