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Rapid Detection of Ammonia-oxidizing Bacteria in Activated Sludge Based on 16S-rRNA Gene by Using PCR and Fluorometry

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Abstract To detect whole ammonia-oxidizing bacteria in the activated sludge, group-specific primers targeting the 16S-rRNA gene of ammonia-oxidizing bacteria were used. The electrophoresis pattern of the PCR products seemed to produce a single band of approximately 1.0 k bp for the bacteria in activated sludge and *Nitrosomonas europaea*. No band was observed for nitrite-oxidizer *Nitrobacter winogradskyi* and heterotrophs such as *Pseudomonas putida*. Then direct measurement of the PCR product was made by fluorometry using the reagent Hoechist 33258, so that the fluorescent intensity was in proportional to the cell number of the sample up to 240. Total time required for the test was about 4 h including DNA extraction. The DNA fragments produced were cloned and their sequences showed high similarity to those of *Nitrosomonas* spp. This study showed the feasibility to detect ammonia-oxidizing bacteria and to estimate their population rapidly for the control of the nitrogen elimination process.

Keywords: ammonia-oxidizing bacteria, activated sludge, 16S-rRNA, PCR, fluorometry

In order to prevent water pollution so-called eutrophication, activated sludge process is applied to remove nitrogenous compounds in waste water. The process consists of the degradation of organic nitrogen to release ammonia, the oxidation of ammonia via nitrite to nitrate and the reduction of nitrate to eliminate nitrogen as molecular nitrogen. These biochemical conversions are performed by various micro-organisms inhabiting in activated sludge, therefore it is important to maintain the population of individual microorganisms required for the each step. One of major problem is that ammonia-oxidizing bacteria tend to be inhibited by variety of compounds in waste water and sensitive to pH and temperature shifts resulting in operating failure. Once their population decreases, long time is required to return to the original state because of their extremely low growth rate, so that the rapid detection of the bacteria is strongly desired for the operation of the process. The conventional technique based on cultivation is unsuitable because it requires long time. PCR methods have been applied to detect special species of microorganisms by using primers to amplify specific gene [1]. In this study group-specific primers NSO 190 and NSO 1225r targeting the 16S-rRNA gene of ammonia-oxidizing bacteria belong to β-subdivision were employed, because species of ammonia-oxidizers to be detected were usually unknown [2]. The product of the PCR reaction was directly measured by fluorescent method to make the test simple and rapid.

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Nitrosomonas europaea IFO 4298 was cultured under aerobic conditions at 30°C for 30 days in a 500 mL flask containing 50 mL of a medium consisted of 0.3% $(NH_4)_2SO_4$, 0.05% K_2HPO_4, 50 ppm $MgSO_4$, 20 ppm $FeCl_3$, 4 ppm $CaCl_2$ and 0.05 ppm cresol red. The pH during the cultivation was maintained at about 8.0 by adding sterilized 0.5 M K_2CO_3 according to the color of the indicator (cresol red). The medium for *Nitrobacter winogradskyi* IFO 14297 was consisted of 0.1% $NaNO_2$, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% NaCl, 5 ppm $Fe_2(SO_4)_3$, 2 ppm $MnSO_4 \cdot 5H_2O$ and the initial pH was adjusted to 7.5. Its cultivation was carried out in the same conditions as *N. europaea* except that pH control was needless. *Escherihia coli* IFO 3301 and *Pseudomonas putida* IFO 3738 were aerobically cultured for 24 h at 30°C in a medium containing 1.0% polypepton (Wako Chemical), 0.2% yeast extract and 0.1% $MgSO_4 \cdot 7H_2O$ (pH 7.0). Activated sludge was cultured in a vessel (3 L of liquid volume) with two kinds of medium, the medium 1 of the same composition described above except for 0.06% $(NH_4)_2SO_4$ and the medium 2 containing 0.12% glucose and 0.04% polypepton (Wako Chemical). The culture vessel was automatically operated by repeating the following three stages: nitrification (160 min), pumping the medium 1 and 2 at a flow rate of 20 mL/h, respectively, under the aerobic conditions (1/3 VVM); de-nitrification by endogenous respiration (290 min), gentle stirring without aeration and feeding the medium 1 and 2; settling and effluence (30 min), precipitating the activated sludge followed by effusing the supernatant to keep the liquid volume to 3 L. The pH and the temperature were maintained through the cultivation at 8.0 and 30°C, respectively.

