Direct Profiling of rRNA in Saline Wastewater Treatment Samples Using an Oligonucleotide Microarray

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(Received October 12, 2006—Accepted January 23, 2007)

Fifteen oligonucleotide probes, targeting a broad range of microorganisms, were spotted on a DNA microarray. Specificity and reproducibility were evaluated using fluorescently labeled rRNA from 16 bacterial and eukaryal strains. The oligonucleotide microarray was then used to analyze activated sludge samples of saline industrial wastewater in which microbial communities had been characterized previously using 16S rRNA clone libraries. The results obtained were partially consistent with the results of cloning, and demonstrate the possibility of using oligonucleotide microarrays for the monitoring and characterization of saline industrial wastewater populations.

Key words: oligonucleotide microarray, saline industrial wastewater, ribosomal RNA

To elucidate the population dynamics of microorganisms in a natural environment, it is necessary to investigate their diversity, community structures, abundance, and functions. Now, however, it is widely recognized that most environmental microbes are unculturable or resistant to traditional culture techniques1,5,7). Currently, to inspect microbes in natural environments, ribosomal RNA (rRNA)-based molecular techniques are used1). Generally, rRNA sequences contain both conserved and variable regions, which are ideal for the design of primers for PCR and probes for hybridization. In addition, they are suitable to utilize in detection because rRNAs (large and small subunits) are components of the ribosome and exist in large numbers inside the cell. Because of these features, in principle, one can analyze microbial communities using hybridization techniques targeting rRNA, which is directly extractable from environments or which can be visualized using fluorescently labeled probes3,18). Ultimately, a DNA microarray technology that targets a variety of microorganisms that are archaeal and eukaryal strains might be useful for studying microbial populations and dynamics simply and quickly, without the need for PCR amplification4,6,9). However, when DNA microarray technology is applied to rRNA, it is necessary to distinguish a small number of mismatches between the target and non-target microorganisms because of the highly conserved nature of rRNA sequences1). The optimization of hybridization and washing conditions is required for robust experimental frameworks14,19,20). Optimized microarrays can provide a rapid and useful tool for monitoring complex microbial communities.

In this study, we designed an oligonucleotide microarray that targets a variety of microorganisms. Then, hybridization and washing conditions were optimized using cultured reference microorganisms. Furthermore, samples of saline industrial wastewater, which were previously characterized using molecular-based approaches22–25), were examined with the small-scale oligonucleotide microarray.

Materials and Methods

Bacterial strains and environmental samples

To evaluate the specificity of the oligonucleotide on a microarray, 16 bacterial and eukaryal strains were chosen. The reference strains used for hybridization were Paracoccus denitrificans JCM 6892T, Roseobacter denitrificans IAM 14592T, and Roseobacter litoralis IAM 14593T as

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Alphaproteobacteria, Achromobacter xylosoxidans JCM 5490T, Brachymonas denitrificans JCM 9216T, and Nitrosomonas europaea NBRC 14298 as Betaproteobacteria, Pseudomonas stutzeri NBRC 14165T, Pseudomonas putida NBRC 14164T, and Escherichia coli IAM 12119T as Gammaproteobacteria, Streptomyces flavidovirens NBRC 13039T and Rhodococcus erythropolis IAM 12122T as Actinobacteria, Enterococcus faecalis NBRC 3971 and Bacillus subtilis IAM 12118T as Firmicutes, Cytophaga arvensicola JCM 2836T and Cytophaga aurantiaca JCM 8511T as Bacteroidetes, and Saccharomyces cerevisiae JCM 7255T as Eukarya.

Environmental samples used in this study were activated sludge samples from saline wastewater (1 to 13% [w/v] Na₂SO₄). The saline wastewater was drained from a factory recovering precious metals (e.g., gold, platinum, and silver) from industrial wastes. The denitrification system used consisted of an anoxic reactor with stirred activated sludge (0.5 l) and sedimentation tank. The sludge was returned to the reactor daily to maintain about 8,000 mg/l mixed liquor volatile suspended solids (MLVSSs). After operation of the reactor for 21 days and 130 days, sludge samples were collected. The nitrogen removal rate was 0.5 kg/m³/day on day 21 and 1.4 kg/m³/day on day 130. The samples had been characterized previously using 16S rRNA gene cloning and the terminal-restriction fragment length polymorphism (T-RFLP) technique²².

