Design of 16S rRNA-targeted oligonucleotide probes and microbial community analysis in the denitrification process of a saline industrial wastewater treatment system

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Abstract

Three 16S rRNA-targeted oligonucleotide probes, namely, PSMg437 targeting several members of the genus *Pseudomonas*, Hlm474 targeting several members of the genus *Halomonas*, and Clw844 targeting several members of the genus *Colwellia*, were designed. The microbial community structure and nitrogen removal ability of nitrate-containing saline wastewater treatment systems with anaerobic packed bed and fluidized bed were monitored. Direct cell counting using fluorescence in situ hybridization (FISH) images revealed that various phylogenetic groups were evenly distributed in the anaerobic packed bed whereas members of the genus *Halomonas* were dominant particularly in the anaerobic fluidized bed. These results suggest that the microbial communities produced by different flow conditions correlated with denitrification ability in saline industrial wastewater treatment systems.

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Keywords: Fluorescence in situ hybridization; Saline industrial wastewater; Denitrification; *Halomonas*; *Colwellia*

1. Introduction

The biological removal of nitrogen compounds through nitrification and denitrification is an integral part of most wastewater treatment systems for preserving water resources. Some industrial wastewaters and seawaters contain a large amount of nitrates and salt [1–3]. Van der Hoek et al. [4] and Clifford and Liu [5] showed that denitrification combined with ion exchange was possible under 3 wt% NaCl conditions. However, the denitrification rate of saline wastewaters decreased under highly saline conditions [6]. Therefore, nitrogen removal from saline wastewater has been considered to be difficult.

Metallurgic wastewater is one of the nitrate-containing saline wastewaters discharged during the recovery process of precious metals from industrial wastes. We have already demonstrated that biological treatment can remove nitrogen from this unique metallurgical wastewater [2]. However, nitrite and/or nitrate sometimes accumulated in the denitrification processes of these systems. Therefore, knowledge of the microbial ecology in saline wastewater is required to determine the factors influencing the efficiency and stability of metallurgical wastewater treatment systems (MWTSs). Although aerobic halophilic species have been reported so far [7,8], the microbial ecology of the denitrifying moderately halophilic bacteria has been barely reported. Labbe et al. revealed that *Methylophaga* members are dominant in a methanol-fed denitrification reactor used for a marine aquariums where seawater is recycled in a closed circuit [3]. Grguric et al. suggested that *Pseudomonas* spp. are involved in denitrification in a closed...
seawater system [9]. In our previous study, the microbial community in MWTSs has been described on the basis of 16S rRNA gene by DGGE and a cultivation method, and subsequent phylogenetic analysis [10,11]. The microbial community in MWTS consisted of Cytophaga–Flavobacterium, Gram-positive bacteria, β-Proteobacteria including Alcaligenes sp. and Acidovorax sp., and γ-Proteobacteria including Pseudomonas sp., Halomonas sp. and Colwellia sp. However, DGGE analysis can only identify the individual members of the complex microbial community but not quantify each member. To determine the microbial community shift and analyze its relationship with nitrogen removal ability, quantitative analyses of individual microbial members are thus required. These studies, which are considered as the “full-cycle rRNA approach” [12,13], have been reported for some wastewaters, but not for saline wastewaters. Furthermore, although the microbial community shift is important for industrial wastewaters, whose compositions frequently vary, most of the previous studies determined only microbial communities given by temporary sample. To maintain high nitrogen removal efficiency from saline wastewater, control of the microbial community is important.

In this study, we designed new oligonucleotide probes specific for the three phylogenetic groups of γ-Proteobacteria that had been detected in our previous study [10,11] (Fig. 1). Quantitative analysis of the microbial community in MWTSs was conducted by FISH direct counting using the newly designed probes. The changes in the microbial community were correlated with observed changes in the efficiency and stability of denitrification was clarified through the long-time operation of two kinds of MWTSs, which are circulating bioreactor systems equipped with an anaerobic packed bed or an anaerobic fluidized bed, with different flow conditions.

2. Materials and methods

2.1. Metallurgic wastewater treatment system and sludge samples

Sludge samples adhering and not adhering to biofilm-supporting media were randomly collected from two laboratory-scale anaerobic bioreactors used as MWTSs on days 167, 258 and 397 as described previously [10]. One of the anaerobic reactors, designated as R1, was packed with cubic media made of sponge without mixing. The other anaerobic reactor, designated as R2, was a completely mixed fluidized bed with poly(vinylalcohol) particles coated with activated carbon. Acetic acid was added as a carbon source for denitrification. Inlet composition was NOx-N (nitrate and nitrite) (1800–2500 mg l\(^{-1}\)), NH4-N (320–1690 mg l\(^{-1}\)), and salinity (15,000–35,000 mg l\(^{-1}\)). For FISH analysis, sludge samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (137 mM NaCl, 8.10 mM Na2HPO4·12H2O, 2.68 mM KCl, and 1.47 mM KH2PO4; pH 7.4) for 2 or 16 h at 4 °C, and then washed with PBS. The samples were stored in a 1:1 mixture of PBS and 96% ethanol at −20 °C. For positive references for FISH analysis, isolated bacteria from MWTSs in our previous study [10] were grown on trypticase soy agar (TSA) (Becton Dickinson, USA), fixed, and stored as described above.