Population of ammonia-oxidizing bacteria was estimated by the most-probable-number (MPN) method because conventional colony counting method was difficult to be applied [3]. Ten fold serial dilutions of a sample were prepared and each 0.5 mL of them was inoculated into 4.5 mL of the medium consisting of 0.05% $(NH_4)_2SO_4$, 1.0% K_2HPO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.03% NaCl, 0.0003% $FeSO_4 \cdot 7H_2O$ and 0.75% $CaCO_3$ (pH 8.0) in a test tube. After the cultivation by shaking for 45 days, the formation of nitrite was detected by the diazotization method [4]. When no nitrite was detected, zinc powder was added to reduce nitrate to nitrite. Nitrite might be consumed by nitrite-oxidizers to form nitrate. By using the MPN table, the most-probable-number (MPN) of ammonia-oxidizing bacteria was estimated from the number of the test tubes which produced nitrite or nitrate [3].

Prior to PCR, the DNA of bacteria was extracted and purified as follows. Bacterial cells were harvested from homogenized sample by centrifugation and washed twice. After re-suspending them in 100 μL of 10 mM Tris-EDTA buffer, extraction and purification of DNA were made with E.Z.N.A^R Bacterial DNA Kit (Omega Bio-tek Inc.) including treatment with lysozyme (from chicken egg white, ICN Co.) for 5 min at 30°C and proteinase K (Type 4, Ambion Co.) for 1 h at 55°C. The DNA was eluted into 0.8 mL sterile distilled water and stored in a freezer at –20°C.

The sequences of the forward primer NSO 190 (5′-GGAGAAAAGCAGGGGATCG-3′) and the reverse primer NSO 1225r (5′-CGCCATTGTATTACGTGTGA-3′) were based on the published sequences of the probes for the 16S-rRNA gene of ammonia-oxidizing bacteria belong to β-subdivision [2]. They were purchased from Espec Oligo Service Co. The PCR amplification were carried out in 50 μL of final volume containing 2 to 8 μL of DNA template solution, 0.8 μM of each primer, 0.2 mM each dNTP, 2 mM $MgCl_2$ and 1.25 U of ExtraTaq^R polymerase (Takara Co.) in 10 mM Tris-HCl and 50 mM KCl buffer (pH 8.3) with a thermal cycling apparatus Progene^R (Nalge Nunc Int. Co.) by the following program: de-naturation for 30 sec at 94°C; annealing for 20 sec at 52°C; extension 60 sec at 72°C.

The amplified DNA fragments from the activated sludge were inserted into pGMT^R-T Easy vector (Promega Co.) and introduced into *E. coli* XL1-Blue. The DNA fragments were extracted from three colonies of the transformed *E. coli*, respectively, and purified with QIA prep Spin Miniprep Kit (Qiagen Inc.). The DNA sequences were analyzed with the dideoxy method using 5′-FITC labeled primer by Bex Co. Similar searches were made with the BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

The quantitative assay of the DNA in the PCR product was made by a fluorescent method. Twenty •L of the product was added to 0.6 mL of 10 mM TE buffer (pH 7.4) containing 200 mM of NaCl and 0.6 μg of fluorescent reagent Hoechist 33258 (Molecular Probes Inc.) which bound to the AT rich regions of the double strand DNA [5]. The fluorescent intensities at 450 nm were measured by a Fluorometer (M 200, Ajinomoto Co.) with exciting at 350 nm after normalization at 5000 fluorescent unit for 0.1 mg/L quinine sulfate in 0.1 N H_2SO_4.

