Analysis of the Microbial Community in an Activated Sludge Enriched with an Inorganic Nitrite Medium

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(Received January 24, 2000—Accepted April 24, 2000)

To enrich nitrite-oxidizing bacteria, an activated sludge sample was transferred serially into an inorganic nitrite medium. Following the transfer, the culture maintained nitrite-oxidizing activity for over three months. Nitrite-oxidizing bacteria slightly decreased, but maintained their number at $10^4$ MPN/ml, and $10^5$–$10^6$ cfu/ml of heterotrophic bacteria were also detected. Random cloning and analysis of amplified 16S rDNA using a universal primer set for bacteria showed that a culturable Pseudomonas putida-related strain was dominant in the culture, though the bacterium did not oxidize nitrite. The most dominant bacterial group estimated from the proportion of clones that showed identical pattern of restriction fragment length polymorphism belonged to the $\gamma$-subdivision of Proteobacteria. This was partly consistent with the results from whole-cell hybridization using group-specific fluorescent probes. Further limiting dilutions of the enriched culture produced a nitrite-oxidizing system with low numbers of heterotrophs. Molecular analysis suggested that the members were different from those in the enriched culture, and several kinds of Proteobacteria belonging to the $\beta$, $\gamma$, and $\alpha$-subdivision, as well as bacteria in the high G+C Gram-positive phylum, existed. But in 16S rDNA sequence, none showed close similarity to any known autotrophic nitrite oxidizers. These results indicated that the population in serially transferred culture and limiting dilution culture is rather diverse, with some heterotrophic bacteria, and suggested the occurrence of an unidentified species of nitrite-oxidizing bacteria.

Key words: activated sludge, nitrification, nitrite-oxidizing bacteria, 16S rDNA, molecular analysis

The nitrogen cycle is of fundamental importance in all ecosystems, and is predominantly controlled by microorganisms. Microbial nitrification, the oxidation of ammonia to nitrate, is the key process in the removal of ammonia from wastewater. The nitrification process consists of two steps: conversion of ammonia to nitrite and formation of nitrate from nitrite. These steps are carried out by ammonia- and nitrite-oxidizing bacteria, respectively. Analysis of the structures and diversity of communities of nitrifying bacteria in natural environments is, however, severely limited by technical problems, such as the difficulties in isolating pure cultures of nitrifying bacteria, and in identifying and discriminating between isolated strains. In addition, populations counted by the most probable number (MPN) technique or other methods are often too small to explain bacterial activities$^{3,27}$. Community analysis of autotrophic nitrifying bacteria has, therefore, been hampered by both the time required and the limited amount of information that may be obtained through using such

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tivated sludge with an inorganic nitrite medium, and investigated the microflora in the enriched culture, as well as MPN counts, by various methods including the amplification and the cloning of bacterial 16S rDNA.

Materials and Methods

Enrichment of nitrite-oxidizing bacteria

An activated sludge sample was collected from the Miyazaki Waste Water Treatment Facility in Oita City. The sample (40 ml) was washed once with saline and inoculated to 160 ml of the inorganic nitrite (ION) medium\(^\text{20}\). The ION medium contained NaNO\(_2\) (60 mg-N/liter), KH\(_2\)PO\(_4\) (100 mg/liter), EDTA-Fe (6 mg/liter), MgSO\(_4\)·7H\(_2\)O (50 mg/liter), NaHCO\(_3\) (200 mg/liter), and trace amount of CaCO\(_3\) (pH 7.5). After shaking for 1 week at 30°C in the dark, concentrations of ammonia, nitrite, and nitrate, and cell numbers were measured, and then the culture (40 ml) was transferred to another 160 ml of medium. This process was successively repeated over 18 weeks.

