Activity and Community Composition of Denitrifying Bacteria in Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-Using Solid-phase Denitrification Processes

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Two laboratory-scale solid-phase denitrification (SPD) reactors, designated reactors A and B, for nitrogen removal were constructed by acclimating activated sludge with pellets and flakes of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) as the sole added substrate under denitrifying conditions, respectively. The average denitrification rate in both reactors was 60 mg NO₃⁻-N g⁻¹ (dry wt) h⁻¹ under steady-state conditions, whereas washed sludge taken from the reactors showed an average denitrification rate of 20 mg NO₃⁻-N g⁻¹ (dry wt) h⁻¹ with fresh PHBV as the sole substrate. The difference in the denitrification rate between the two might be due to the bioavailability of intermediate metabolites as the substrate for denitrification, because acetate and 3-hydroxybutyrate were detected in the reactors. Most of the predominant denitrifiers isolated quantitatively by the plate-counting method using non-selective agar medium were unable to degrade PHBV and were identified as members of genera of the class Betaproteobacteria by studying 16S rRNA gene sequence information. nirS and nosZ gene clone library-based analyses of the microbial community from SPD reactor A showed that most of the nirS and nosZ clones proved to be derived from members of the family Comamonadaceae and other phylogenetic groups of the Betaproteobacteria. These results suggest that the efficiency of denitrification in the PHBV-SPD process is affected by the availability of intermediate metabolites as possible reducing-power sources as well as of the solid substrate, and that particular species of the Betaproteobacteria play the primary role in denitrification in this process.

Key words: Solid-phase denitrification, poly(3-hydroxybutyrate), nirS gene, nosZ gene, microbial community

Biological denitrification is a series of distinct bioenergetic reactions in which nitrate is reduced to dinitrogen gas through nitrite, nitric oxide and nitrous oxide. This biochemical process is important not only as a key step in the nitrogen cycle in nature but also as a means of nutrient removal in engineered wastewater treatment processes. Biological denitrification processes using biodegradable solid polymers, termed the solid-phase denitrification (SPD) process, have received attention as promising methods for removing nitrogen from water and wastewater. The SPD process has some advantages over conventional nitrogen removal systems using soluble substrates, e.g., a constant supply of reducing power and ease in handling and operation. Promising solid substrates for denitrification are poly-hydroxyalkanoates (PHAs) and some other biodegradable aliphatic polyesters. To date, SPD processes using poly(3-hydroxybutyrate) (PHB) and its copolymer, poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV), have been most intensively studied. The application of poly(ε-caprolactone) (PCL) to SPD processes has also been reported.

Functional and structural analyses of the microbial communities involved in SPD processes are necessary to provide a basis for their practical application to nitrogen removal. In this connection, several strains of PHB- and PHBV-degrading denitrifying bacteria have been isolated and characterized from activated sludge and PHA-utilizing...
SPD processes\textsuperscript{2,3,24,26,30,38}. A representative of PHB-degrading denitrifiers is \textit{Diaphorobacter nitroreducens}\textsuperscript{25}. Our previous study on a 16S rRNA gene clone library constructed from a PHBV-utilizing SPD reactor has shown that members of the family \textit{Comamonadaceae}, a major phylogenetic group of the \textit{Betaproteobacteria}, predominated in this process\textsuperscript{26}. However, the question of what kinds of microorganisms are actually involved in denitrification in PHBV-SPD processes has remained unanswered.

Although 16S rRNA genes are powerful molecular markers for studying the entire prokaryotic community structure in an environment, they can provide no direct information about the physiologic and functional nature of the community. In the present study, therefore, \textit{nirS} and \textit{nosZ} genes, encoding cytochrome \textit{cd}, nitrite reductase and nitrous oxide reductase, respectively\textsuperscript{45}, were used as molecular markers to assess the community composition of denitrifying bacteria in the PHBV-SPD process. These denitrifying-enzyme genes as well as the Cu-containing nitrite reductase gene, \textit{nirK}, have been used for the molecular characterization of denitrifying microbial communities in different environments including wastewater treatment systems\textsuperscript{2,13,32,44,45}. In addition, the predominant culturable denitrifying bacteria from PHBV-SPD reactors were isolated, phylogenetically identified, and studied in terms of their substrate specificity. Relationships between the activity and community structure of denitrifiers in the PHBV-SPD process are discussed.