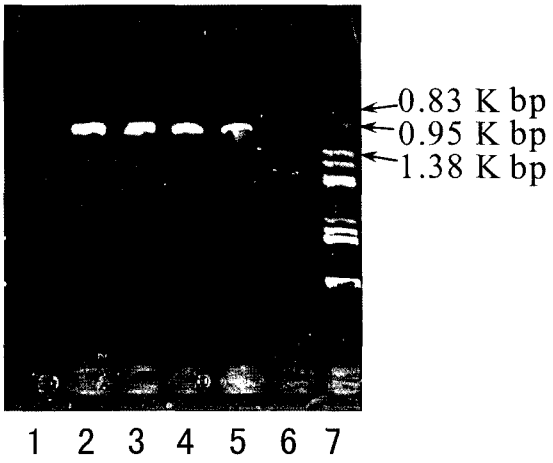

Fig. 1. Agarose gel electrophoresis of the PCR products with NSO 190 and NSO 1225r primers. 8 μL of the reaction samples were electrophoresised through 0.5% agarose gel in 90 mM Tris-borate 2 mM EDTA buffer. The bands were stained in 0.5 g/mL of ethidium bromide for 30 min and recorded digitally with Printgraph (AE-6911 CX, Atto Co,). Lane: 1 blank; 2, 3, 4 and 5 the amplification products from the activated sludge containing 160, 120, 80, and 40 cells of ammonia-oxidizer, respectively; 6 the amplification product from *P. putida*; 7 maker.

As shown in Fig. 1, the PCR product using the primers NSO 190 and NSO 1225r showed a single band of approximately 1.0 k bp for the activated sludge on the agarose gel. No band was observed for heterotrophic bacterium *P. putida*. A single band was also obtained at the same position for the typical autotrophic ammonia-oxidizer *Nitrosomonas europaea*, whereas no band was recognized for the nitrite-oxidizer *Nitrobacter winogradskyi* and the heterotrophic bacterium *E. coli* as shown in Fig. 2. These results suggested that the primers annealed selectively to the 16s-rRNA gene of ammonia-oxidizing bacteria to produce 1.0 k bp of the DNA fragment.

To elucidate this hypothesis sequence analysis and homology search of the DNA fragments were carried out. The fragments were introduced to *E. coli* XL-Blue and three colonies of the transformants were used for sequencing. Fig. 3 indicates the partial nucleotide sequence of the fragment 1 from the colony 1 which showed highest similarity to that of the 16S-ribosomal gene of *Nitrosomonas* sp. strain NM 41 (Genbank accession no. AF272421). There were 13 discrepancies includeing 10 mismatches and 3 gaps in 784 nucleotides so that they exhibited 98% similarity. The sequence of fragment 1 indicated the same high similarity to that of *Nitrosomonas* sp. strain NM33 (Genbank accession no. AF272419), as listed in Table 1 (the nucleotide sequence

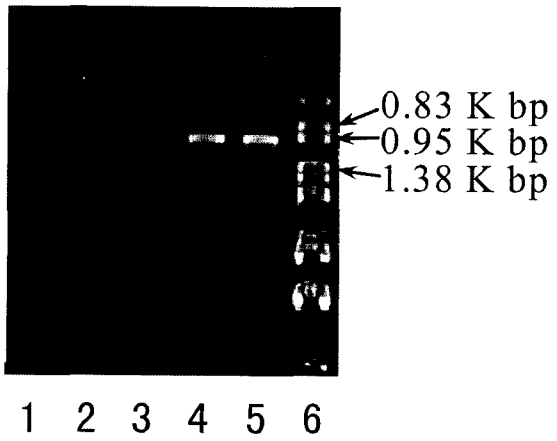

Fig. 2. Agarose gel electrophoresis of the PCR products with NSO 190 and NSO 1225r primers. Experimental conditions were the same as described in Fig. 1. Lane: 1 blank; 2, 3, 4, and 5 the amplification products from *E. coli*, *N. winogradski*, *N. europaea*, and the activated sludge, respectively; 6 maker.