Bacterial counting

The biomass in the enrichment culture was harvested by centrifugation (10,000 g, 20 min) and resuspended in phosphate-buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na\(_2\)HPO\(_4\), and 0.2 g/l KH\(_2\)PO\(_4\)) containing 10 mM EDTA (pH 7.0) and 0.01% Tween 80. To disperse the activated sludge, the suspension from the early stage of the enrichment was treated with a Potter Homogenizer (10 times), and sonicated for 30 sec (20 kHz, 40 W). After the fourth or fifth transfer, when the biomass showed no flocculation, a dilution series was prepared in PBS. Total cell numbers were estimated by an acridine orange direct count (AODC) method. The diluted cell suspension was filtered on an isopore filter (Millipore, R = 0.75, porosity = 0.2 μm), and then the filter was stained with 0.01% acridine orange\(^\text{11}\). The number of heterotrophic bacteria was estimated by spreading the serially diluted sample onto the standard agar medium (0.5% peptone, 0.25% yeast extract, glucose 0.1%, and agar 1.5%, pH 7.0). The spread plates were incubated at 30°C for 2 days.

The nitrite-oxidizing bacteria were enumerated by...
the most-probable number technique (MPN). The
serially-diluted samples were cultured in ION medi-
num (five tubes for each dilution) by shaking at 30°C.
After 4 weeks’ incubation, the production of nitrate in
each culture was checked colorimetrically\(^{32}\) and the
most-probable numbers were estimated using
Cochran’s Table\(^{23}\).

**Chemical analysis**

Nitrite and nitrate were routinely assayed by HPLC
(JASCO PU980): column, Shodex Asahipak NH-2P-
504E; solvent, 0.71 g Na\(_2\)HPO\(_4\), 0.78 g NaH\(_2\)PO\(_4\), and
14.04 g NaClO\(_4\)-H\(_2\)O in 1 liter of distilled water; de-
tection, UV absorption at 210 nm. In the MPN
counting of nitrite-oxidizing bacteria, nitrate was as-
sayed as nitrite by reducing nitrate with zinc powder,
after degrading the endogenous nitrite in a sample with
aniline acetate\(^{30}\). Ammonia was measured by the
method of Scheiner\(^{23}\).

**PCR amplification of 16S rDNA**

To extract DNA from pure culture, a bacterial
colony was suspended in 100 \(\mu\)l of 50 mM Tris-HCl/20
mM EDTA buffer (pH 8.0, HTE buffer) and was
treated by boiling (15 min) and chilling (5 min).
The treated cells were centrifuged at 10,000 g for 10 min,
and the supernatant was used as a template for PCR
amplification, directly or after 10-fold dilution by
H\(_2\)O. The near-complete 16S rRNA gene (16S rDNA)
from the extracted DNA was obtained by employing the
bacterial conserved primers 70F (5'-TAACA-
CATGCAAGTCGA-3') and 1387R (5'-GGGAACCT-
TATTCACCG-3') in a PCR\(^{19}\). The reaction mixture
consists of 10 munit/\(\mu\)l of ExTaq (Takara Shuzo Co.
Ltd, Shiga Japan), reaction buffer, 200 \(\mu\)M dNTPs, 40 nM of each primer set, and extracted DNA
(2 \(\mu\)l) in a final volume of 50 \(\mu\)l. The reaction mix-
ture was overlaid with a drop of mineral oil and placed
in a thermal cycler. The cycling program was: 30 cy-
cles of 94°C for 30s, 45°C for 30s, and 72°C for 120s
with an initial denaturation of one cycle of 98°C for
30s and a final extension of one cycle of 72°C for 300s.
The reaction mixture was separated by electrophore-
sis on 1% agarose gel and visualized with 0.01% ethidium bromide.

**PCR-TA cloning of 16S rDNA from enriched cul-
ture**

The biomass in the enriched culture (40 ml) was
harvested by centrifugation (10,000 g, 10 min),
washed once with 40 ml PBS, resuspended in HTE (1
ml), and treated by boiling and chilling as described
above. The extract was then diluted 100-fold and used
as the template mixture for 16S rDNA amplification.
After the PCR, the mixture was separated by electrophoresis on 1% agarose gel and amplified fragments
about 1.3 kbp long were recovered from the gel using
a DNA recovery unit (SupRec-01, Takara Shuzo).
The recovered sample was ligated with pGEM-T vector
(TA Cloning Kit, Invitrogen, California) at 16°C for
16 h. The ligated mixture was introduced into com-
petent Escherichia coli JM109 cells\(^{33}\). Transform-
ants were screened on Luria-Bertani (LB) agar plates
containing 50 mg/ml ampicillin, 0.125 mM 5-bromo-
4-chloro-indolyl-\(\beta\)-D-galactopyranoside, and 0.1 mM
isopropyl-\(\beta\)-D-thiogalactopyranoside. The resulting
white colonies, indicating they were carrying pGEM
inserted with ampiclon, were individually trans-
ferred onto another ampicillin-containing LB agar
plate.