2.2. Oligonucleotide probes

The oligonucleotide probes used in this study are listed in Table 1. Newly designed oligonucleotide probes for clusters I–III described in our previous study [10,11] (Fig. 1) were as follows: (i) PSMg437 (16S rRNA position, 436–452), modified PSMg described by Braumann-Koerber et al. [20] to be specific for several members of genera Pseudomonas, (ii) Clw844 (16S rRNA position, 844–863), specific for several members of genus Colwellia and (iii) Hlm474 (16S rRNA position, 474–493), specific for several members of genus Halomonas. The specificities of these designed probes were checked using the probe match program with the RDP II (Ribosomal Database Project II) [20]. The optimal formamide concentration was determined using a formamide gradient of 0–60% in hybridizations with fixed cells of the positive and negative reference strains to achieve optimal stringency: (i) For PSMg437, the positive reference was R1-Dec-MIB-5 and the negative reference was Microbacterium NCIMB 1141 (two mismatches). (ii) For Clw844, the positive reference was R2-July-MIB-2. (iii) For Hlm474, the positive reference was R3-Apr-MIB-1 and the negative references were Halomonas subglaciescola NCIMB 12994 (three mismatches) and H. marina NCIMB 1966 (three mismatches).

2.3. In situ hybridization

Fixed samples were immobilized on glass slides and dehydrated by successive passages through 50%, 80% and 100% ethanol. According to the standard hybridization protocol described by Amann [21], hybridization was performed at 46 °C for 2 h in a hybridization buffer containing NaCl (900 mM), formamide, Tris–HCl (20 mM, pH 7.4), and sodium dodecyl sulfate (SDS) (0.01%). The formamide concentrations are listed in Table 1. The probe concentration was 0.5 μg l\(^{-1}\). Hybridization was followed by a stringent washing step at 48 °C for 20 min in a washing buffer containing Tris–HCl (20 mM, pH 7.4), probe-dependent concentrations of NaCl (Table 1), and SDS (0.01%). Then, the samples were counterstained with SYBR Green I (1:10000 dilution; Molecular Probes Inc., USA), mounted in

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**Table 1**

<table>
<thead>
<tr>
<th>Probe Description</th>
<th>Positive Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSMg437</td>
<td>R1-Dec-MIB-5</td>
</tr>
<tr>
<td>Clw844</td>
<td>R2-July-MIB-2</td>
</tr>
<tr>
<td>Hlm474</td>
<td>R3-Apr-MIB-1</td>
</tr>
</tbody>
</table>

---

**References**

[9]...

[10]...

[11]...

[12]...

[13]...

[14]...

[15]...

[16]...

[17]...

[18]...

[19]...

[20]...

[21]...
FluoroGuard Antifade Reagent (Bio-Rad, USA), and observed under a confocal laser scanning microscope (TCS4D; Leica Lasertechnik, Germany) equipped with an Ar–Kr ion laser (488, 568 and 647 nm).

2.4. Quantification

Image integration and analysis were performed using the software package Mac scope (Mitani Corp., Japan). Quantification of cells hybridized with specific probes relative to the number of EUB338-hybridized cells was carried out.

3. Results

3.1. Probe design and in situ detection with newly designed probes

For the several members of genera *Pseudomonas* (cluster I), *Colwellia* (cluster II) and *Halomonas* (cluster III), the probe sets and their short names were designed, as listed in Table 1. The sequences of *Aquifex pyrophilus* was used as an outgroup to root the tree. The bootstrap numbers indicate the value of 1000 replicate trees supporting order [14], and values below 500 are omitted. Scale bar = 10% nucleotide substitution.
III) (Fig. 1), three probes were newly designed according to the sequences obtained in our previous study [10,11]. Clusters II and III were new clusters included in the genera *Colwellia* and *Halomonas*, respectively. By checking the specificity using the probe match program [20], probe Clw844 showed no mismatch to target sequences obtained from the isolated bacteria and DGGE bands. Probe Hlm474 showed no mismatch to target sequences derived from the isolated bacteria, *H. campisalis* [AF054286] and *Zymobacter palmae* [D14555]. For cluster I affiliated with the genus *Pseudomonas*, probe PSMg (16S rRNA position, 440–454) [19] which is specific for the genus *Pseudomonas* showed one mismatch to the sequences cR1-00A-1 and cR2-99O-1 obtained in our previous study [11]. Therefore, probe PSMg was modified by displacing the probe region to the 16S rRNA position 437–453 (PSMg437). Search with the probe match program found that the specificity of probe PSMg437 is almost the same as that of probe PSMg.