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    GGAGAAAAGCAGGGGATCGCAAGACCTTGCGCTTTTTGAGCGGCCGATGCCTGATTAGCT
159 GGAGGAAAGCAGGGGATCGAAAGACCTTGTGCTTTTAGAGCGGCCGATGCCTGATTAGCT

    AGTTGGTAAGGTAAAGGCTTACCAAGGCAACGATCAGTAGCTGGTCTGAGAGGACGACCA
219 AGTTGGTGGGGTAAAGGCCTACCAAGGCAACGATCAGTAGCTGGTCTGAGAGGACGACCA

    GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTG
279 GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTG

    GACAATGGGCGAAAG-CTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGT
339 GACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGT

    AAAGCTCTTTCGGTCGGGAAGAAATAGTTGTGGCTAATATCCACAATGAATGACGGTACC
399 AAAGCTCTTTCGGTCGGGAAGAAATAGTTGTGGCTAATATCTACAATGAATGACGGTACC

    GACATAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGC
459 GACATAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGC

    GTTAATCGGAATTATTGGGCGTAAAGGGTGCGCAGGCGGTTGTATAAGTCAGATGTGAAA
519 GTTAATCGGAATTATTGGGCGTAAAGGGTGCGCAGGCGGTTATATAAGTCAGATGTGAAA

    TCCCTGGGCTTAACCTAGGAATTGCGTTTGAAACTATATAACTAGAGTGTGACAGAGGGG
579 TCCCTGGGCTTAACCTAGGAATTGCGTTTGAAACTATATAACTAGAGTGTGACAGAGGGG

    AGTGGAATTCCATGTGTAGCAGTGAAATGCGTAGAGATGTGGAAGAACACCGATGGCGAA
639 AGTGGAATTCCATGTGTAGCAGTGAAATGCGTAGAGATGTGGAAGAACACCGATGGCGAA

    GGCAGCTCCCTGGGTTAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATT
699 GGCAGCTCCCTGGGTTAACACTGACGCTCAGGCACGAAAGCGTGGGGAGCAAACAGGATT

    AGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTCGGATCTATATAAAGA
759 AGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTCGGATCTATATAAAGA

    TTAGGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGAAGTACGGTCGC-AGATTAAA
819 TTAGGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGAAGTACGGTCGCAAGATTAAA

    ACTCAAAGGAATTGACGGGGACCCGCACAAGC-GTGGATTATGTGGATTAATTCGATGCC
879 ACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCA

    ACGC Fragment 1
939 ACGC Nitrosomonas sp. strain NM 41
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Fig. 3. Comparison of the partial nucleotide sequences of the fragment 1 and the 16S-rRNA gene of *Nitrosomonas* sp. strain NM 41. The lower row is the partial sequence nucleotide of the 16S rRNA gene of *Nitrosomonas* sp. strain NM 41 from 159 to 942 and the upper row is that of the fragment 1. Grey background indicates discrepancy. The nucleotide sequence of the primer NSO 190 is underlined. Symbol "–"means a gap.

is not indicated). As to the fragment 2 and 3, both the search results were identical to that of the fragment 1 with slightly lower similarity as shown in Table 1. The nucleotide sequence of the fragment 3 corresponded to the different position of the *Nitrosomonas* spp., because it was inversely inserted into the vector. These facts supported that the primers NSO 190 and NSO 1225r were specific to ammonia-oxidizing bacteria.