**Restriction length fragment polymorphism analy-
sis**

Isolated bacterial strains and clones were classified
into operational taxonomic groups by restriction
length fragment polymorphism (RFLP) analysis\(^{9}\). The inserted DNA fragments were amplified using
70F and 1387R oligonucleotide as the primers, and
plasmids purified from E. coli transformants as tem-
plates. The amplicons were then digested by Cfo I at
30°C, and Taq I (Boehringer Mannheim, GmbH,
Germany) at 50°C overnight. Electrophoresis was
conducted in a horizontal slab gel containing 3%
agarose. One of two molecular weight marker, Gene
Ruler 100 bp DNA Ladder (MBI Fermentus, Lithu-
ania) or PCR Markers 50-2000 bp (Takara Shuzo),
was also subjected to the electrophoresis. Photographs of
gels were taken under UV light (302 nm) after the gel
was stained with 0.1 mg/ml ethidium bromide and
destained with water.
DNA sequencing and phylogenetic analysis

The whole DNA sequence of amplicons was determined according to Lane et al.\textsuperscript{15}, using an automatic DNA sequencer (ABI 310) and some modifications: primers used were 70F, 519F (5'-AGCAGCCGCGGTAATACG-3'), 700R (5'-CGTATTCACTCGTAC-3'), 923F (5'-ACGGGGRCCCGCAACCAGC-3'), 1102R (5'-CGCTCGTGCTCGGACTTA-3'), and 1387R. Partial DNA sequences were determined using 70F as a primer. Sequence similarity searches (300 bps of the VI region) were done using the BLAST program in Genbank\textsuperscript{4} (http://www.ncbi.nlm.nih.gov).

The phylogenetic relationship was analyzed using Clustal W\textsuperscript{25}, with databases from the Ribosomal Database Project\textsuperscript{16} (http://rdp.life.uiuc.edu) and Genbank\textsuperscript{40} for the 300 bps sequences in the VI region.

![Graph](image)

Fig. 1. Changes in the concentration of nitrogen compounds and microbial populations in an activated sludge during serial transfer in ION medium. (A) Concentrations of nitrate (▲), nitrite (▲), and ammonia (●), (B) Cell numbers of total bacteria (○), heterotrophs (□), autotrophic nitrite oxidizers (○), and autotrophic ammonia oxidizers (●).

Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) analysis of whole cells in the enriched culture was done as follows. The cultures were fixed with paraformaldehyde (4 g/100 ml PBS, pH 7.2) at 4°C for 3 hr and then washed twice with PBS and once with 50% ethanol. The washed samples (10\textsuperscript{5}–10\textsuperscript{6} cells/3 µl) were applied onto an HT Coating Slide Glass (M5279BL, Higashi Kyushu Shimadzu, Oita Japan) and then dehydrated through a series of increasing ethanol concentrations (50, 80, and 98%). Fixed samples were hybridized according to the method of Amann\textsuperscript{23} with the following fluorescein isothiocyanate (FITC)-labeled oligonucleotides: EUB338 for all bacteria\textsuperscript{28}, ALF71b, BET42a, GAM42a for the α-, β-, γ-subdivisions of the Proteobacteria, HGC for the high G+C Gram-positive phylum, and FC for the Flexibacter-Cytophaga phylum\textsuperscript{29}, respectively. FITC-labeled oligonucleotides were purchased from Hokkaido System Science (Sapporo, Japan). Fluorescent stained cells were observed under a microscope (Olympus BX50, Olympus Japan). The population of each group was expressed as a percentage of the EU-B338-stained cells. FITC-EUB338 gave 74–78% positive reaction against cells in the enriched culture as observed by phase contrast microscopy.

