Thauera and *Azoarcus* as functionally important genera in a denitrifying quinoline-removal bioreactor as revealed by microbial community structure comparison

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Abstract

Structural shifts associated with functional dynamics in a bacterial community may provide clues for identifying the most valuable members in an ecosystem. A laboratory-scale denitrifying reactor was adapted from use of nonefficient seeding sludge and was utilized to degrade quinoline and remove the chemical oxygen demand. Stable removal efficiencies were achieved after an adaptation period of six weeks. Both denaturing gradient gel electrophoresis profiling of the 16S rRNA gene V3 region and comparison of the 16S rRNA gene sequence clone libraries (LIBSHUFF analysis) demonstrated that microbial communities in the denitrifying reactor and seeding sludge were significantly distinct. The percentage of the clones affiliated with the genera *Thauera* and *Azoarcus* was 74% in the denitrifying reactor and 4% in the seeding sludge. Real-time quantitative PCR also indicated that species of the genera *Thauera* and *Azoarcus* increased in abundance by about one order of magnitude during the period of adaptation. The greater abundance of *Thauera* and *Azoarcus* in association with higher efficiency after adaptation suggested that these phylotypes might play an important role for quinoline and chemical oxygen demand removal under denitrifying conditions.

Introduction

With the advent of sophisticated molecular techniques, many uncultured bacteria have been identified via cloning and sequencing of their phylogenetically meaningful sequences, such as the 16S rRNA gene (Pace, 1997), yet the biological functions of most remain uncertain. Linking bacterial phylogenetics and biological functionality represents an ongoing effort in the field of microbial ecology (Torsvik & Ovreas, 2002). However, many studies are finding that the composition and function of the microbial community often shift together in response to environmental stimuli (Fernandez, 2000; Kaplan & Kitts, 2004; Norman, 2004). This new paradigm in microbial ecology may provide valuable insight into the functional dynamics of a microbial community.

Many diverse bacteria, some archaea and even some fungi perform denitrification (Shoun & Tanimoto, 1991; Zumft, 1997). This is an important process in the global nitrogen cycle, and an essential step for nitrogen removal in many wastewater treatment plants. Denitrification also has the potential to offset the global surfeit of fixed nitrogen (Moffat, 1998). The denitrifying process has been studied in many ecosystems. Although some denitrifying bacteria have been isolated (Mihelcic & Luthy, 1988; McNally *et al.*, 1998; Mechichi, 2002; Shinoda, 2004), the techniques involved generally only recover a low proportion of the total present (Wagner, 1993; Kaeberlein *et al.*, 2002). Furthermore, traditional phenotypic characterization methods are not suitable for assessing the population diversity of denitrifying bacteria, as many of these microorganisms belong to the *Betaproteobacteria*, which do not grow on traditional media (Etchebehere, 2002; Khan, 2002; Mechichi, 2002). Therefore, structural information on the microbial community gathered to date for many denitrifying ecological systems, and for denitrifying reactors in particular, remains nonsystematic (Etchebehere, 2002).

A remarkable feature of the process of denitrification is that it is often associated with the decomposition of organic matter under anaerobic conditions (Zoh, 1999). Environmental scientists have used this characteristic to degrade certain highly complex compounds (van Schie & Young, 1998; Song *et al.*, 2000; Khan, 2002; Probian *et al.*, 2003; Shinoda, 2004), such as monocyclic alkylbenzenes (Evans, 1991; Heider, 1998), halobenzoate (Coschigano *et al.*, 1994; Song *et al.*, 2000), phenol (van Schie & Young, 1998) and some polycyclic aromatic hydrocarbons (PAHs) (Rockne, 2000). Quinoline is one of the main nitrogen-containing PAHs (NPAHs), a class comprising strong pollutants present in soils and waters. Previous studies have shown that quinoline and some of its derivatives possess toxic, carcinogenic, mutagenic and recalcitrant properties (Minako, 1977; Azhar & Stuckey, 1994). In recent years, NPAH pollution has become a public health concern (Stuermer *et al.*, 1982; O'Loughlin *et al.*, 1996). Removal of quinoline under denitrifying conditions has been demonstrated (Li, 2001) and the degradation pathway has been proposed (Johanson, 1997), but little is known about the microbial diversity underlying this process. Key members of the functional microbial population that are responsible for the removal process have not been identified in many ecosystems.

In this study, a denitrifying reactor (DR) treating quinoline-containing synthetic wastewater was set up and high removal efficiencies for quinoline and chemical oxygen demand (COD) were achieved after 6 weeks of adaptation from seeding sludge (SS). The objective of this study was to identify the functionally important microorganisms for quinoline and COD removal under denitrifying conditions through comparative molecular analyses of the microbial communities in the SS and the biofilm samples.

Materials and methods

Reactor operation

An 18-L tank was used to construct the bench-scale continuous-flow biofilm DR. Semisoft media, constructed from plastic ring and synthetic fibre strings, were packed in the tank. Configuration of the media has been previously described (Zhang, 1998). SS was collected from the secondary sedimentation tank of the wastewater treatment plant in the Shanghai Coking & Chemical Factory (Wujing, Shanghai). The supporting medium was incubated with SS for 10 h to provide ample time for microbial colonization. The synthetic wastewater was composed of quinoline, glucose, $NaNO_3$ and K_2HPO_4. The concentration of quinoline and glucose was approximately 40 and 180 mg L^{-1}, respectively, and the C/N/P concentration ratio was 150 : 30 : 1. An electromagnetic metering pump (ES series, Iwaki Co., Tokyo, Japan) was used to pump the wastewater into the tank and keep the hydraulic retention time at 24 h. Temperature was maintained at 30 °C by an electric heater (Yongxing Co.,YH, China). After a 6-week adaptation period, the reactor reached a stable stage and was monitored for five consecutive days for quinoline and COD removal efficiency. COD was determined by the standard method outlined by Greenburg *et al.* (1992). The quinoline concentration was determined by high-performance liquid chromatography (GC7890, Techcomp, Shanghai, China) according to the manufacturer's instructions. The pH and dissolved oxygen (DO) levels were measured with pH (pHS-3C, Leici, China) and oxygen (Oxi330i, WTW, Germany) meters, respectively.

