PCR-DGGE analysis of denitrifying bacteria in a metallurgic wastewater treatment process

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Abstract The wastewater generated from the processes of recovering precious metals from industrial wastes contains high concentrations of acids such as nitric acid and of salts. Biological nitrogen removal from this wastewater was attempted by using a circulating bioreactor system equipped with an anoxic packed bed or an anoxic fluidized bed and an aerobic three-phase fluidized bed. The system was found to effectively remove nitrogen from the diluted wastewater (T-N; 1,000–4,000 mg litre⁻¹). The microbial population structure of activated sludge in an anoxic reactor was analyzed by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S ribosomal DNA (rDNA) fragments. DGGE analysis under different operating conditions demonstrated the presence of some distinguishable bands in the separation pattern, which were most likely derived from many different species constituting the microbial communities. Furthermore, the population diversity varied in accordance with the nitrate-loading rate, water temperature and reactor condition. Some major DGGE bands were excised, reamplified and directly sequenced. It was revealed that the dominant population in the anoxic reactor were affiliated with the β subclass of the class Proteobacteria.

Keywords Denitrification; metallurgic wastewater; microbial community; PCR-DGGE; 16S rDNA

Introduction
It is important to recover precious metals such as gold, silver, platinum and palladium in order to efficiently utilize natural resources and reduce industrial wastes. However, the wastewater that is produced during the recovery of precious metals from industrial waste contains a large amount of nitrogen as well as high salinity and various base metals. Therefore, to prevent eutrophication in lakes and inland seas, it is essential to develop a process of removing nitrogen from saline wastewaters such as metallurgic wastewater. So far, Hirata et al. (2001) have performed biological treatment of such wastewater using an anoxic-aerobic circulating process. However, the microbial community in this process has not been clarified. Analysis of the microbial population is important for domination and immobilization of microorganisms possessing denitrification abilities under saline conditions.

DGGE has been used to resolve PCR-amplified regions of genes coding for 16S rDNA based on differences in nucleotide sequence (Muyzer et al., 1993; Kowalchuk et al., 1997). This has proven to be a simple approach to obtain profiles of microbial populations that can be used to identify temporal or spatial differences in population structure that occur in response to environmental factors.

In this study, we used the DGGE method to determine the relative genetic complexity of microbial populations in metallurgic wastewater treatment systems. The technique was also used to monitor population changes over time on different reactor conditions. The taxonomic affiliation of some dominating members of the population was determined by sequencing DGGE bands.

Methods
Metallurgic wastewater treatment system and sludge samples
Three series of anoxic-aerobic circulating bioreactors were used as metallurgic wastewater
treatment systems. These systems consisted of an anoxic packed bed or an anoxic fluidized bed, an aerobic fluidized bed and sedimentation tank. The first system was of laboratory scale with an anoxic packed bed (volume of anoxic packed bed was 2 l; Run 1), the second one was of laboratory scale with an anoxic fluidized bed (volume of anoxic fluidized bed was 2 l; Run 2) and the third one was of pilot plant scale (volume of anoxic packed bed was 50 l; Run 3). Treated wastewater in the sedimentation tank was circulated into the anoxic reactor. The volume of the circulated wastewater was adjusted to fivefold (Runs 1 and 2) or fourfold (Run 3) that of the influent. The metallurgic wastewater used in this study was exhausted from a factory recovering precious metals from industrial wastes. Composition of the wastewater was NO$_2$-N (1,000–4,000 mg litre$^{-1}$), NH$_4$-N (200–1,000 mg litre$^{-1}$), TOC (500 mg litre$^{-1}$), salinity (10,000–50,000 mg litre$^{-1}$). To investigate temporal variations of microbial populations, sludge samples were collected from the anoxic reactors of the three systems at different times (July, October, December 1999 and April, May 2000).

**DNA extraction**

DNA was extracted from a 0.5 g (wet weight) sludge pellet. The sludge was harvested by centrifugation at 10,000 g for 10 min. Harvested cells were sonicated for 30 s in sucrose-lysis buffer. After sonication, it was centrifuged at 2,000 g for 10 min. The supernatant was at 55°C in the presence of sodium dodecyl sulfate, proteinase K and hexadecylmethylammonium bromide. DNA was extracted by applying phenol, chloroform and isooamyl alcohol and precipitated by the addition of ethanol.