**Materials and Methods**

**Biodegradable plastics**

PHBV pellets and powder containing 5\% poly(3-hydroxyvalerate) (PHV) were obtained from Japan Monsanto Co., Tokyo, Japan. PHBV sheets (5\% PHV) were kindly provided by the Mikawa Textile Research Institute, Aichi Prefectural Government, Gamagori, Japan. The PHBV sheets were cut into small flakes (5 mm×5 mm). All PHBV pellets and flakes were washed with ethanol and then pure water prior to use.

**Construction of PHBV-acclimated reactors**

Activated sludge samples were collected from the main aerobic treatment tank of a sewage treatment plant in Toyo-hashi, Japan, and used as the seed for constructing denitrification reactors. Two glass flasks (2 L capacity) containing 1,000 ml each of culture medium was inoculated with the sludge and semi-anaerobically incubated at 25°C for more than 2 months in the presence of nitrate added. One of the reactors was acclimated with PHBV pellets (reactor A) and the other, with PHBV flakes (reactor B). At the beginning, mineral base RM2 (pH 7.0)\textsuperscript{39}, 20 g of PHBV and 20 mM KNO\textsubscript{3} were added to the reactors. During the first 4 weeks, half of the supernatant in the reactor was exchanged with fresh mineral medium containing 40 mM KNO\textsubscript{3} every 3–4 days of operation. During the subsequent period, the reactors were loaded daily with the same medium by exchanging half of the supernatant and also supplemented with 5 g each of PHBV every week. The concentration of microbial sludge was adjusted to ca. 700 mg dry wt L\textsuperscript{-1} every week. During the acclimation, the reactors were gently stirred at 70 rpm with a magnetic stirrer; under these conditions, the dissolved oxygen (DO) tension in the core of the reactors was less than 0.5 mg L\textsuperscript{-1}, indicating that the reactors were continuously operated under semi-anaerobic conditions.

**Measurement of denitrifying activity**

The rate of denitrification in the reactors was measured by monitoring the change in the concentration of nitrate and nitrite in each batch cycle, for which ion chromatography was used as described previously\textsuperscript{24,26}. Also, the denitrification rate was measured separately using washed sludge and fresh PHBV powders as the sole substrate. For this, sludge samples from the reactors at the end of each batch cycle were collected by centrifugation, washed twice with 50 mM phosphate buffer (pH 7.0), and concentrated in a small volume of this buffer. An aliquot of the concentrated sludge suspension was introduced into rubber-plugged test tubes (30-ml capacity) containing 20 mM KNO\textsubscript{3}, 0.5 g of PHBV powder, and 25 ml of RM2 mineral medium (pH 7.0) from which \((\text{NH}_4)_2\text{SO}_4\) was eliminated. The tubes were sparged with argon to establish anoxic conditions and incubated on a reciprocal shaker at 25°C for 24–48 h. Then, the concentrations of nitrate, nitrite, nitrous oxide, and N\textsubscript{2} gas were monitored by ion chromatography and gas chromatography as described previously\textsuperscript{24,26}. Preliminary experiments showed that the amounts of nitrite and nitrous oxide produced as intermediates both in the reactors and in the test tubes were negligible in almost all cases. Therefore, the nitrate removal rate was considered as the denitrification rate in this study.

**Measurement of organic acids**

Samples were taken from the reactors at appropriate intervals and centrifuged at 12,600×g for 10 min. The resultant supernatant samples were filtered through a membrane filter (pore size, 0.2 μm) and stored at −20°C until analyzed. Lower fatty acids and 3-hydroxybutyrate (3HB) were measured by ion-pair HPLC as described previously\textsuperscript{33}. 

