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Denitrifying degradation of dimethyl phthalate

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Abstract Results of batch experiments on the denitrifying degradation of dimethyl phthalate (DMP) was most favorable at pH 7–9 and 30–35°C. DMP was first degraded to monomethyl phthalate (MMP), which was in turn degraded to phthalate before complete mineralization. There was no fatty acid residue in the mixed liquor throughout the experiments. The maximum specific degradation rates were 0.32 mM/(gVSS·h) for DMP, 0.19 mM/(gVSS·h) for MMP, and 0.14 mM/(gVSS·h) for phthalate. About 86% of available electron in DMP was utilized for denitrification; the remaining 14% was presumable conserved in the new biomass with an estimated yield of 0.17 mg/mg DMP. Based on 16S rDNA analysis, the denitrifying sludge was mainly composed of β-subdivision and α-subdivision of *Proteobacteria* (33 and 5 clones out of a total of 43 clones, respectively), plus some *Acidobacteria*. Using a primer set specifically designed to amplify the denitrification *nirK* gene, 10 operational taxonomy units (OTUs) were recovered from the clone library. They clustered into a group in the α-subdivision of *Proteobacteria* most closely related to denitrifier *Bradyrhizobium japonicum* USDA110 and several environmental clones.

Keywords Phthalic esters · Denitrification · 16S rDNA · Dimethyl phthalate · *nirK* gene · Phylogenetic analysis

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Introduction

Phthalic esters are most widely used as plasticizers in the manufacture of plastics for the improvement of processability. They are also used as additive in paints, adhesives, cardboard, lubricants and fragrances (Crosby 1998). Because phthalic esters are not chemically bound to plastics, they are easily leached out and released into the environment. They were detected in landfill leachate (Marttinen et al. 2003a), surface water, sewage sludge, and sediment (Fromme et al. 2002). Phthalates and their degradation intermediates, are toxic, mutagenic and potentially endocrine disruptive on aquatic species (Knudsen and Pottinger 1999) and were classified as priority pollutants. Thus, the removal of phthalates from the environment has aroused much attention in recent years.

In the natural environment, such as surface water, soil or sediment, phthalates are mainly degraded biologically (Staples et al. 1997). A number of studies were conducted for the degradation of phthalates in wastewater and leachate. It was found that phthalates could be effectively degraded under methanogenic condition (Shelton et al. 1984; Kleerebezem et al. 1999a; Wang et al. 2000), but their removal in aerobic treatment systems was mostly due to sorption by the primary and secondary sludge (Marttinen et al. 2003b). Denitrifying degradation of phthalates was conducted mostly for pure cultures. Aftring and Taylor (1981) described a *Bacillus* sp. that could convert *o*-phthalate in nitrate medium through decarboxylation to benzoate. Nozawa and Maruyama (1988) isolated an *o*-phthalate-degrading denitrifier from soil, *Pseudomonas* sp. strain P136, which produced metabolites, including benzoate, cyclohex-1-ene-carboxylate, 2-hydroxycyclohexanecarboxylate and pimelate. Wang et al. (1999) isolated a di-butyl phthalate (DBP)-degrading denitrifying bacterium from an activated sludge treating coke wastewater, which converted

DBP sequentially to monobutyl phthalate (MBP), phthalate and protocatechuate. However, denitrifying degradation of phthalic esters by mixed culture, as in wastewater treatment, was overlooked so far.

The microbial diversity of a mixed culture is now often analyzed according to the 16S rDNA extracted from the community. However, phylogenetic diversity resulting from the 16S rDNA analysis may not be relevant to the physiological diversity of the community. On the other hand, phylogenetic analysis may also be analyzed according to the functional genes, which are better reflection of the community's certain functionality, such as nitrification/denitrification (Purkhold et al. 2000; Okano et al. 2004), dissimilatory sulfate reduction (Wagner et al. 1998), and methane production (Hales et al. 1996). Among all the denitrifying enzymes, a copper-containing nitrite reductase encoded by the functional gene *nirK* was widely used to characterize denitrifying bacteria in environmental samples. This was due to the central role of nitrite reductase in the denitrification pathway (Velasco et al. 2001; Henry et al. 2004; Qiu et al. 2004).