RNA extraction and labeling

The cells were harvested by centrifugation (4000xg, 20 min). Total RNA was isolated from the cells using the bead-beating disruption method, as described previously¹⁸. In addition, RNA samples were prepared from the activated sludge using the same procedure. The extracted RNA (≥1 µg/µl) was labeled using the ULYSIS® Alexa Fluor® 546 Nucleic Acid Labeling Kit (Molecular Probes Inc., Eugene, OR, USA) according to the manufacturer’s instructions.

Microarray manufacturing and processing

Fifteen oligonucleotide probes, targeting a wide range of bacterial and archaean groups, were chosen and spotted on the microarray (Table 1). Specificities and reference information are all described in probeBase¹¹. The oligo probes were ordered from NIPPN TechnoCluster Inc. (Tokyo, Japan) and used for producing microarrays by the company. Approximately 600-µm-diameter spots were arrayed on CodeLink Activated slides (GE Healthcare Bio-Sciences KK, Little Chalfont, UK) using bubble-jet technology¹³. The residual reactive groups of the microarray were blocked at 50°C for 15 min in a pre-warmed blocking solution (0.1 M Tris, 50 mM ethanalamine [pH 9.0], 0.1% SDS). Washing was then carried out at 50°C for 15 min in pre-warmed

<table>
<thead>
<tr>
<th>Probe*</th>
<th>Sequence 5’-3’</th>
<th>Length [base]</th>
<th>GC content [%]</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEG</td>
<td>GCT AAG TCT AGA ACT GGC</td>
<td>18</td>
<td>50.0</td>
<td>Negative control</td>
</tr>
<tr>
<td>UNIV1390</td>
<td>GAC GGG CGG TGT GTA CAA</td>
<td>18</td>
<td>61.1</td>
<td>All organisms</td>
</tr>
<tr>
<td>UNIV910</td>
<td>CCC CGT CAA TTC CTT TGA G</td>
<td>19</td>
<td>52.6</td>
<td>All organisms</td>
</tr>
<tr>
<td>EUK502</td>
<td>ACC AGA CTT GCC CTC C</td>
<td>16</td>
<td>62.5</td>
<td>Eukarya</td>
</tr>
<tr>
<td>EUB927</td>
<td>ACC GCT TGT GCG GGC CC</td>
<td>17</td>
<td>76.5</td>
<td>Most Bacteria</td>
</tr>
<tr>
<td>EUB338</td>
<td>GCT GCC TCC CTT AGG AGT</td>
<td>18</td>
<td>66.7</td>
<td>Most Bacteria</td>
</tr>
<tr>
<td>ARCH915</td>
<td>GTG CTC CCC CGC CAA TCC CT</td>
<td>20</td>
<td>65.0</td>
<td>Archaea</td>
</tr>
<tr>
<td>EURY498</td>
<td>CTT GCC CRG CCC TT</td>
<td>14</td>
<td>64.3</td>
<td>Most Euryarchaeota</td>
</tr>
<tr>
<td>CREN499</td>
<td>CCA GRC TTG CCC CCC GCT</td>
<td>18</td>
<td>72.2</td>
<td>Most Crenarchaeota</td>
</tr>
<tr>
<td>GPOS1192</td>
<td>TAA GGG GCA TGA TGA TTT GAC G</td>
<td>22</td>
<td>45.5</td>
<td>Gram-positive bacteria</td>
</tr>
<tr>
<td>HGC69a</td>
<td>TAT AGT TAC CAC CGC CGT</td>
<td>18</td>
<td>50.0</td>
<td>Most Actinobacteria</td>
</tr>
<tr>
<td>LGC353b</td>
<td>GGA AGA TTC CCT ACT GCT</td>
<td>18</td>
<td>50.0</td>
<td>Firmicutes</td>
</tr>
<tr>
<td>CF319a</td>
<td>TGG TCC GTG TCT CAG TAC</td>
<td>18</td>
<td>55.6</td>
<td>Cytophaga-Flavobacterium branch</td>
</tr>
<tr>
<td>ALF968</td>
<td>GGT AAG GTT CTG CGC GCT</td>
<td>18</td>
<td>55.6</td>
<td>Alphaproteobacteria</td>
</tr>
<tr>
<td>BET42a</td>
<td>GCC TTC CCA CTT CGT TT</td>
<td>17</td>
<td>52.9</td>
<td>Betaproteobacteria</td>
</tr>
<tr>
<td>GAM42a</td>
<td>GCC TTC CCA CAT CGT TT</td>
<td>17</td>
<td>52.9</td>
<td>Gammaproteobacteria</td>
</tr>
</tbody>
</table>

* The NEG probe is designed not to hybridize to any known organisms. Specificity and reference information are all described in probeBase¹¹.