Positive and negative reference cells were used for the in situ visualization of target bacteria using the newly designed probes. The optimum formamide percentages were determined by comparing the fluorescence intensities of the positive and negative reference cells at various formamide concentrations. The highest formamide concentrations which resulted in a distinguishable positive reference from a negative reference were 20%, 10% and 45% for probes PSMg437, Clw844 and Hlm474, respectively. Next, the designed probes were applied to isolated bacteria affiliated with each genus (Table 2) at appropriate formamide concentrations to confirm their specificities. The specific detection was performed for all tested strains.

### Table 1.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>Sequence (5'-3')</th>
<th>NaCl conc. (mM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>Bacteria, several members of delta subclass of Proteobacteria</td>
<td>GCTGCCTCCCGTAGGAGT</td>
<td>0</td>
<td>[15]</td>
</tr>
<tr>
<td>ALFlb</td>
<td>Alpha subclass of Proteobacteria, several members of delta subclass of Proteobacteria</td>
<td>CGTTCGYTCTGAGCCAG</td>
<td>20</td>
<td>[16]</td>
</tr>
<tr>
<td>BET42a</td>
<td>Beta subclass of Proteobacteria, members of Cytophaga-Flavobacterium-Bacteroides phylum</td>
<td>GCCTTCCCACTTCGTTT</td>
<td>35</td>
<td>[16]</td>
</tr>
<tr>
<td>GAM42a</td>
<td>Gamma subclass of Proteobacteria, members of Cytophaga-Flavobacterium-Bacteroides phylum</td>
<td>GCCTTCCCACATCGTTT</td>
<td>35</td>
<td>[16]</td>
</tr>
<tr>
<td>CF319a</td>
<td>Cytophaga-Flavobacterium-Bacteroides phylum</td>
<td>TGGTCCGTGTCTCAGTAC</td>
<td>35</td>
<td>[17]</td>
</tr>
<tr>
<td>LGC354A</td>
<td>Gram-positive bacteria with low G+C content</td>
<td>TGGAAGATTCCCTACTGC</td>
<td>35</td>
<td>[18]</td>
</tr>
<tr>
<td>LGC354B</td>
<td>Same as LGC354A</td>
<td>CGGAAGATTCCCTACTGC</td>
<td>20</td>
<td>[18]</td>
</tr>
<tr>
<td>LGC354C</td>
<td>Same as LGC354A</td>
<td>CCGAAGATTCCCTACTGC</td>
<td>35</td>
<td>[18]</td>
</tr>
<tr>
<td>PSMg437</td>
<td>Several members of <em>Pseudomonas</em> sp.</td>
<td>CTTCCTCCCAAC</td>
<td>20</td>
<td>This study</td>
</tr>
<tr>
<td>Clw844</td>
<td>Several members of <em>Colwellia</em> sp.</td>
<td>GCTGCGTTACTCACTTCATT</td>
<td>10</td>
<td>This study</td>
</tr>
<tr>
<td>Hlm474</td>
<td>Several members of <em>Halomonas</em> sp.</td>
<td>CTGTGGGTGATGTCCTTCCT</td>
<td>45</td>
<td>This study</td>
</tr>
</tbody>
</table>

a FA, formamide.
Hlm474-hybridized cells are important particularly for NO\textsubscript{x} removal in R\textsubscript{2}. The Clw844-hybridized cells comprised 26\% of the EUB338-hybridized cells in R\textsubscript{2} only on day 258, when NO\textsubscript{x} removal rate was most stable throughout the MWTS operation time.

### 4. Discussion

The metallurgic wastewater used as an influent of the anaerobic reactors contained 1.5–3.5 wt\% NaCl, similar to that of seawater. We found that bacteria affiliated

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**Table 2**

Detection of isolated bacteria, positive and negative references using new designed oligonucleotide probes