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Stoichiometric analyses

The overall mixture of microbial sludge and PHBV pellets remaining in the reactors were collected at the end of operations. The sludge and PHBV were separated manually from each other using tweezers, washed twice with pure water, and subjected to a dry weight analysis as described previously\(^{14}\) to estimate the growth yield coefficient \(Y_{X/s}\). The substrate consumed/oxidant consumed (S/O) ratio was calculated based on the amounts of PHBV and nitrate consumed. The theoretical relationship between \(Y_{X/s}\) and the S/O ratio was based on information from Hiraishi and Khan\(^{17}\) (see also chemical reaction formula \([1]\) and \([2]\) given below).

Direct cell counting

Ten milliliters of sludge suspension from the reactors at the end of batch cycles was sonicated on ice for 90 sec with 2-sec intermittent bursts (20 kHz; output power, 50 W) and diluted with filter-sterilized phosphate-buffered saline (PBS). Aliquots (10–50 \(\mu\)l) of these diluted samples were taken and used for direct cell counting. Total bacterial counts were measured by epifluorescence microscopy with ethidium bromide (EtBr) or SYBR Green II (Molecular Probes, Inc., Eugene, USA) staining as described previously\(^{15,43}\). Detection of viable cells using a LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes) was performed according to the manufacturer’s instructions and as described previously\(^{43}\). Stained specimens were observed under an Olympus BX-50 epifluorescence microscope equipped with a Flovel FD-120 M digital CCD camera (Flovel Co., Tokyo, Japan). The number of stained cells was counted using the image analysis program WINROOF (Flovel): 10–15 fields per sample and a total of 1,000–2,000 cells per sample.

Enumeration, isolation and characterization of denitrifiers

Sludge and mixed liquor samples taken from the reactor were diluted with PBS as described above, and appropriate dilutions were used for the enumeration of viable bacteria. Plate counts of aerobic heterotrophic bacteria were obtained by the smear-plating method using 1/10-diluted PBY\(^{12}\) agar medium or 1/10-diluted tryptic soy agar medium. Denitrifying bacteria were enumerated by the pour-plating method using agar media containing mineral base RM2, 20 mM KNO\(_3\), vitamin solution PV\(_1\)\(^{31}\) (1 ml L\(^{-1}\)) and 1.8% agar as the basal medium. The agar media were supplemented with either 0.2% tryptic soy broth, 10 mM acetate, 10 mM 3HB or 2% PHBV powder (w/v) as the sole carbon and energy source: designated TSN, ACN, 3HB and PHBN agar media, respectively. For plating, 1 ml of each of the diluted samples noted above was plated and incubated anaerobically at 25°C using the AnaeroPak system (Mitsubishi Gas Chemicals, Niigata, Japan). Inoculated plates were incubated for 2 to 4 weeks before the final reading of CFUs. Few if any detectable colonies appeared on the 4 agar media without nitrate added, thereby confirming that colonies recovered on the test media with nitrate were denitrifiers. For counting CFUs of PHBV-degrading denitrifiers, colonies exhibiting a cleared halo formation were taken as being positive. Colonies recovered on the TSN plates were randomly selected and purified by repeatedly streaking on the same agar medium under anaerobic conditions. Since all isolates thus obtained were aerobic chemoorganotrophic denitrifying bacteria, they were maintained aerobically on PBY agar slants.

16S rRNA gene amplification and sequencing

Crude cell lysates as the DNA source were prepared for PCR use as described previously\(^{19}\). 16S rRNA gene fragments that corresponded to positions 8 to 1543 in Escherichia coli 16S rRNA\(^{8}\) were PCR-amplified from the cell lysate with a set of bacterial universal primers, fD1 and rP1\(^{42}\), as described previously\(^{10}\). PCR products were purified by the polyethylene glycol precipitation method\(^{16}\), sequenced with a SequiTTherm Long Read cycle sequencing kit (Epicentre Technologies, Madison, USA), and analyzed with an Amersham-Pharmacia ALFexpress DNA sequencer.

Quinone profiling

Quinones from sludge samples and the isolates were extracted with a chloroform-methanol mixture and partially purified by column chromatography. Quinone components were separated and identified by reverse-phase HPLC and photodiode array and mass spectrometric detection with external ubiquinone (Q-\(n\)) and menaquinone (MK-\(n\)) standards. Detailed information on the analytical procedures has been given previously\(^{14,15}\).