Sampling and DNA extraction

The SS sample was processed and collected by centrifugation at 8000 ***g***. Biofilm samples were collected by scraping the sludge from the surface of the supporting medium 2 and 6 weeks after the start of the period of adaptation. For each sample, 50 mg of sludge was collected in an Eppendorf tube. Two hundred microlitres of extraction buffer (100 mM Tris, 100 mM EDTA, 200 mM NaCl, 1% PVP, 2% CTAB, pH 8.0) was added to the sample. The sample was then vortexed for 5 min, and 200 μL of SDS buffer (100 mM Tris, 100 mM ethylenediamine tetraacetic acid [EDTA], 200 mM NaCl, 1% polyvinylpyrrolidone [PVP], 2% cetyltrimethyl ammonium bromide [CTAB], pH 8.0) was added. The tube was cooled on ice for 10 mins, and then centrifuged at 13,000 ***g*** for 10 min at 4 °C. The supernatant was transferred to a new Eppendorf tube. DNA was extracted from the supernatant with equal volumes of phenol, phenol–chloroform–isoamyl alcohol (25 : 24 : 1, by volume) and chloroform–isoamyl alcohol (24 : 1, by volume). The aqueous phases were precipitated with two volumes of ethanol at −20 °C overnight, and centrifuged at 15 000 ***g*** for 20 min at 4 °C to collect the DNA pellet. The pellet was washed with 70% ethanol once and vacuum-dried. Finally, the dried DNA pellets were resuspended in 30 μL TE buffer (pH 8.0).

DGGE analysis of the V3 region

The SS, a sample collected when the system had adapted for 2 weeks (TW) and a sample collected when the system had reached a stable stage (DR) were subjected to denaturing gradient gel electrophoresis (DGGE) analysis. Every sample was tested in triplicate. The V3 region of the 16S rRNA gene was amplified using the primers described by Muyzer *et al.* (1993). The 50 μL reaction mixture contained 1U *Taq* DNA polymerase, 5 μL of the corresponding 10× buffer, 4 μL of 25 mM dNTP mixture (TaKaRa Co., Shiga, Japan), 25 pmol of each primer, and 10 ng of genomic DNA. The samples were amplified in a thermocycler PCR system (PCR Sprint,

Thermo Electron Corp., UK) using the following programme: 94 °C for 4 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; and 72 °C for 6 min.

Parallel DGGE was performed with a Dcode System apparatus (Bio-Rad, Hercules, CA) as per the manufacturer's instructions. Amplification products were separated in 8% (weight in volume, w/v) polyacrylamide gels containing a linear 35–60% denaturant gradient (100% denaturant corresponds to 7 M urea and 40% deionized formamide). Electrophoresis was performed in 1× Tris-acetate–EDTA (TAE) buffer at a constant voltage of 200 V and a temperature of 60 °C for 200 min. The DNA bands were stained by using SYBR green (Amresco, Solon, OH) and were photographed with a UVI gel documentation system (UVItec, Cambridge, UK).

Four dominant DGGE bands were excised from the gels, and were eluted by incubation in 50 μL of sterilized distilled water overnight at 4 °C. Using 5 μL of the liquid as template, the bands were re-amplified with the primers described above. The PCR products were evaluated by agarose (1%, w/v) gel electrophoresis, purified and concentrated with UltraClean™ 15 DNA Purification Kits (Mo Bio Inc., Solana Beach, CA). The purified products were ligated into a pGEM-T easy vector (Promega, Madison, WI) and were electrotransformed into competent *Escherichia coli* DH5α cells. Plasmid DNA was extracted from randomly selected clones and screened for inserts of the expected size and correct DGGE migration properties. The cloned plasmid insert was sequenced by a commercial biology company (BioAsia Co., Shanghai, China) using the ABI 377 sequencer (Applied Biosystems, Foster City, CA).

16S rRNA gene sequence amplification and library construction

16S rRNA gene sequence clone libraries were constructed to examine microbial diversity in the SS and DR samples. PCR was performed to obtain most of the near-full-length 16S rRNA gene using the universal bacterial primers P0 (5′-GAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′) (Weisburg, 1991; Di Cello, 1997), which were designed on the basis of the conserved bacterial regions at the 5′ and 3′ ends of the 16S rRNA gene (positions 27f and 1495r, respectively, on the *E. coli* rRNA gene sequence), which allowed amplification of almost the entire gene (Grifoni, 1995). The 50 μL reaction mixture contained 1 U *Taq* DNA polymerase and 5 μL of the corresponding 10 × buffer, 4 μL of 25 mM dNTP mixture (TaKaRa Co.), 25 pmol of each primer and 10 ng of genomic DNA. Amplification was performed using the following programme: initial denaturation at 94 °C for 4 min; 30 cycles consisting of 94 °C for 45 s, 55 °C for 1 min and 72 °C for 1 min; and final extension at 72 °C for 6 min. PCR amplification was performed with a thermocycler PCR system (PCR Sprint, Thermo Electron Corp.). The PCR products were evaluated by agarose (1%, w/v) gel electrophoresis, stained with ethidium bromide, purified and concentrated with UltraClean™ 15 DNA Purification Kits (Mo Bio Inc.). Purified products were ligated into a pGEM-T easy vector with T4 ligase and were electrotransformed into competent *E. coli* DH5α cells according to the manufacturer's instructions (Promega). Ampicillin (100 μg mL^{-1}; Amresco) was added to Luria-Bertani (LB) medium to select recombinants, and isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) and 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-Gal) (80 μg mL^{-1}) were included in the broth for recombinant identification.

Sequence analysis of the 16S rRNA gene clone libraries

Cloned 16S rRNA genes were sequenced by a commercial company (BioAsia Co.) using the ABI 377 sequencer for partial sequences and ABI 3730 DNA sequencer (Applied Biosystems) for near-full-length sequences. Partial sequencing was performed with a single primer, P0, which generated sequences of about 500 bp, which were used to group the sequences at a 99% standard to provisional operational taxonomic units (OTUs, phylotypes).