Results

Enrichment culture

To enrich nitrite-oxidizing bacteria, an activated sludge sample was transferred serially into the ION medium. Fig. 1 shows the change in nitrite-oxidizing activity and microflora during the transfer. Because of carriage of organic nitrogen compounds from the activated sludge, the concentration of accumulated nitrate just after the first transfer was somewhat higher than that of added nitrite as a substrate (4 mM). Nitrite-oxidizing activity was maintained and a corresponding amount of nitrate was accumulated throughout the transfers. After the seventh transfer, nitrite-oxidizing activity decreased temporarily, since the reciprocal shaker failed and the culture remained static for 2 days. However, after the tenth
transfer, the activity increased again. Total cell number as estimated by the AODC method was $10^7$–$10^8$ cells/ml during the transfers. The number of nitrite-oxidizing microorganisms was $10^6$ MPN/ml initially, and gradually decreased through the serial transfers and became stable after the sixth transfer at about $10^4$ MPN/ml. The number of ammonia-oxidizing microorganisms decreased rapidly through the transfers and became stable at about $10^1$–$10^2$ MPN/ml. On the other hand, heterotrophic bacteria maintained their number at $10^6$–$10^7$ cfu/ml throughout the serial transfer. None of the isolated heterotrophs oxidized nitrite in ION medium, or in organic medium. This indicates that certain numbers of nitrite-oxidizing bacteria had been maintained in the culture, but heterotrophic bacteria, which do not oxidize nitrite autotrophically or heterotrophically, still dominated the enrichment culture.

Isolation of an autotrophic nitrite oxidizer from the enriched culture was tried after 16 weeks using ION agar medium and ION Gelrite medium. Several colonies appeared on the ION Gelrite plate, but all of these also grew on standard nutritional agar medium and did not show nitrite-oxidizing activity either in the inorganic or organic liquid medium.

Molecular analysis of the enriched culture

A mixture of 16S rDNAs was amplified from the 16-week enriched culture. Using a TA-cloning vector system, 100 transformants, which carried approximately 1.3 kb of amplified fragments, were randomly cloned. These clones were divided into three groups (E1～E3) by RFLP analysis of their amplicon with Cfo I (Fig. 2A). When digested by Taq I, 10 clones randomly selected from group E1 also showed identical RFLP patterns (data not shown). The percentage of group E1, E2, and E3 clones in the total analyzed was 67, 27 and 6%, respectively (Table 1). E1-1, one of the clones in group E1, carried the 16S rDNA fragment (DDBJ accession number: ABO40418) most similar in sequence to Pseudomonas putida ATCC12633 (Genbank accession number (ac): D37923, 95% homology). Representative transformants of group E2 and

### Table 1. Homology analysis of isolated microorganisms and cloned 16S rDNA from the enriched culture.

<table>
<thead>
<tr>
<th>No.</th>
<th>Frequency (%)</th>
<th>Nearest neighbor (Similarity %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spread plating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>64</td>
<td>Pseudomonas putida (95) Proteobacteria (γ)</td>
</tr>
<tr>
<td>H2</td>
<td>25</td>
<td>Rastoria eutropha (90) Proteobacteria (γ)</td>
</tr>
<tr>
<td>H3</td>
<td>5</td>
<td>Rastoria eutropha (90) Proteobacteria (γ)</td>
</tr>
<tr>
<td>H4</td>
<td>5</td>
<td>Pseudomonas stutzeri (90) Proteobacteria (γ)</td>
</tr>
<tr>
<td>Random cloning</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>67</td>
<td>Pseudomonas putida (95) Proteobacteria (γ)</td>
</tr>
<tr>
<td>E2</td>
<td>27</td>
<td>Cytophaga sp. (84) Flexibacter-Cytophaga</td>
</tr>
<tr>
<td>E3</td>
<td>6</td>
<td>Thermonema lapsum (80) Flexibacter-Cytophaga</td>
</tr>
</tbody>
</table>
E3 carried fragments that showed similarity to *Cytophaga* sp. (ac: PPU70977, 84% homology) and *Thermanema lapsum* ATCC 43542 (ac: L11703, 80% homology), respectively.