DNA extraction and purification

Bulk DNA was extracted from sludge samples as described previously\(^{28}\). The crude DNA extracted was further purified by a standard method including deproteinization with chloroform-isoamylalcohol and RNase treatment\(^{29}\). The DNA solution thus obtained was diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) as needed and used for PCR experiments.

Amplification and sequencing of denitrifying enzyme genes

Fragments of nitrite reductase genes, nirK and nirS, from
the bulk DNA purified from sludge samples were PCR-amplified with the oligonucleotide primer pairs nirK1F (GG[A/C]ATGGT[G/T][C/C/G]TGGCA)-nirK5R (GCC-TGATCAG[A/G][T[T][A/G][TG]-1006 and nirS2F (5'-GAAT[G/A/C/T][C/T][C/C/G][GAGCCG-3']-nirS6R (5'-A[C/G][A/G]CGTGGACTT[A/G][GCCGTT-3'], respectively. The primer pair nirS2F’-nirS6R’ was a modification of the nirS2F-nirS6R pair described by Braker et al. The expected sizes of PCR-amplified nirK and nirS fragments were 0.51 and 0.80 kb, respectively. For PCR amplification of nosZ fragments, we first used previously described primers8, but this attempt gave unsatisfactory results. In this study, therefore, we designed a new pair of PCR primers, nosZ2f (5'-GT-GGCCGAAGAACCC[G/C]CACGG-3') and nosZ3r (5'-T[C/G]GCCGGGATGTGCAGCGA-3'), based on nosZ gene sequences retrieved from databases (see Fig. 4). The sequences of nosZ2f and nosZ3r correspond to positions 1006 to 1025 and 1466 to 1484 in the sequences of nosZ2f and nosZ3r, but this attempt gave unsatisfactory results. In this study, therefore, we designed a new pair of PCR primers, nosZ2f (5'-GT-GGCCGAAGAACCC[G/C]CACGG-3') and nosZ3r (5'-T[C/G]GCCGGGATGTGCAGCGA-3'), based on nosZ gene sequences retrieved from databases (see Fig. 4). The sequences of nosZ2f and nosZ3r correspond to positions 1006 to 1025 and 1466 to 1484 in the nosZ gene of Raistonia eutropha strain H16 (database accession number AY305378), and the expected size of PCR-amplified nosZ fragments was 0.48 kb. All thermocycling reactions were performed by the touchdown PCR method10 using an rtTaq DNA polymerase kit (Takara, Otsu, Japan), one of the primer sets and a Takara Thermal Cycler. The first half of the PCR procedure included an initial preheating step of 2 min at 94°C and 20 cycles of a touch-down procedure consisting of denaturation for 1 min at 94°C, annealing for 1 min at a temperature decreasing from 60 to 51°C with 1°C decremental steps of 2 cycles each and extension for 1 min at 72°C. Following this, additional 20 cycles of the thermocycling reaction with annealing at 50°C was performed. The final step was followed by extension at 72°C for 5 min. PCR products were separated by agarose gel electrophoresis, cut from the gel, and then purified using a GENECLEAN Spin kit (Bio 101, Vista, USA). Purified PCR fragments were subcloned using a pT-Blue Perfectly Blunt cloning kit (Novagen, Madison, USA). Transformation of Escherichia coli competent cells was carried out according to a standard manual of molecular cloning13). Plasmid DNA was isolated and purified by using the Wizard Plus Mini prep DNA Purification System (Promega Inc., Madison, USA) according to the manufacturer’s instructions. Nucleotide sequences of nirS and nosZ gene clones were determined as described for 16S rRNA gene sequences. For comparison, nosZ genes from the previously described denitrifying bacteria Brachymonas denitrificans strain AS15220) and Diaphorobacter nitroreducens strain NA108724,25 were PCR-amplified, subcloned and sequenced as described above. In addition, Aquitalea sp. strain PGP-1 and Comamonas sp. strain TSL-h, both of which were isolated from SPD reactor A in this study, were subjected to the nosZ gene analysis. This study is the first to determine the nosZ gene sequences of these 4 denitrifying species.