A representative clone of each provisional OTU was selected and bidirectionally sequenced to obtain the near-full-length sequence using the T7 and SP6 primers, which are located in the pGEM-T easy vector (Promega). All near-full-length sequences were checked for chimera formation with the Chimera check program (version 2.7; Ribosomal Database Project, www.rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU) (Cole, 2003).

Partial sequences were aligned using Clustal X version 1.81 (Thompson, 1997) and OTUs were identified based on a 99% similarity criterion. In order to determine whether the libraries we constructed were large enough to provide stable phylotype richness, the progressive sampling approach described by Kemp & Aller (2004a) was utilized. Pseudolibraries of varying sizes were constructed by randomly subsampling clones from the two libraries. Two nonparametric estimators, S_{Chao1} (Chao, 1987, 1984) and S_{ACE} (Chao *et al.*, 1993), were calculated for each subset using an online program (http://www.aslo.org/lomethods/free/2004/0114a.html) and the output data were treated by the method described by Kemp & Aller (2004b).

Two statistical indices, the Shannon Wiener index (H) and the reciprocal of Simpson's index ($1/D$), were used for the two libraries. These were calculated by using the following functions:

$$H = -\sum p_i \ln p_i$$

where p_i is the decimal fraction of individuals (clones) of the *i*th OTU (Krebs, 1989), and

$$D = \sum p_i^2$$

where p_i was calculated as follows:

$$p_i = n_i/N$$

where n_i is the number of clones in a OTU and N is the total number of clones (Simpson, 1949).

Similarities between the clone libraries were calculated using the LIBSHUFF computer program (Singleton, 2001) (http://www.arches.uga.edu/~whitman/libshuff.html), which was based on the homologous and heterologous coverage (Good, 1953) curves generated from the 16S rRNA gene libraries. Sequences were randomly shuffled 999 times between libraries before the distance between the curves was calculated using the Cramér–von Mises test statistic (Pettitt, 1982). The matrix analysed by LIBSHUFF was generated by the PRELIBSHUFF program (see url above), and the DNADIST program of PHYLIP (http://evolution.genetics.washington.edu/phylip.html) using the Jukes–Cantor model for nucleotide substitution.

All near-full-length 16S rRNA gene sequences matched their nearest relatives using a BLAST search of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The 16S rRNA gene sequences of the two libraries were aligned with the retrieved sequences from the GenBank databases using the Clustal X (version1.81) program. Phylogenetic trees were constructed using a neighbour-joining method using the MEGA2 program (Kumar, 2001). A bootstrap analysis of 100 replicates was also performed using the same software.

Real-time quantitative PCR (RTQ-PCR)

Primers F948 and ATD1420R were used to quantify specifically the *Thauera* and *Azoarcus* group of organisms. Primer F948 was specifically designed for *Betaproteobacteria* amplification (Gomes, 2001) and ATD1420R was a specific primer for genera *Azoarcus*, *Thauera* and *Denitromonas* (Loy, 2005). The 50-μL amplification mixture contained 1U *Taq* DNA polymerase and 5 μL of the corresponding 10× buffer, 4 μL of 25 mM dNTP mixture (TaKaRa Co.), 25 pmol of each primer, 10 ng of genomic DNA and 1× SYBR Green I (Amresco). PCRs were performed in 8-Strip Low Profile tubes (TLS-0851; MJ Research, Watertown, MA) and closed with Ultra Clear caps (TCS-0803; MJ Research). The following RTQ-PCR program was performed: an initial denaturation step at 94 °C for 7 min followed by 40 cycles consisting of 94 °C for 45 s, 60 °C for 45 s, 72 °C for 1 min and 82 °C for 6 s. At the end of each cycle (at 82 °C), the fluorescent signal was measured. The samples were run by using a DNA Engine Opticon 2 system (MJ Research); data were recorded, and analysed with the corresponding Monitor software (Version 1.1).

The clone (DR-80) from the most dominant OTU of DR samples was used to establish a standard curve that was included in each RTQ-PCR run. Plasmid DNA was extracted and purified using a 3S kit (Biocolor, Shanghai, China), and the plasmid DNA concentration was measured fluorimetrically using a Hoefer DyNA Quant 200 apparatus (Amersham Biosciences Corp, Uppsala, Sweden); calf thymus DNA (Pharmacia Biotech, Uppsala, Sweden) was used as a standard. The purified recombinant plasmid DNA was serially diluted in double-distilled water (ddH_2O) to a final concentration ranging from 2.54×10^3 to 2.54×10^9 copies μL^{-1}. Two-microlitre aliquots of each dilution were used for RTQ-PCR to generate the standard curve and used as quantification standards for SS and DR samples.

Nucleotide sequence accession numbers

DNA sequences representative of each OTU were deposited in GenBank under accession numbers AY945863–AY945900 (SS) and AY945901–AY945924 (DR). The sequences of the four excised DGGE bands (A–D) were assigned accession numbers AY945925–AY945928.

Results

Performance of the denitrifying reactor

Quinoline removal was not detectable in the SS. After a start-up period of 6 weeks, the reactor reached a steady state. Inside the tank the pH was 7.0 and the DO levels was less than 0.1 mg L^{-1}. Removal efficiencies for quinoline and COD on five consecutive days (2–6 June 2003) are shown in Fig. 1. The average quinoline removal efficiency was 90.2% and the average COD removal efficiency was 81.1% during this steady-state period.