**PCR**

The oligonucleotide primers 341f and 907r (Muyzer et al., 1998) were applied to selectively amplify 16S rDNA genes of all eubacteria. A 40-nucleotide GC-rich sequence was attached to the 5’ end of the primer 341f to improve the detection of sequence variation in amplified DNA fragments by subsequent DGGE (Myers et al., 1989). The mixtures used for PCR amplification of bacterial sequences contained 25 ng extracted DNA, 0.5 µM each primer, 200 µM each deoxynucleoside triphosphate, 1.5 mM MgCl$_2$, 1.25 U of KOD DNA polymerase (TOYOB), 5 µl of 10× PCR buffer for KOD and sterile water to a final volume at 50 µl. PCR amplification was carried out with the following program: 94°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min; and a single final extension at 72°C for 2 min. The presence of PCR products was confirmed by analyzing 8 µl of product on 2% agarose gels stained with ethidium bromide.

**DGGE**

DGGE was performed with a D-CODE Multi-system (Bio-Rad). PCR products were loaded onto 6% (wt/vol) polyacrylamide gels in 1.0× TAE (40 mM Tris, 40 mM acetate, 1 mM EDTA [pH 8.4]). The polyacrylamide gels were made with denaturing gradient ranging from 30 to 50% (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run at 60°C, for 4 hours at 130 V. After electrophoresis, the gels were soaked for 30 min in SYBR Green nucleic acid gel stain (1:10,000 dilution; Molecular Probes) and photographed on a UV transillumination table with a DC120 Zoom Digital Camera (KODAK).

**Sequencing of DGGE fragments**

DNA fragments to be nucleotide sequenced were punched from the gel with sterile tips and placed in sterile vials. DNA extraction from gels was performed with a QIAEX-DNA extraction kit (QIAGEN). Extracted DNA was used as template DNA in a cycle sequencing reaction with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) in accor-
dance with the manufacturer’s instructions. Sequencing of 16S rDNA fragments was done with an ABI PRISM 377 DNA Sequencing System (Applied Biosystems). Database searches were conducted by the sequencing match program with the RDP II (Ribosomal Database Project II). The sequences determined in this study and those retrieved from the databases were aligned by Clustal W with DDBJ (DNA Data Bank of Japan). Neighbor-joining trees were constructed by Clustal W with DDBJ and Tree View.

Results and discussion
Nitrogen removal profile in metallurgic wastewater treatment system
Figure 1 shows the time courses of nitrite and nitrate concentration in the effluent of the anoxic reactors of Runs 1, 2 and 3. The NOx-N concentration of influent was about 2,000 mg/l, which included mostly nitrate. The NOx removal rate in anoxic reactors of Runs 1 and 3 were almost 90% or more. Nevertheless, accumulation of nitrite was observed in that of Run 2 for about 200 days from the first operational day. It was caused by the denitrifying bacteria not being fixed in the reactor of Run 2.

Temporal variations in microbial populations by DGGE analysis
Figure 2 shows DGGE band profiles of the PCR amplification products obtained from sludge samples in the anoxic reactors. Temporal differences in microbial populations in the anoxic reactors of Runs 1 and 2 were hardly observed at the different sampling times. On the other hand, the samples of Run 3 showed temporal variations in microbial populations from July to October 1999. Runs 1 and 2 were operated at a constant water temperature, whereas Run 3 was operated at the outside which caused water temperature change. It was supposed that the temporal variations in microbial populations of Run 3 were caused by the temperature variations. On comparing DGGE band profiles of Runs 1, 2 and 3, those of Run 1 were similar to those of Run 3, although not to those of Run 2. It was suggested that the microbial community was affected by the difference in fluidizing condition rather than the difference in the scale and water temperature.

Figure 1 Time courses of NO₂-N (○) and NO₃-N (●) concentration in the effluent of the anoxic reactors of metallurgic wastewater treatment systems. The arrows indicate the sampling days for DGGE analysis.
Sequencing and identification of DGGE fragments

DGGE fragments were excised from gels and amplified by PCR and the nucleotide sequences were determined. None of the sequences obtained precisely matched those found in the database, suggesting that previously uncharacterized populations may have been present in these samples. The analysis of the microbial community by PCR-DGGE clarified that the microbial community in the metallurgic wastewater treatment system consisted of two groups (the *Alcaligenes defragrans* group and the *Pseudomonas* group).

Conclusions

1. DGGE analysis could detect the microbial community change caused by difference of water temperature and reactor conditions.
2. It was revealed that microbial community in the metallurgic wastewater treatment system consisted of mainly *Alcaligenes defragrans* and *Pseudomonas* groups.
3. High denitrification ability of nitrate and nitrite in the anoxic packed bed of metallurgic wastewater treatment systems was confirmed in Runs 1 and 3.

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References


Figure 2 DGGE band profile of PCR products from anoxic reactors of RUNs 1, 2 and 3