Sequence comparisons and phylogenetic analyses

All sequence data were compiled with the GENETYX-MAC program (Software Development Co., Osaka, Japan). 16S rRNA gene sequence data were compared with those retrieved from Ribosomal Database Project II19. Nucleotide sequences and translated amino acid sequences of nirS and nosZ clones were analyzed using the BLAST homology search system1,27). The multiple alignment of sequences and calculation of the nucleotide substitution rate with Kimura’s two parameter model27) were performed using the CLUSTAL W program28). Distance matrix trees were constructed by the neighbor-joining method28), and the topology of the trees was evaluated by bootstrapping with 1,000 resamplings19).

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited with the DDBJ under the accession numbers AB277845 to AB277852 for 16S rRNA genes, AB278071 to AB278090 for nirS genes, and AB278091 to AB278115 for nosZ genes.

Results

Denitrifying activity of PHBV-using reactors

PHBV-containing reactors A and B were operated at 3- to 4-day batch intervals during 30 days of start-up and with a 24-h batch cycle thereafter. Both the reactors exhibited similar denitrifying activity during the overall period of operation. As an example, changes in denitrifying activity in reactor A are shown in Fig. 1. Nitrate remained in detectable amounts at the end of each batch cycle during the first 2 weeks of operation but was not detected thereafter (Fig. 1a). Also, little nitrite was detected after 2 weeks of operation. During the first 40 days of operation, the denitrification rate exhibited by the reactor in each batch cycle increased markedly. After that, the denitrification rate exhibited by the reactor became constant at around 60 mg NO3−-N g−1 (dry wt) h−1 (Fig. 1b). To estimate the actual denitrification rate with PHBV more accurately, sludge samples were taken from the reactors at the end of each batch cycle, washed and separately tested for denitrification with fresh PHBV as the sole substrate. The denitrification rate with the fresh PHBV also increased linearly during the first 30 days of operation and reached around 20 mg NO3−-
acetate and 3HB were constantly produced in both the reactors under steady-state conditions. The concentrations of acetate and 3HB detected in the supernatant at the end of operation were 2.9–4.8 and 1.9–2.4 mmol L\(^{-1}\), respectively.

Thus, the marked difference in the denitrification rate between the reactor itself and the washed sludge with fresh PHBV was possibly due to the bioavailability of intermediate metabolites, e.g., organic acids, as a usable substrate for denitrification. In fact, the washed sludge taken from the reactors at the end of operation exhibited 2.0–2.5-fold higher denitrification rates with acetate than with PHBV (data not shown).

Table 1. Stoichiometric estimation of microbial biomass, PHBV and nitrate in the reactor

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Measured value for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PHBV added (g)</td>
<td>45.0</td>
</tr>
<tr>
<td>PHBV consumed (g)</td>
<td>20.1</td>
</tr>
<tr>
<td>Total NO(_3)−N added and removed (g)</td>
<td>7.28</td>
</tr>
<tr>
<td>Excess biomass produced (g dry wt)</td>
<td>8.7</td>
</tr>
<tr>
<td>(Y_{xs}) (g g(^{-1}))</td>
<td>0.43</td>
</tr>
<tr>
<td>S/O ratio (g g(^{-1}))</td>
<td>0.624</td>
</tr>
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Based on the biochemical reactions \((1)\) and \((2)\), it can be predicted that a \(Y_{xs}\) value of 0.43–44 corresponds to a S/O ratio of 0.58–0.59\(^{17}\). However, the experimental values of the S/O ratio obtained (0.624–0.640) were considerably higher than the predicted values. This suggests that PHBV as the substrate was consumed not only for denitrification but also for other biochemical processes, e.g., aerobic respiration.

Quinone profiling of microbial communities

Sludge samples were collected from reactors A and B under steady-state conditions (on days 48, 56 and 66) for quinone profiling. In both the reactors, ubiquinone Q-8 accounted for 83–85% of the total quinone content. The remaining fractions were composed mainly of Q-9 (3–5%) and Q-10 (7–9%). Menaquinones constituted only minor proportions of the total content. In light of the available information on microbial quinone systems\(^{14}\), it was evident that Q-8-containing proteobacterial species, especially those of the Betaproteobacteria, constituted the overwhelming majority of the microbial communities in the reactors. The
Denitrifying Microbial Communities with PHBV

results of quinone profiling are in agreement with those of a previous study on a PHBV-acclimating denitrification process.