PCR–DGGE fingerprinting of microbial communities in the denitrifying reactor and seeding sludge

The DGGE profiles revealed that the composition of the microbial community in the reactor was dramatically shifted during the period of adaptation (Fig. 2). Those for the DR samples showed that the most dominant band was band A. A band with the same electrophoretic mobility as band A was also detectable in the profile of the sample taken after 2 weeks of adaptation (TW). However, this band was less dominant in the TW sample. After 2 weeks, quinoline removal efficiency was 67.5% and COD removal efficiency was 53.4%. The abundance of band A therefore appeared to be correlated with quinoline and COD removal efficiency in

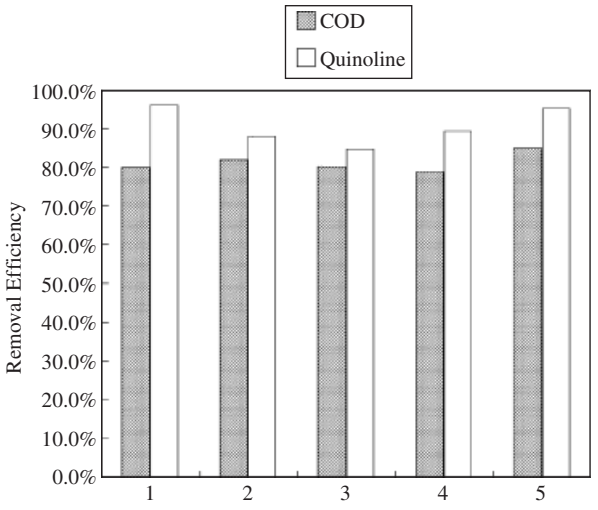

Fig. 1. Quinoline and chemical oxygen demand (COD) removal efficiencies over 5 consecutive days of monitoring. 1–5: 2–6 June 2003

the reactor. Band A was not observed in the DGGE profile of SS. This suggested that the population represented by band A was enriched during adaptation.

Four dominant bands (A–D) in the DGGE profile were sequenced and identified. Their closest relatives found in the GenBank database are listed in Table 1. The most dominant band (band A) from the DR sample was related most closely to *Thauera aminoaromatica* (AJ315677), with a sequence similarity of 100%. From the SS samples, it was determined that the three dominant bands are related to three uncultured bacteria. One of these bands (band C) is affiliated with *Bacteroidetes*. The other bands (bands B and D) have highest sequence similarities (95% and 97%, respectively) to an uncultured *Betaproteobacterium* (AY133064) and an uncultured bacterium (AF314435), respectively.

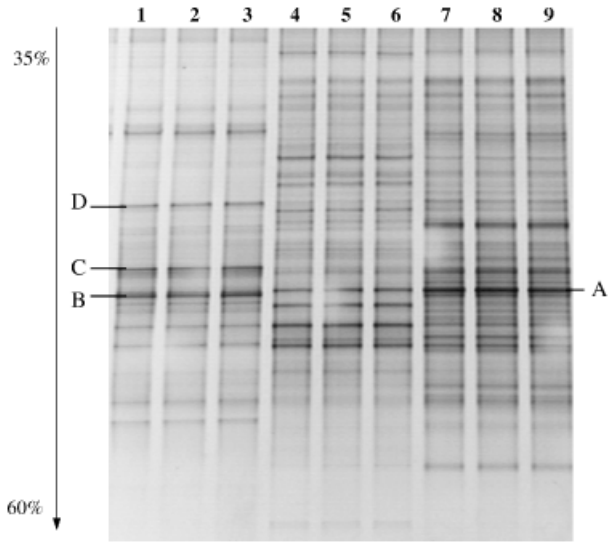

Fig. 2. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V3 region amplified from the total DNA of the seeding sludge and two biofilm samples in the denitrifying reactor. The denaturing gradient was from 35% to 60%. Lanes 1–3: seeding sludge (SS); lanes 4–6: biofilm sample collected after a period of adaptation for 2 weeks (TW); lanes 7–9: biofilm sample collected at the stable stage (DR).

Statistical analysis of the 16S rRNA gene clone libraries

Partial sequencing of the clones using the P0 primer generated fragments of approximately 500 bp. By using an arbitrarily defined limit of 99% gene sequence similarity, all clones in the SS and DR libraries were grouped into provisional OTUs (phylotypes). A representative clone from each OTU was sequenced for the near-full-length category. Six near-full-length sequences were positively identified (one from DR and five from SS) as possible chimeras with the CHECK_CHIMERA program at RDP, and were not analysed further. The corresponding partial sequences were also discarded and were not utilized in the statistical analysis.

The remaining 95 and 125 clones from the SS and DR libraries, respectively, were used for statistical comparisons. Two nonparametric richness estimators (S_{Chao1} and S_{ACE}) were used to assess whether the two libraries were of sufficient size to obtain meaningful stable richness estimates. The number of phylotypes that appeared at different times was input into the online program. Evaluation data files were generated for the SS and DR clone libraries. The data in these files were transferred into an Excel spreadsheet, 'largeenough.xls', which can be downloaded from the same website; graphs of S_{Chao1} and S_{ACE} vs. subsample size were then displayed. For the two libraries we constructed, the S_{Chao1} and S_{ACE} all reached an asymptote (Fig. 3). This indicated that the libraries were large enough to yield stable phylotype richness estimates (Kemp & Aller, 2004a, b). The predicted number of phylotypes suggested that the diversity in the SS library was higher than that in the DR library.

The Shannon Wiener index (H) and the reciprocal of Simpson's index ($1/D$) were also calculated (Table 2). Both of these statistical indices suggested that the phylotype diversity in the SS library was higher than that in the DR library. This result is consistent with those calculated by the richness estimators, indicating that during the period of adaptation, microbial diversity decreased in the DR library as compared with that in the SS library.