<table>
<thead>
<tr>
<th>Genus</th>
<th>Bacterial strain</th>
<th>PSMg437</th>
<th>Clw844</th>
<th>Hlm474</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas</em></td>
<td>R\textsubscript{1}-Apr-MIB-5[AB126964]</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>R\textsubscript{1}-Dec-MB-5[AB126965]</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Colwellia</em></td>
<td>R\textsubscript{1}-Dec-MIB-6[AB126967]</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>R\textsubscript{1}-July-MIB-2[AB126968]</td>
<td>ND</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>Halomonas</em></td>
<td>R\textsubscript{1}-Apr-MIB-3[AB126969]</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>R\textsubscript{1}-Apr-MIB-7[AB126970]</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>R\textsubscript{1}-July-MIB-1[AB126971]</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>R\textsubscript{2}-Apr-MIB-4[AB126972]</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>R\textsubscript{2}-Apr-MIB-2[AB126973]</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>R\textsubscript{2}-Apr-MIB-5[AB126974]</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td><em>Others</em></td>
<td>R\textsubscript{1}-Apr-MIB-4(Alcaligenes sp.) [AB126975]</td>
<td>–</td>
<td>–</td>
<td>+*</td>
</tr>
<tr>
<td></td>
<td>R\textsubscript{1}-Dec-MIB-3(Cytophaga–Flavobacterium) [AB126976]</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Halomonas subglaciescola</em> NCIMB 12994[M93358]</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td><em>Halomonas marina</em> NCIMB 1966[D11177]</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td><em>Microbulbifer elongatus</em> NCIMB 1141[AB021368]</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*Distinguishable from positive; +, positive for hybridization, –, negative for hybridization; ND, not determined; gray square, strains used for positive references; open square, strains used for negative references.

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**Fig. 2.** Nitrogen removal rate (upper) and quantification by FISH (lower) of the composition of the bacterial community in anaerobic reactors of MWTSs using oligonucleotide probes shown in Table 1: (a) R\textsubscript{1} and (b) R\textsubscript{2}. The percentage of the specific signal was calculated in relation to the number of EUB338-stained cells: PSMg437 (white), Hlm474 (gray), Clw844 (small strips), BET42a (black), CF319a (large strips) and LGC354 (light gray) (shown in this order). Bar shows maximum and minimum values.
with the genus *Halomonas* constitute a large fraction of EUB338-hybridized cells in R2 on days 167 and 258 by FISH direct counting. The bacteria affiliated with the genus *Halomonas* have been reported as moderately halophilic and some of them reduce nitrate and nitrite [8]. *H. campisalis* can reduce nitrate at 125 g l⁻¹ NaCl, and its reduction rate is highest in culture amended with acetate [22,23]. *H. desiderata* isolated from municipal wastewater treatment system expresses all enzymes necessary for the complete denitrification [24]. The isolated bacteria, R1-Apr-MIB-3, 7, July-MIB-1, R2-Apr-MIB-1, 2 and 5 (Fig. 1), all of which are Hlm474-positive, were found to be denitrifying bacteria by an activity test [10]. Therefore, it was suggested that these species play the important role of denitrification in MWTS. For Clw844-hybridized cells, not all of the isolated strains have denitrification ability [10]. Therefore, it was also suggested that a part of Clw844-hybridized cells are responsible for denitrification in MWTS. The β-Proteobacteria was reported as the largest subdivision in both municipal and industrial wastewater treatment systems [12,25]. In contrast, the microbial community structure in MWTS indicated that γ-Proteobacteria were more important members than β-Proteobacteria in saline wastewater treatment. This was also observed in the methanol-fed denitrification process of saline wastewater [3]. The NaCl concentrations of the influent were 3.2%, 1.4% and 1.9% on days 167, 258 and 397, respectively. On day 258, when the fraction of β-Proteobacteria increased in R1, the NaCl concentration was the lowest throughout the operation time. These results confirmed that γ-Proteobacteria was the key division for saline wastewater treatment systems.

In general, the quantitative evaluation of microbial community shifts based on DGGE band profiles was difficult, because of the PCR bias derived from 16S rRNA gene-targeted universal primers [26,27]. However, new probe design and direct counting revealed that the microbial community structure in MWTS clearly varies according to flow conditions and correlates with NO₃⁻ removal efficiency. The microbial community shift in R2 indicates that the microbial community structure in a reactor is easily disrupted by environmental fluctuation, because the fluidized bed reactor might tend to favor a less diverse community structure. In fact, the compositions of actual metallurgic wastewater used in this study were different on every sampling day, suggesting that community shift might have been correct the environmental fluctuation. On the other hand, the community shift is relatively less in R1. This might be because the biofilm, which was enhanced to develop in a packed bed, was responsible for protecting the microbial community against environmental stress. Thus, the difference in the stability of community structure might correlate with nitrogen removal efficiency.

This study demonstrated that simultaneous monitoring of microbial community shifts and nitrogen removal efficiencies over time is more effective than analysis of microbial communities given by temporary sample in estimating key species in denitrification of saline wastewater treatment systems. Furthermore, an approach as represented by full-cycle rRNA analysis, which analyzes qualitatively and quantitatively microbial communities, is necessary for the complete understanding of microbial ecology in wastewater treatment systems.

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**References**


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