Enumeration and isolation of denitrifiers

Population densities of denitrifying bacteria as CFUs as well as direct total and viable counts were measured in reactors A and B under steady-state conditions (Fig. 2). No marked differences were noted in these bacterial counts between the two reactors. Among the media used for enumeration, TSN agar medium yielded the highest counts of denitrifiers, in the order of $10^7$ to $10^8$ CFU ml$^{-1}$. The TSN count accounted for 7.5% of the direct total count, 10% of the total viable (LIVE/DEAD) count and 83% of the plate count of aerobic heterotrophic bacteria on average. Comparable or slightly lower counts were obtained with ACN agar medium, accounting for 7.2% of the total count and 80% of the aerobic plate count. The average denitrifying bacterial numbers obtained with 3HBN and PHBN agar media accounted for 1.7 and 0.34% of the direct total counts, respectively. These data suggest that most of the viable heterotrophic bacteria in the reactors detected as CFUs were acetate-utilizing denitrifiers, whereas PHB-degrading denitrifiers constituted rather minor populations.

Several single colonies were chosen at random from the highest countable plates of TSN agar used for the enumeration and subjected to the standard purification procedure. Thus, a total of 50 strains were isolated from reactors A and B and further examined as described below.

Phenotypic and phylogenetic characterization of isolates

Results of phylogenetic and phenotypic characterization of the isolates are summarized in Table 2. The isolates were categorized into 12 groups (groups 1 to 12) at the species or a similar taxonomic level, all of which were assigned to the phylum Proteobacteria by 16S rRNA gene sequencing and quinone profiling. Most of the isolates (90%) were assigned to genera of the Betaproteobacteria, especially those of the family Comamonadaceae (i.e., Acidovorax, Brachymonas, Comamonas, Diaphorobacter and Simplicispira). The major established genera to which more than 15% of the isolates were assigned were Brachymonas, Comamonas and Diaphorobacter. The Brachymonas and Diaphorobacter isolates, designated groups 5 and 9, respectively, were affiliated with previously known species, B. denitrificans and D. nitreducens, respectively. The Comamonas isolates were classified into two groups, one of which (group 7) was most closely related to a previously described uncultured bacterium, clone OS1L-16, and one of which (group 8) was most similar to a denitrifying bacterium, Comamonas sp. strain PD-14. The group-7 Comamonas isolates was also closely associated with Comamonas denitrificans (strain 123, accession no. AF233877) at a 99% similarity level, whereas the group-8 isolates showed less than 97% similarity to any established species of the genus Comamonas. In addition, the isolates belonging to the recently described genus Aquitalea (group 4), whose closest relative was an uncultured bacterium, clone OS1L-24, accounted for 16% of all the isolates.

The uncultured bacteria OS1L-16 and OS1L-24 noted above as well as many other Comamonas-related clones were detected previously as the major uncultured clones from a PHBV-SPD process. Therefore, the PHBV-SPD reactors reported previously and in this study might share a similar bacterial community.

All of the test isolates were aerobic chemoorganotrophic bacteria that grew well on ACN and 3HB agar media under anaerobic denitrifying conditions, thereby confirming them to be acetate- and 3HB-utilizing denitrifiers. In all isolates, the formation of nitrogen gas from nitrate was confirmed by gas chromatography (not shown). With the exception of the group-9 Diaphorobacter isolates and the group-8 Comamonas isolates, no degradation of PHBV by the denitrifying isolates was observed (Table 2).
Results of testing under denitrifying conditions.

NT, not tested.

A trace amount of demethylmenaquinone-8 was present in addition.