Comparison of the libraries by LIBSHUFF analysis

Partial sequences were aligned using ClustalX, and evolutionary distances were calculated using the Jukes–Cantor algorithm in the DNADIST program (http://evolution.genetics.washington.edu/phylip.html). The distance matrices were then used to determine compositional

Table 1. Sequence identity of the dominant bands obtained from denaturing gradient gel electrophoresis (DGGE) analysis from the bacterial communities of the seeding sludge and denitrifying reactor

Band*	Related bacterial sequences	Similarity (%)	Accession no.
A	*Thauera aminoaromatica* (AJ315677)	100	AY945925
B	Uncultured betaproteobacterium (AY133064)	95	AY945926
C	Uncultured *Bacteroidetes* bacterium (AJ575722)	97	AY945927
D	Uncultured bacterium PHOS-HE36 (AF314435)	97	AY945928

*Bands were excised from the DGGE gel shown in Fig. 2.

differences between the two libraries. The result of the LIBSHUFF (Singleton, 2001) analysis is shown in Fig. 4. The solid line indicates the value of $(C_X–C_{XY})^2$ for the original samples at each value of *D*. *D* is equal to the Jukes–Cantor evolutionary distance determined by the DNADIST program in PHYLIP. The broken line indicates the 950th value (or $P = 0.05$) of $(C_X – C_{XY})^2$ for the randomized samples. The heterologous coverage was significantly different ($P = 0.001$) from the homologous coverage, indicating that most sequences from the SS library had low similarity to sequences in the DR library (Fig. 4). This result was similar to that determined in the DGGE analysis, and suggested that a significant population composition shift in the microbial communities occurred during the period of adaptation.

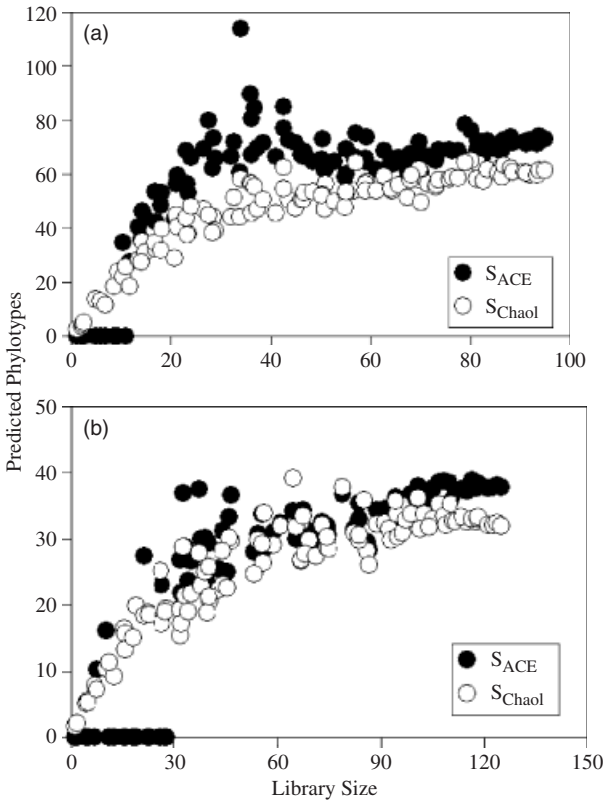

Fig. 3. Predicted numbers of phylotypes based on values of S_{ACE} (•) and S_{Chao1} (○) vs. library size. The two libraries reached an asymptotic maximum, indicating that they were large enough to yield stable estimates of phylotype richness. (a) Seeding sludge, (b) denitrifying reactor libraries.

Phylogenetic analysis of the 16S rRNA gene libraries

Phylogenetic trees were constructed for the two libraries using the near-full-length 16S rRNA gene sequences of the representative clones and reference sequences retrieved from GenBank using the MEGA2 program (Kumar, 2001).

Composition of the SS library

In the SS library, 38 phylotypes were identified and clustered into 11 different bacterial groups, eight of which were affiliated with the classes *Proteobacteria* (*Alpha*, *Beta*, *Gamma* and *Delta*), *Acidobacteria*, *Chlorobi*, *Clostridia* and *Bacteroidetes*. The remaining three groups were not closely related to any recognized microorganisms, but were affiliated with the uncultured bacteria (unaffiliated groups SS I, SS II and SS III; Fig. 5).

The most abundant group (represented by 48% of the SS clones) was affiliated with the class *Bacteroidetes*. The largest OTU (SS1, comprising 26% of the SS clones) of this library was affiliated with *Flavobacteriaceae genomosp* at a 98.5% sequence similarity, as calculated by the Jukes–Cantor model in MEGA2 program (Kumar, 2001). Fourteen OTUs and their neighbours were included in the *Betaproteobacteria* group. Three OTUs in this group (SS33, SS31 and SS9), representing 4% of the total clones, were affiliated with the *Thauera* and *Azoarcus* cluster (Fig. 5).

Composition of the DR library

All clones from the DR library were closely affiliated with the *Betaproteobacteria* group, in which 24 OTUs were classified (Fig. 6). *Thauera* and *Azoarcus* were represented most abundantly, with 73.6% of the total DR clones (representing 11 OTUs) constituting this lineage. The most dominant OTU (DR1), representing 56% of the total clones, shared a 99.9% sequence similarity with *Thauera aminoaromatica*, which was

Table 2. Comparison of diversity indices of the seeding sludge (SS) and denitrifying reactor (DR) libraries

Library	No. of clones	No. of OTUs observed	1/*D**	*H*†
SS	95	38	10.68	3.07
DR	125	24	3.05	1.94

*$D = \sum p_i^2$, where p_i was calculated as follows: $p_i = n_i/N$, n_i is the number of clones in an operational taxonomic unit (OTU) and N is the total number of clones.

†Shannon–Wiener index, H, defined as $-\Sigma p_i \ln p_i$, where p_i is the decimal fraction of individuals (clones) of the ith OTU.

recently identified as a denitrifying species capable of growing with amino-aromatic compounds (Mechichi, 2002).

Many phylotypes in the DR library were related to recognized denitrifying bacteria. OTUs DR2, DR3 and DR4 were most closely related to *Sterolibacterium denitrificans*, a recently described cholesterol-oxidizing denitrifying species (Tarlera & Denner, 2003). OTUs DR7, DR18 and DR 22 were allied with an uncultured *Betaproteobacteria* clone (AY118150), which was reported to be the most dominant nitrate-reducing bacteria in a benzene-degrading culture (Ulrich & Edwards, 2003).

Real-time quantitative PCR

RTQ-PCR indicated that the 16S rRNA gene copy number of the genera *Thauera* and *Azoarcus* increased by approximately one order of magnitude from the SS to DR samples. The assay had a linear range of detection spanning six orders of magnitude (Fig. 7). The average threshold cycles (C_t) of SS and DR samples were 22.9 ± 0.2 and 19.1 ± 0.5, respectively. The corresponding copy numbers were $3.70 \pm 0.16 \times 10^5$ and $3.69 \pm 0.97 \times 10^6$ copies mg^{-1} of wet weight of biofilm samples.