As no PCR product was assumed to be produced from the activated sludge except for ammonia-oxidizer by using the primers NSO 190 and NSO 1225r, the cell number of ammonia-oxidizer may be estimated from the quantity of the PCR product, which is known to be in proportional to the template quantity in the log phase of the PCR amplification [6]. The quantity of the product measured with the fluorescent method was plotted against the cycling number in Fig. 4. It was found that the log phase amplification initiated above 24 cycles for the template extracted from 80 cells of ammonia-oxidizing bacteria. Practically the cycling number 30 and 36 were employed for further tests to raise the sensitivity. The templates containing various quantity of DNA were prepared from the DNA solution extracted from the activated sludge containing 4.1×10^4 cells of ammonia-oxidizer per milliliter. When the cycling number was 30, the fluorescent intensity in creased with increase of the cell as shown in Fig. 5, but the scattering of the data was too wide to estimate the cell number. In the case of 36 cycles, the fluorescent intensity was in proportion to the cell number up to 240 and reached a plateau. The plateau is presumed due to inhibition substances in the template or the limitation of certain reagents for the PCR amplification. The drawback of this method is narrow range-ability. If a result is out of the range, re-test is necessary by changing dilution rate of sample. It is important for the process control to know quickly whether ammonia-oxidizing bacteria exist to a certain desired extent. For this purpose the proposed method is considered to be useful. This method can be applied to specify the species of ammonia-oxidizing bacteria by using species-specific

Table 1. Similarity analysis of the DNA fragments obtained from the activated sludge

Fragment number	Similarities (%) and nucleotide sequence number referred			
	Nitrosomonas sp. NM 41		*Nitrosomonas* sp. NM 33	
1	98	159-942	98	177-960
2	97	159-932	96	177-950
3	97	431-1215	97	449-1233

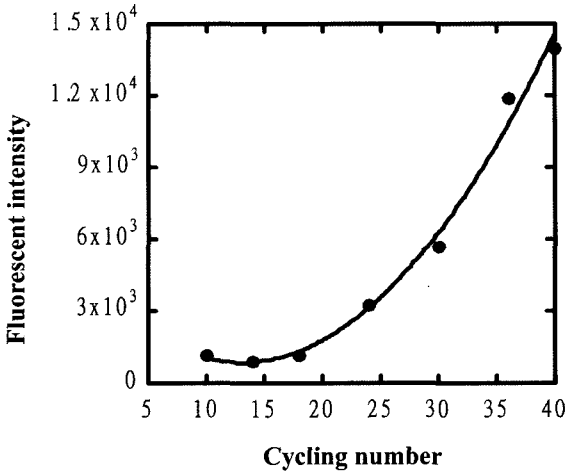

Fig. 4. Relationship between the cycling number and the logarithm of the fluorescent intensity. 80 cells of ammonia-oxidizing bacteria in the activated sludge were amplified with the NSO 190 and NSO 1225r primers.

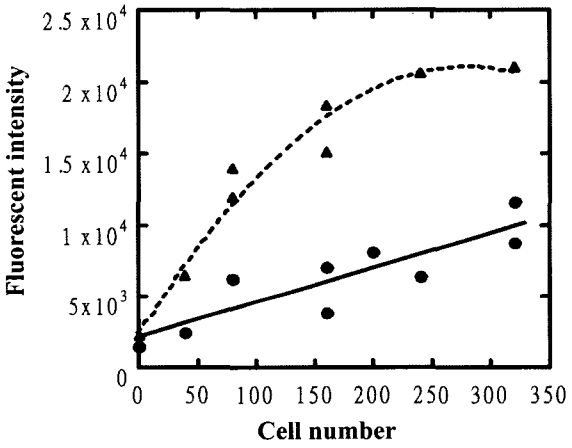

Fig. 5. Relationship between the cell number of ammonia-oxidizer and the fluorescent intensity for the PCR product. Amplifications were carried out under the same conditions as in Fig. 1. Solid line: cycling number 30, Least square method equation: $Y=2020+26X-0.004\ X^2$, dotted line: cycling number 36, Least square method equation $Y=2330+133\ X-0.237\ X^2$.

primers, for example, targeting ammonia monooxigenase gene [7].

In conclusion this fundamental study showed the feasibility to detect ammonia-oxidizing bacteria in the activated sludge and to estimate their population. No expensive apparatus is required such as real-time PCR. The time required for the test was within 4 hours whereas it needs more than a month by the traditional cultivation method. Further work is desired to clarify the reproducibility. The method expected to contribute to the stable operation of the nitrogen elimination process based on the activated sludge.

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