Community analysis based denitrifying-enzyme gene clones

PCR amplification of the denitrifying-enzyme genes was performed for the microbial community in SPD reactor A, the previously described denitrifiers Brachymonas denitrificans strain AS152 and Diaphorobacter nitroreducens strain NA10B and the 2 isolates in this study, Comamonas sp. strain TSL-h (group 8) and Aquitalea sp. strain PGP-1 (group 4). PCR with primer pairs nirS2F7-nirS6R’ and nosZ2f-nosZ3r resulted in the successful amplification of nirS and nosZ gene fragments of expected sizes from the SPD reactor and all of the test organisms, whereas the primer pair nirK1F-nirK5R failed to produce nirK gene fragments from any of the test organisms (data not shown). Also, nirK-targeted PCR with the reactor-community DNA gave a weak signal of the product (not shown). These results suggest that microorganisms containing cytochrome cd, nitrite reductase rather than Cu-type nitrite reductase were the major constituents of the denitrifying microbial community in the PHBV-SPD process.

Based on the aforementioned results, we constructed nirS and nosZ gene clone libraries from the microbial community in SPD reactor A and determined the sequences of 32 nirS clones and 34 nosZ clones as well as of the nosZ gene from the 4 isolates. As shown in Fig. 3, the phylogenetic tree based on the nirS gene sequence showed that the nirS clones from the reactor were classified into three major clusters designated I, II and III. The nirS clones of clusters I and II accounted for 63 and 22% of all the clones detected and formed a major cluster together with the nirS genes of Comamonadaceae genera, including Alcylphilus, Acidovorax and Comamonas, with nucleotide sequence similarities of more than 80%. In particular, the cluster I nirS clones grouped with the uncultured nirS clone R2-s02 (accession no. AB118885) as their nearest neighbor, which was detected previously from a wastewater treatment system (44). The nucleotide sequence similarity between the cluster I nirS clones and the R2-s02 clone ranged from 89 to 94%, and the deduced amino acid sequence similarity ranged from 91 to 97%. The remaining nirS clones (cluster III) were most closely associated to the nirS sequence of the betaproteobacterial species Azoarcus tolyticus.

The phylogenetic tree based on the nosZ gene sequence gave a similar topology to that based on the nirS sequence (Fig. 4). The nosZ clones detected were separated into four major clusters designated I to IV. The majority (68%) of the clones fell into cluster I and grouped with the nosZ genes of the betaproteobacterial species Azoarcus sp., Burkholderia
malei and Rhodoferax ferrireducens and group-4 isolate PGP-1 as their closest relative; the similarity to the PGP-1 nosZ gene was 77–78% in nucleotide sequence and 75–77% in translated amino acid sequence. Thus, unlike the case of the nirS clones noted above, nosZ genes showing more than 80% similarity to the major clones detected were not found in the databases. The cluster II nosZ clones accounted for 21% of the total and were associated with the nosZ genes of Acidovorax sp. strain JS42, Diaphorobacter nitroreducens strain NA10B and the group-8 Comamonas sp. strain TSL-6. The nosZ clones of clusters III and IV grouped with the genes of some species of the Alphaproteobacteria and the Gammaproteobacteria, respectively.

**Discussion**

The two PHBV-SPD reactors studied herein exhibited high rates of denitrification (ca. 60 mg NO₃⁻-N g⁻¹ [dry wt] h⁻¹) under steady-state conditions. These rates are much higher than those recorded for activated sludge with various soluble substrates. However, the SPD reactor-derived washed sludge showed approximately one-third lower denitrification rates with fresh PHBV as the substrate than did the reactors themselves. This difference between the two is possibly due to the difference in the bioavailability of soluble intermediate metabolites that could serve as electron donors for denitrification. Similar results have been obtained with a PCL-utilizing SPD process. Results of organic acid analyses suggest that, in the PHBV-SPD pro-
cess, acetate and 3HB as intermediate metabolites become available as good substrates for denitrification by non-PHBV-degrading microorganisms. In fact, the plate counts of denitrifying bacteria with acetate or 3HB as the carbon and energy source were much higher than those with PHBV. In view of this, together with the fact that the majority of the denitrifying strains isolated were unable to degrade PHBV, it is likely that the predominant denitrifiers in the PHBV-SPD process denitrify with intermediate metabolites (e.g., acetate and 3HB) as electron donors constantly produced by co-existing microorganisms.