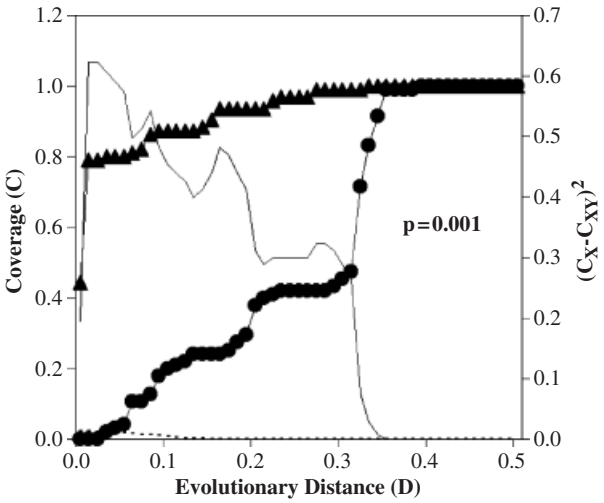

Fig. 4. Results of LIBSHUFF comparison of the homologous coverage curve (▲) and heterologous coverage curve (●) for the seeding sludge (SS, *X*) and denitrifying reactor (DR, *Y*) 16S rRNA gene sequence libraries. The solid line indicates the value of $(C_X - C_{XY})^2$ for the original samples at each value of *D*. *D* is equal to the Jukes–Cantor evolutionary distance determined by the DNADIST program of PHYLIP. The broken line indicates the 950th value (or $P = 0.05$) of $(C_X - C_{XY})^2$ for the randomized samples.

Discussion

Molecular techniques provide powerful tools to monitor structural shifts of microbial communities as their functions change in response to environmental changes. This systematic analysis can help identify populations whose shifts are associated with functional dynamics of the community (Bond, 1995; Watanabe, 1998). This strategy can lead to identification of functionally important populations in a community, although the actual physiological process carries out by the population cannot be elucidated in this way.

PCR-based approaches, using 16S rRNA genes as markers, are powerful tools for the characterization of complex bacterial communities without cultivation and isolation. These methods have provided valuable information on bacterial diversity in wastewater treatment systems and in other ecologically important environments. The reliability of these techniques is mainly dependent upon the efficiency of DNA extraction and PCR bias (von Wintzingerode *et al.*, 1997; Qiu, 2001). In this study, between-sample comparisons were made by applying identical treatments to all samples. Furthermore, noise created by technical bias may be decreased by using three different primer pairs (the V3 region primers for DGGE, full-length primers for library construction and group-specific primers for RTQ-PCR) per sample. This combined approach should yield a more reliable indication of biological variability within and between community structures.

In this study, 99% gene sequence similarity was used as a criterion to define an OTU. The sequences within an OTU defined in this manner may have originated from different species (Stach, 2003). Limitations in the application of OTUs have been discussed in previous publications (Hughes, 2001; Keswani & Whitman, 2001). However, when comparing the relative richness among different libraries, as mentioned by Stach *et al.* (2003), it is still reasonable to use data sets representing the same length and region of the 16S rRNA gene.

Changes in two estimators (S_{Chao1} and S_{ACE}) with library size were used to determine whether the libraries were sufficiently large. The two libraries were also subjected to rarefaction analysis, and all of the curves were below the saturation point (data not shown), indicating that the libraries were not exhaustively sampled. However, estimators such as S_{Chao1} and S_{ACE} are more efficient and less subjective (Kemp & Aller, 2004a). According to Kemp &