Heterotrophic microorganisms isolated from the 16-week enriched culture were also classified into four groups (H1 ~ H4) from the morphology of colonies and cells (Table 1). The most dominant bacterial group, H1, were Gram negative, motile rods (1.5 × 0.7 µm) comprising 64% of total colonies. Ten strains randomly selected from group H1 showed identical RFLP patterns (Fig. 2B), which indicated that the group consisted of closely-related species. The partial 16S rDNA sequence (300 bp) of one member of group H1 (strain H1-1) was identical to that of E1-1. The second most predominant group, H2, composed 25% of the colonies. They were also Gram-negative, motile rod (1.5 × 0.6 µm). One of them, strain H2-1, showed 90% homology to *Ralstonia eutropha* (*Alcaligines eutrophus* ATCC 17697, ac: L40413) in its partial 16S rDNA sequence. The nearest neighbors of representative strains of the other groups, H3 (5% of total colonies) and H4 (5% of total colonies), were *Rastoria eutropha* (ac: AFO19037, 89% homology) and *Pseudomonas stutzeri* (ac: PSU26261, 98% homology), respectively. The dominance of the γ-subdivision of the *Proteobacteria* was confirmed by in situ analysis. The biomass from the 16-week enriched culture was fixed and stained with various kinds of group-specific FITC-oligonucleotide probes. Cells were stained by probes of ALF71b, BET42a, GAM42a, HGC and FC at frequencies of 3%, 14%, 37%, 5%, and 14% of EUB-338 stained cells, respectively. Cells stained by BET42a or FC were secondary dominant.

**Molecular analysis of limiting dilution culture**

To study the populations of nitrite-oxidizing bacteria, an analysis of the limiting dilution culture, i.e., the culture for MPN counting, was performed. Fig. 3 shows a typical time course of limiting dilution cultures of 16-week enriched activated sludge. By the inoculation of a 10⁻³-diluted sample, nitrite was oxidized completely over 2 weeks. The number of heterotrophic microorganisms found during culture reached 10⁸ cfu/ml. In the 10⁻⁵-dilution culture, in which there was further dilution of organic substances and biomass transferred, quite low numbers (10¹⁻¹₀² cfu/ml) of heterotrophic bacteria were detected after incubation for 2 and 3 weeks. Heterotrophs grown on nutrient agar plates looked homogeneous and morphologically similar to those of H1-1. Analysis of RFLP and partial sequences of amplified 16S rDNA revealed that they were identical to E1-1. In the case of 10⁻⁵-dilution culture, 40% of the nitrite remained, even after 4 weeks.

Using a DNA mixture obtained from 2-week cultures of MPN at the 10⁻⁵-dilution level, where nitrite oxidation activity is high, bacterial 16S rDNAs were amplified and cloned randomly. Ninety-eight clones were classified into 14 groups (M1 ~ M14) from the RFLP pattern of their cloned fragments (Fig. 4). None of their patterns were identical to those of heterotrophs clustered into groups H1 ~ H4 or groups E1 ~ E3. Group M1 clones that showed an M1 pattern on RFLP analysis were dominant (49% of all clones). By sequence analysis of the representative clones of

![Fig. 3](image-url)  
**Fig. 3.** Time course of the concentration changes of nitrogen compounds and heterotrophic bacteria in the MPN cultures. The enriched culture of 16-times transfer shown in Fig. 1 was diluted 10³-fold (A) or 10⁵-fold (B) and incubated in ION medium. Changes in concentrations of nitrate (△) and nitrite (▲), as well as in numbers of heterotrophic bacteria (□) were monitored.
each group, it was found that they were comprised of β- (57% of total clones), γ- (27%) and α-subdivisions (8%) of Proteobacteria, with some members in a high G+C Gram positive phylum (10%). The sequence of the class M1 (DDJB ac: ABO40416) indicated that they belong to the β-subdivision, and the nearest neighbour was 16S rDNA from Hydrogenophaga sp. (ac: H13266, 95% homology). The second most dominant group, M2, was cloned with 18% frequency and one clone might belong to the γ-subdivision of the Proteobacteria (DDJB ac: ABO40417), showing 91% homology to Escherichia coli (ac: L10328). Fragments of representative clones from all other minor groups were also partially sequenced (300 bp) and the results of phylogenetic analysis are summarized in Table 2.