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Table 1. Oligonucleotide probes on the microarray
4×SSC with 0.1% SDS.

Hybridization and washing conditions

To obtain optimum hybridization conditions, the formamide concentration in the hybridization buffer (10–40% formamide [FA], 0.9 M NaCl, 20 mM Tris/HCl [pH 8.0]) and salt concentration in the washing buffer (4–216 mM NaCl, 5 mM EDTA, 0.01% SDS, 20 mM Tris/HCl [pH 8.0]) were examined for *P. stutzeri* and *S. flavidovirens*. For hybridization, 40 µl of hybridization buffer containing labeled rRNA (ca. 3 µg per reaction) was added on a microarray. The microarray was covered with a large hybridization slip (24×60 mm) (Spaced Cover Glass XL; Takara Bio Inc., Otsu, Shiga, Japan) and hybridized overnight at 20°C in the dark. The microarray was immersed into 50 ml of washing buffer in a plastic centrifugation tube. Washing was carried out for 15 min at 20°C in a water bath. Validation of hybridization and washing conditions was evaluated using the optimization index obtained from the difference between the sum of positive signal intensities and the sum of false positive signal intensities.

Scanning and data analysis

The microarrays were scanned at a resolution of 3.25 µm using an arrayWoRx 2-colar biochip reader (Applied Precision, Issaquah, WA, USA) at a wavelength of 532 nm for Alexa 546. To determine signal fluorescence intensities for each spot, 16-bit TIFF scanned images were analyzed using ArrayVision™ Version 8.0 software (GE Healthcare). Positive and negative spots were defined based on the signal intensities of the negative probe and background. Signal intensities were calculated by subtracting the local background intensities from the raw signal intensities. Spots that had signal-to-noise ratios greater than 2.0 were considered positive.

Results and Discussion

Optimization of hybridization and washing conditions

To obtain optimum hybridization conditions, the formamide concentration in the hybridization buffer (10, 20, and 40%) and salt concentration in the washing buffer (4, 46,
and 215 mM NaCl) were examined systematically. Three hybridization categories were defined as follows in terms of signal intensity and specificity: (i) positive signal, (ii) false positive signal, and (iii) false negative signal. Among the three hybridization conditions, 20% formamide was chosen as the optimum in terms of signal intensity and specificity (data not shown). Therefore, all further hybridizations were done using 20% formamide. Furthermore, signal intensities in each salt concentration show that the number of probes showing positive signals was maximum for both bacteria in 46 mM NaCl (Fig. 1). In addition, the optimization index, which is calculated from the difference between the sum of positive signal intensities and false positive signal intensities, was maximized at 46 mM NaCl (Fig. 2). Therefore, 46 mM NaCl was used for washing in subsequent experiments.

Hybridizations with cultured microorganisms

Probe specificity and hybridization reproducibility were evaluated through the hybridization of fluorescently labeled rRNAs from 16 bacterial and eukaryal species. Seven probes (UNIV1390, UNIV910, EUB927, EUB338, EUK502, HGC69a and LGC353b) generated acceptable signals for respective target bacteria (Fig. 3). The BET42a and GAM42a probes elicited signals from both the Betaproteobacteria and Gammaproteobacteria because the sequence has only a single base-pair difference (Table 1). However, the signal intensity of BET42a was higher when the target was a betaproteobacterium, and the signal inten-

![Fig. 2. Evaluation of washing conditions for Pseudomonas stutzeri (A) and Streptomyces flavovirens (B). The optimization index is calculated from the difference between the sum of positive signal intensities and the sum of false positive signal intensities. Data are means ± standard errors (n=4).](image-url)

Fig. 3. Hybridization patterns obtained from reference microorganisms. For each strain, one to three microarrays (n=4–12) were analyzed.
sity of GAM42a was higher when the target was a gammaproteobacterium (Fig. 4). The probes were therefore able to discriminate between Betaproteobacteria and Gammaproteobacteria when the reference microorganisms were tested. On the other hand, four probes (GPO51192, CF319a, ALF968, and ARCH915) gave no reasonable signals for any target bacterium. Notably, the archaea-targeting probe ARCH915 showed a strong false positive signal for all bacteria tested. Non-specific binding between ARCH915 and non-target microorganisms was also reported. A high GC content and consecutive GC sequences were considered to be causative of non-specific binding (Table 1). For that reason, further characterization of probes such as ARCH915, EURY498, and CREN499 targeting Archaea was omitted.