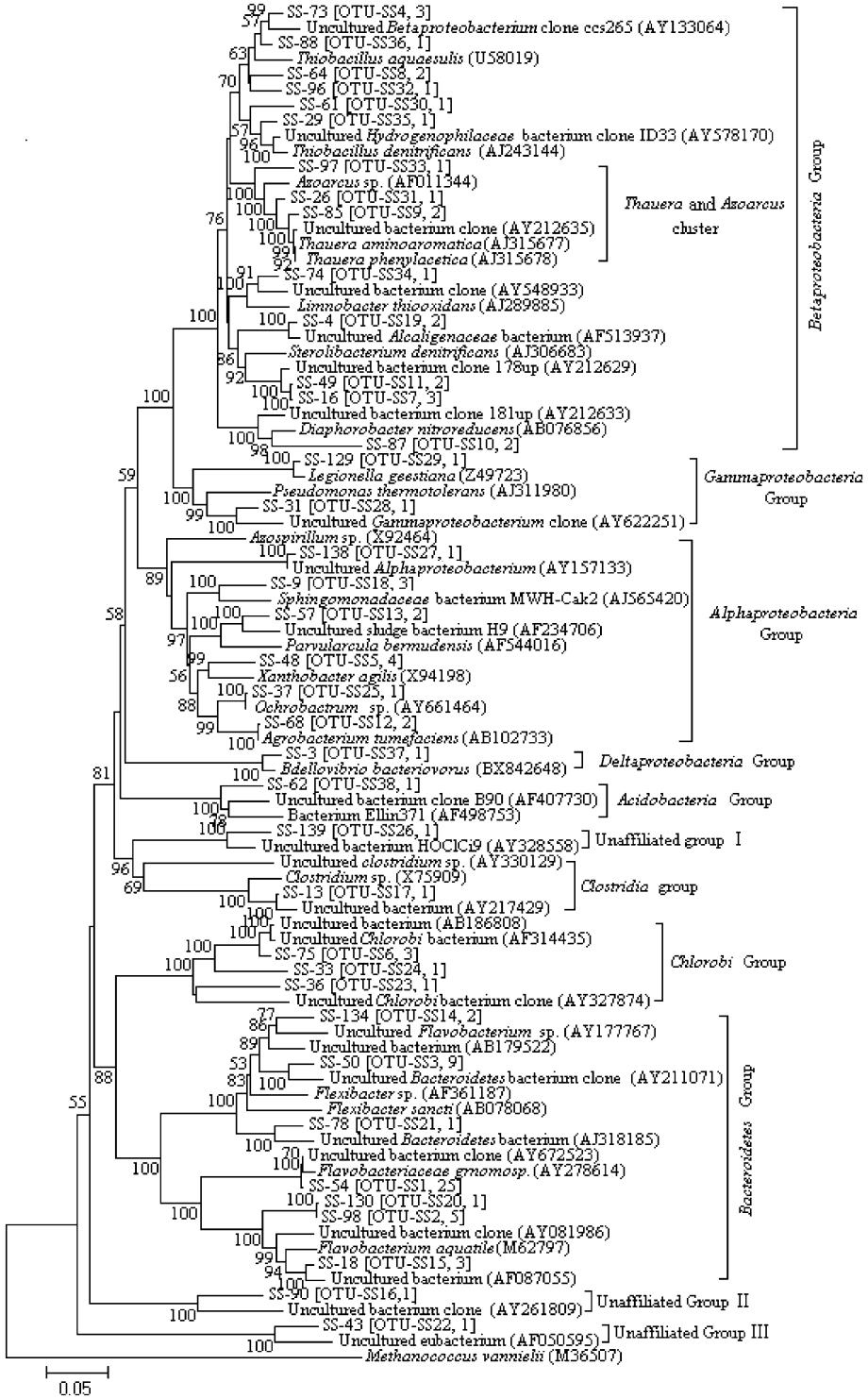

Fig. 5. Dendrogram of 16S rRNA gene sequences showing the phylogenetic affiliation of the operational taxonomic units (OTUs) from the seeding sludge. The neighbour-joining tree was constructed from the near-full-length 16S rRNA gene sequences of representative clones of each OTU and sequences retrieved from the GenBank database. *Methanococcus vannielii* (M36507) was selected as the outgroup. The OTU name and the number of clones belonging to that phylotype are shown in square brackets. The scale bar represents five nucleotide substitutions per 100 nucleotides. Bootstrap values (100 replicates) above 50% are shown at branch nodes.

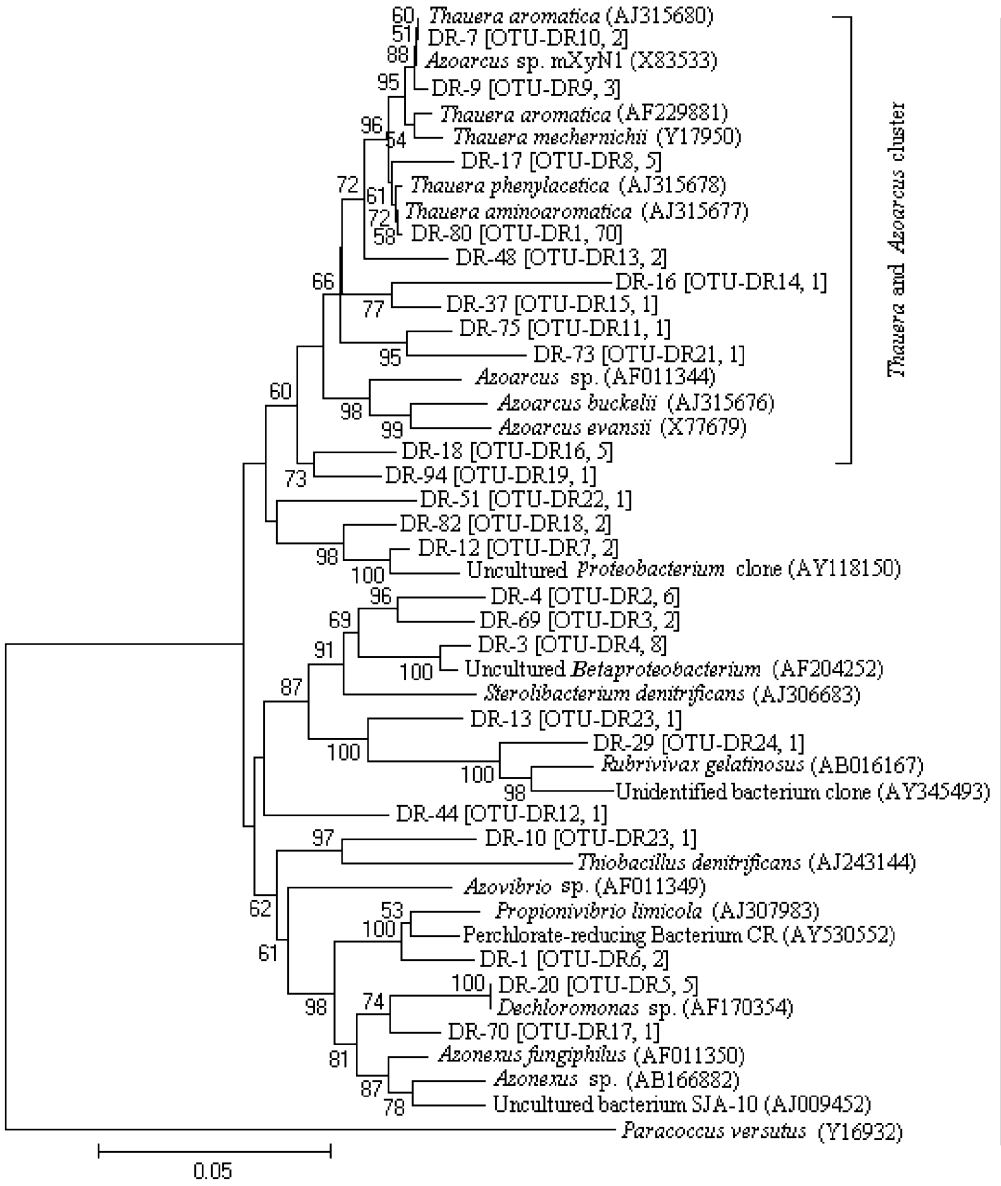

Fig. 6. Dendrogram of 16S rRNA gene sequences showing the phylogenetic affiliation of the operational taxonomic units (OTUs) from the denitrifying reactor. *Paracoccus versutus* (Y16932) was used as the outgroup. Other details as in the legend to Fig. 5.