Fig. 5 shows that the phylogenetic positions of the clones belonging to the Proteobacteria were distributed not only to the α-subdivision, but also to the β- and γ-subdivisions. Members in each subdivision, especially M1, M10, M12 and M13 in the β-subdivision, M11 and M14 in the α-subdivision, and M2 and M5 in the γ-subdivision, clustered closely. But clones M2-1 and M3-1 were phylogenetically far from known nitrite-oxidizing bacteria, showing 80% and 88% homology to Nitrococcus mobilis (ac: M96403), and Nitrobacter winogradsky (ac: L11661), respectively.

Table 2. Homology analysis of cloned 16S rDNA from limiting dilution culture.

<table>
<thead>
<tr>
<th>No.</th>
<th>Frequency (%)</th>
<th>Nearest neighbor (Similarity %)</th>
<th>Phylum</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>49</td>
<td>Hydrogenophaga sp. (95)</td>
<td>Proteobacteria (β)</td>
</tr>
<tr>
<td>M2</td>
<td>19</td>
<td>Escherichia coli (91)</td>
<td>Proteobacteria (γ)</td>
</tr>
<tr>
<td>M3</td>
<td>2</td>
<td>Xantobacter agilis (95)</td>
<td>Proteobacteria (α)</td>
</tr>
<tr>
<td>M4</td>
<td>2</td>
<td>Rhizobium gallicum (95)</td>
<td>Proteobacteria (α)</td>
</tr>
<tr>
<td>M5</td>
<td>6</td>
<td>Escherichia coli (88)</td>
<td>Proteobacteria (γ)</td>
</tr>
<tr>
<td>M6</td>
<td>2</td>
<td>Micrococcus sp. (93)</td>
<td>Gram-positive High-G+C</td>
</tr>
<tr>
<td>M7</td>
<td>4</td>
<td>Corynebacterium cellulare (96)</td>
<td>Gram-positive High-G+C</td>
</tr>
<tr>
<td>M8</td>
<td>2</td>
<td>Microbacterium sp. (93)</td>
<td>Gram-positive High-G+C</td>
</tr>
<tr>
<td>M9</td>
<td>2</td>
<td>Microbacterium sp. (97)</td>
<td>Gram-positive High-G+C</td>
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<tr>
<td>M10</td>
<td>2</td>
<td>Hydrogenophaga pallonii (92)</td>
<td>Proteobacteria (β)</td>
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<tr>
<td>M11</td>
<td>2</td>
<td>Mycoplana segnis (90)</td>
<td>Proteobacteria (α)</td>
</tr>
<tr>
<td>M12</td>
<td>2</td>
<td>Hydrogenophaga pallonii (97)</td>
<td>Proteobacteria (β)</td>
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<tr>
<td>M13</td>
<td>4</td>
<td>Hydrogenophaga pallonii (96)</td>
<td>Proteobacteria (β)</td>
</tr>
<tr>
<td>M14</td>
<td>2</td>
<td>Ochrobacterium antropi (97)</td>
<td>Proteobacteria (α)</td>
</tr>
</tbody>
</table>

Fig. 4. RFLP analysis of 16S DNA amplicons cloned from the limiting dilution culture. The cloned fragments were amplified with primers of 70F and 1387R and digested by Cfo I. The digested samples from clones M1-1-M14-1 were applied to gel (Lane 1-14). A molecular weight marker (Gene Ruler 100 bp DNA Ladder) was also subjected to electrophoresis (Lane M).