Since the SPD reactors were operated under semi-anaerobic conditions, PHBV was possibly degraded not only by anaerobically growing, denitrifying bacteria but also by aerobically growing bacteria. This assumption is supported by our finding that the experimentally obtained value of the S/O ratio was higher than the theoretically predicted value. In the biodegradation of PHBV, extracellular PHB depolymerase is the key enzyme, catalyzing the hydrolysis of the polymer to its monomer 3HB\(^23,41\). Also, extracellular 3HB-oligomer hydrolases may be involved in 3HB production\(^39\). While it is known that 3HB is converted to acetoacetate by 3HB dehydrogenase within the cell, there remains the question of how acetate is produced in the PHBV-SPD process. Further studies on the metabolism of PHBV and the production of intermediate metabolites in the SPD microbial community is necessary to understand how non-PHB-degrading denitrifying bacteria are provided with soluble substrates for denitrification.

Based on the results of 16S rRNA gene sequencing and

Fig. 4. Neighbor-joining phylogenetic tree of nosZ gene clones from microbial community of SPD reactor A and those from denitrifying strains. *"Colwellia psychrerythraea" strain 34H nosZ (CP000083) was used as the outgroup to root the tree. Scale=5% substitution (K_nuc). The nodes supported by more than 80% of bootstrap confidence are shown by solid circles. The strains analyzed in this study are asterisked.*
Denitrifying Microbial Communities with PHBV

quinone profiling, most of the predominant denitrifying isolates recovered quantitatively by the plate-counting method were assigned to genera of the Betaproteobacteria, particularly those of the family Comamonadaceae. Although the culturable population of denitifiers represented a small proportion of the total population (7.5%), this culture-dependent information is relatively consistent with the results of quinone profiling of the whole community and our previous finding that most of the uncultured 16S rRNA gene clones in a PHBV-SPD process belonged to the Comamonadaceae (group 4) and Comamonas (group 7) isolates obtained in this study and the uncultured clones detected previously suggest that PHBV-SPD processes share a common microbial community regardless of the origin of seed sludge.

In general, nirS gene phylogenetic trees have a similar topology to 16S rRNA gene phylogenetic trees. In the present study, consistent with the results of the 16S rRNA gene-based phylogenetic analysis of the isolates, most of the nirS genes from the SPD microbial community (i.e., those of clusters I and II) proved to be derived from members of the Comamonadaceae. This finding is also consistent with the results of our previous 16S rRNA gene-based analysis of a PHBV-SPD microbial community. The cluster 1 nirS clones, comprising the largest group of nirS clones detected, was most closely related to the uncultured nirS clone R2-s02, which was found previously in an activated sludge system. This means that the predominant species of denitrifying bacteria present in the PHBV-SPD process are not specific to this process but are common in wastewater treatment systems.

As in the case of the nirS gene clone library, most of the nosZ clones formed a tight major cluster (cluster I) associated with some members of the Betaproteobacteria. At this time, however, it cannot be concluded that the denitrifying bacteria characterized by the presence of the cluster I nosZ genes correspond to those of the Comamonadaceae containing the cluster I nirS genes, partly because the nosZ genes of different phylogenetic groups of the Betaproteobacteria used for comparison overlapped on the nosZ phylogenetic tree and partly because the cluster I nosZ genes did not show close similarities to any of the Comamonadaceae nosZ genes. The nosZ tree indicates that, at least, the cluster II nosZ genes originated from members of the Comamonadaceae.

This study has not yet provided definite information on what species of denitrifying bacteria are most significant in PHBV-SPD processes. However, on the basis of the collective data obtained in this study and previously, it is logical to conclude that members of the Betaproteobacteria, especially those of the family Comamonadaceae, not only constitute the major population of the microbial community but also actually play the primary role in denitrification in the PHBV-SPD process. The majority of the predominant denitrifying bacteria may be non-PHBV degraders and denitrify with soluble metabolites of PHBV produced by co-existing bacteria. In further studies, denitrifying-enzyme gene expression profiling and the proteomic and metabolomic analyses of the SPD microbial community should clarify the relationships between the ecology of active denitrifying bacteria and nitrogen removal efficiency in the PHBV-SPD process.

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