Overall, the oligonucleotide DNA microarray developed in this study is applicable to analyses of environmental microbial communities, but it is necessary to replace non-functional probes and design additional probes.

**Hybridization with environmental samples**

In DNA microarray hybridization studies targeting complex environmental samples, low hybridization signals can be attributed to the low abundance of perfectly matched targets, but also to non-specific unavoidable or erratic hybridizations. Considering the risk of such cross-hybridization, we judged that the DNA microarray is a more effective tool for population-monitoring of time-course samples than for uncharacterized environmental samples without the help of other comparative methods.

We used a DNA microarray to analyze activated sludge samples of saline industrial wastewater, which had been studied previously. Samples were taken at 21 and 130 days from the start of the reactor’s operation. The Alphaproteobacteria (26% of total clone composition), Betaproteobacteria (35%), Gammaproteobacteria (23%), and Bacteroidetes (13%) were major constituents of the 21 days’ sample, whereas Gammaproteobacteria (73%) predominated, and Bacteroidetes (10%) and Firmicutes (17%) were present in the 130-day sample (Fig. 5).

Results of DNA microarray analyses showed that no significant population change was observed with the UNIV910, UNIV1390, EUB338, or EUB927 probe (Fig. 6).

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**Fig. 4.** Hybridization patterns and signal intensity for a betaproteobacterium, *Nitrosomonas europaea* (A) and a gammaproteobacterium, *Pseudomonas putida* (B). Signal intensity is expressed in arbitrary units (A.U.). Data are means ± standard errors (n=4).

**Fig. 5.** Microbial community composition of saline industrial wastewater samples deduced from 16S rRNA clone analyses. The clone composition of sludge samples was examined on days 21 (n=82) (A) and 130 (n=61) (B). Data are adapted from Yoshie and colleagues.
On the other hand, the signal intensity of BET42a was stronger than that of GAM42a in the 21-day sample. Conversely, the signal intensity of GAM42a was stronger than that of BET42a in the 130-day sample. This result was partially consistent with the result of cloning analyses in which both the Betaproteobacteria and Gammaproteobacteria were present in the 21-day sample, but only the Gammaproteobacteria predominated in the 130-day sample. Although an unignorable signal was detected by BET42a from the 130-day sample, the signal was considered to be given by the Gammaproteobacteria, because of cross-hybridization of the probe.

The number of DNA microarray studies using PCR-amplified rRNA gene targets has increased, but direct profiling of native rRNA on oligonucleotide microarrays is still limited due to the difficulty of array characterization and mismatch discrimination.

One salient technical problem is that hierarchically designed oligonucleotide probes elicit different fluorescent intensities for identical microorganisms because of the different capturing potentials among probes. This phenomenon is partially explainable using the bias of oligonucleotide base composition (GC%), consecutive GC or AT sequences, target locations such as bulges and hairpin loops of rRNA, and target secondary and tertiary structures. It is also possible for probes targeting the same species, but different regions on the rRNA sequences, to compete for a limited number of target fragments if targets used in hybridizations are insufficiently fragmented. For that reason, ideally, quantitative analyses of signal intensities must be carried out for each probe.

An intractable challenge in quantifying microbial populations in natural environments is how to distinguish differences in hybridization intensity between population abundance and cross-hybridization events. Our result suggests that microarray techniques are valuable for studying time-series samples containing minor population changes. We demonstrated the utility of DNA microarrays for monitoring predominant bacterial populations in saline industrial wastewater. This technique is also appropriate for comparison with sequential environmental samples, such as soil depth profiles. The versatility of DNA microarray technology should be evaluated by repeatedly investigating various strategies for the design of probes, characterization of hybridization formats, optimization of hybridization conditions, and the use for different types of environmental samples.

Acknowledgements

This research was partially supported by a Grant-in-Aid for Young Scientists (17680009) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We acknowledge K. Syutsubo at the National Institute for Environmental Studies (NIES) for his early contribution and valuable discussions. We thank S. Kawabata, A. Itsuka, and H. Hashimoto at NIES for technical assistance. We are also grateful to K. Nohara and all members of the toxico-genomics group of NIES for their encouragement.

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