Discussion

In this study, we have demonstrated that activated sludge transferred serially into ION medium maintained nitrite-oxidizing activity for over four months. In culture after 16 transfers, heterotrophic microorganisms still dominated, although spread plating and random cloning of 16S rDNA gave different results to FISH analyses. Further limiting dilution culture produced a nitrite-oxidizing system
with low numbers of heterotrophs. Molecular analysis suggested that the flora were diverse and phylogenetically different species from the enriched culture were dominant in the limiting dilution culture. None of them were closely related to any known autotrophic nitrite oxidizers in their 16S rDNA sequences (<90% similarity).

Relatively large numbers of heterotrophic bacteria survived even on long-term enrichment using ION medium (Fig. 1). Isolation of heterotrophic bacteria indicated that major heterotrophs found in the enriched culture belonged to the β- and γ-subdivision of the Proteobacteria. Random-cloning of 16S rDNA also suggested the dominance of a culturable Pseudomonas sp. and another unisolated Flexibacter-Cytophaga. FISH analysis of the enriched culture using group specific probes partly supported the results described above: the γ-subdivision of the Proteobacteria showed the largest population followed by the β-subdivision of the Proteobacteria and Flexibacter-Cytophaga. Although the cloning probability of each RFLP group is likely biased by the experimental procedures of ligation and PCR, one dominant microbial group in the enriched culture might be culturable heterotrophs such as Pseudomonas putida-related species, and unisolated microorganisms such as members of the Flexibacter-Cytophaga phylum. Of note was that the culturable heterotrophs that do not show autotrophic nitrite-oxidizing activity were stably maintained in the enriched culture. As the ION medium contains trace amounts of an organic compound, EDTA, we then tried an enrichment using an inorganic nitrite medium containing FeCl₂ instead of EDTA-Fe. But heterotrophic bacteria numbered more than 10⁶ cfu/ml after over 30 transfers (data not shown). As shown in Fig. 3, the concentration of nitrate that accumulated in the 10⁻⁵-dilution culture is less than that in the 10⁻³-dilution culture. Considering these differences, the consortium in the enriched culture might be construed as being stable, synergistic and commensual, and the secondary growth of heterotrophic bacteria as also being important. This consortium might have been disrupted in the 10⁻⁵-diluted culture. Further studies
of correlations between population and nitrite-oxidizing activity are needed.

Successive limiting dilution culture produced a nitrite-oxidizing system with low numbers of heterotrophs (Fig. 3). Molecular analysis suggested that the bacteria in the 2-weeks culture, when nitrate oxidizing activity is high, were entirely different from those in the enriched culture. Several kinds of Proteobacteria of the β, γ, and α-subdivisions, as well as bacteria from the high G+C Gram-positive phylum existed, but a homology search revealed no direct relation to known nitrite oxidizers. In spite of this, it is reasonable to infer that some of them are involved in nitrite oxidation, considering that they were molecular ecologically dominant in a limiting dilution culture which showed active nitrite oxidation and where the numbers of heterotrophs were low. Of note is that most dominant 16S rDNA clones belonged to the β-subdivision, constituting about half of the population, because none of the known autotrophic nitrite-oxidizing bacteria were distributed in this phylum.

Mobarry et al.\textsuperscript{10} reported that Nitrobacter spp. were detected in activated sludge flocs, and Burrell et al.\textsuperscript{6} that Nitrospira-related clones dominated in a nitrite-oxidizing sequencing batch reactor. In the present study, we did not obtain any direct information concerning known nitrite oxidizers. Autotrophic nitrite oxidizers are typical fastidious bacteria. Colony development on solid media takes several months; colonies are small and difficult to transfer, and the elimination of heterotrophic contaminants is difficult because of high growth rates. In addition, nitrite-oxidizing genera are polyphyletic\textsuperscript{24}, requiring combinational investigation by molecular ecological analyses. Although the results obtained in this study are limited and even indirect, we believe them to be informative as to microbial consortium involved in nitrite oxidation. Further systematic investigation of oxidation activity and chemical composition, and in situ microbial analysis in activated sludge and enriched cultures should clarify the roles in nitrite oxidation of the unisolated members of the microbial community.

Acknowledgements

This work was supported in part by a Grant-in Aid for Scientific Research (no. 09450306) from the Ministry of Education, Science, Sports and Culture of Japan, and by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of the Japanese Government.

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