Aller, 2004b, the richness estimators underestimate phylotype richness in small libraries, and will be stable only when the library size is large enough (Kemp & Aller, 2004b). The two estimators calculated for the two libraries constructed in this study were both stabilized, indicating that the libraries were large enough to yield stable estimates of phylotype population richness, and therefore that the comparisons based on these libraries are valid.

Both the statistical indices (H and $1/D$) and the estimators (S_{Chao1} and S_{ACE}) indicated decreases in microbial diversity from the SS to the DR libraries. The SS was collected from the coking wastewater treatment plant, and the organic compounds that can be utilized by microorganisms there were of much higher diversity than those in the DR (Li, 2001). This implied that when the original bacterial community was exposed to the quinoline-containing synthetic wastewater, the community profile shifted to one in which synthetic wastewater-utilizing microorganisms predominated under denitrifying conditions. Norman *et al.* (2004) also found a similar diversity decrease toward populations capable of degrading the *n*-alkane component of crude oil. They suggested that when the microbial community was exposed to the crude oil, the *n*-alkane-degrading populations tend to increase and interact with

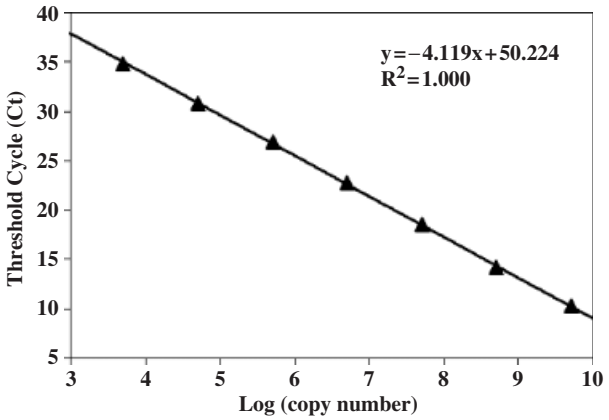

Fig. 7. Standard curve showing the linear relationship between the threshold cycles (C_t) and input copy number of the plasmid from clone DR-80. Linear regression ($R^2 = 1.000$) gives $y = -4.119x + 50.224$.

other species around them, and ultimately alter the community composition and the corresponding degradation potential (Norman, 2004). 'Species diversity is higher in complex environments' is an established hypothesis in traditional ecology, and this phrase may best explain the phenomenon discussed above.

Most clones identified in the DR library were denitrifying bacteria. All DR clones (100%) were affiliated with the *Betaproteobacteria*, whereas in the SS samples the proportion of this subclass was 24%. Khan *et al.* (2002) found that the population of *Betaproteobacteria* in sewage-activated sludges increased from 23–29% to 60–67% during adaptation to poly(3-hydroxybutyrate-co-3-hydroxyvalerate, PHBV) under denitrifying conditions in laboratory-scale reactors. This finding confirms that the *Betaproteobacteria* represents an important cluster for denitrification (Etchebehere, 2002).

Real-time quantitative PCR (RTQ-PCR) was performed to quantify the genera *Thauera* and *Azoarcus* in the biofilm samples. RTQ-PCR has been proved to be an effective method for quantifying bacteria in many different environmental samples down to the genus and species levels (Hein, 2001; Skovhus, 2004; Guilbaud, 2005). Panicker *et al.* (2004) applied the SYBR Green I-based PCR and Taqman assay to detect faecal pathogens and found that the two assays had the same level of sensitivity of detection. An issue that needs to be considered is that the 16S rRNA gene copy number generated from the RTQ-PCR cannot be directly converted into cell counts, as the number of rRNA gene operons per bacterial genome can vary from one to as many as 15 (Klappenbach, 2001). Nevertheless, if it was assumed that the average number of copies of the 16S rRNA gene per cell were the same for SS and DR, our results still indicated that the abundance of *Thauera* and *Azoarcus* increased by approximately one order of magnitude. For instance, if we assume that the average 16S rRNA gene copy number of *Thauera* and *Azoarcus* was four, based on the knowledge that there is an average of four copies of the 16S rRNA gene per cell for the *Betaproteobacteria* (Klappenbach, 2001), then the total cell numbers of *Thauera* and *Azoarcus* in SS and DR would be $9.25 \pm 0.04 \times 10^4$ and $9.23 \pm 0.24 \times 10^5$ per microgram of wet weight of biofilm samples, respectively. The clone library analysis also showed that the proportion of *Thauera* and *Azoarcus* group species increased by one order of magnitude (from 4% to 74%). Thus, the results obtained with the two methods were comparable.

Stable-isotope probing, full-cycle rRNA gene sequence analysis and fluorescence *in situ* hybridization – microautoradiography methods have been used to link phylogenetic identity with physiological function (Lee, 1999; Manefield, 2002; Ginige, 2004). These techniques provide direct evidence on the physiological activities of the members identified. However, this work has demonstrated that classical methods such as DGGE fingerprinting and clone library profiling are still powerful methods for identifying functionally important members from a microbial community through dynamic monitoring of structural shifts in response to different environmental conditions. The study provided strong support for an association between high quinoline and COD removal efficiencies and dominance of the genera *Thauera* and *Azoarcus* in the community.

In conclusion, comparative analysis between the structure – function relationships in the seeding sludge and the adapted denitrifying reactor indicated that, when the original microbial community (SS) was exposed to quinoline-containing waters under denitrifying conditions (DR), the community structure changed and resulted in a significantly different species composition. Diversity decreased and the dominance of denitrifying populations increased in the denitrifying reactor. Furthermore, clones affiliated with the genera *Thauera* and *Azoarcus* significantly increased and became dominant in the DR when its quinoline and COD removal efficiencies reached high and stable levels. This suggests that members of these genera might be the functionally important phylotypes for quinoline and COD removal under denitrifying conditions.

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