This study was conducted to investigate the denitrifying degradation of dimethyl phthalate (DMP), the simplest and most common phthalic ester, by a mixed culture enriched from activated sludge. The objective is to investigate the effects of pH, temperature, nitrate/DMP ratio on DMP degradation. The microbial diversity, based on both 16S rDNA and functional genes, in the denitrifying DMP-degrading community was also studied.

Materials and methods

Culture enrichment

A denitrifying DMP-degrading mixed culture was enriched from activated sludge obtained from a local municipal wastewater treatment plant. Enrichment was carried out in a 1 L reactor operated in a sequencing batch mode, starting with 0.5 L of seed sludge and 0.5 L of feed solution containing potassium nitrate (10 mM) as electron acceptor. For each gram of DMP, the feed solution consisted of the following nutrients:1.73g $NaHCO_3$, 73.6 mg $MgSO_4{\cdot}7H_2O$, 42.9 mg K_2HPO_4, 17.1 mg KH_2PO_4, 22.5 mg $CaCl_2$, 9.2 mg $NiSO_4{\cdot}7H_2O$, 7.1 mg $FeCl_3{\cdot}6H_2O$, 1.9 mg $MnCl_2{\cdot}4H_2O$, 1.0 mg $ZnCl_2$, 1.0 mg $CoCl_2{\cdot}2H_2O$, 0.7 mg $(NH_4)_2MoO_4{\cdot}4H_2O$, 0.5 mg $CuCl_2{\cdot}2H_2O$ and 0.3 mg $NaBO_2{\cdot}10H_2O$. The reactor was initially fed with DMP, plus a co-substrate, methanol, the concentration of which was gradually reduced. The DMP/methanol concentrations (in mM) in the feed solution changed stepwise from the initial 0/5 to 0.2/4, 0.4/3, 0.6/2, 0.8/1, and 1/0. Four batches of acclimation were conducted at each of the first five concentration levels. Ten batches of acclimation were conducted at the end using DMP (1 mM) as the sole carbon source. Each batch lasted 2 to 4 days, until DMP and/or methanol were completely degraded. At the end of each batch, half of the supernatant was replaced by an equal volume of the feed solution before starting the following batch.

Degradation of DMP at various temperatures, initial pH and NO_3^-/DMP ratios

All batch degradation experiments were carried out in duplicate, unless specified otherwise, in 160 ml serum bottles, each of which contained 100 ml of feed solution, leaving 60 ml of headspace. Each bottle was inoculated with 200 mgVSS/L enriched sludge. Before each batch test, the bottle was completely purged by helium and then sealed with a butyl rubber septa. A series of tests were first conducted at five temperature levels from 25 to 45°C at neutral pH for a feed solution containing 1 mM of DMP plus 6 mM of KNO_3. After the optimum temperature had been identified as 35°C, a second series was conducted at five pH levels from pH 5 to 9 for the same feed solution at 35°C.

After identifying the optimum temperature and initial pH, further tests were conducted studying the effect of NO_3^-/DMP ratio from 2 to 50 for NO_3^- and DMP concentrations ranging 2–40 mM and 0.2–2.0 mM, respectively. Each set was conducted in triplicate, of which two were sampled for gas analysis and the third for liquid analysis.

Sample analysis

Gas production from each batch was measured by the volume displacement method using a syringe, and the composition was analyzed by a gas chromatograph (Hewlett Packard-5890, USA) equipped with a thermal conductivity detector. Composition of the mixed liquor was analyzed after centrifugation at 12,000×*g* for 10 min. Volatile fatty acids (VFA) were analyzed by another gas chromatograph (Agilent-6890N, USA) with a flame ionization detector. The detection limit was 1 mg/L for each individual acid. DMP and its potential metabolites, including monomethyl phthalate (MMP) and phthalate, were quantified using a high performance liquid chromatograph (Shimadzu-10A, Japan) with a UV detector (Shimadzu SPD-10AV, Japan) at 254 nm. The mobile phase was a mixture of acetonitrile and 0.05 M KH_2PO_4 at a ratio of 35/65 (v/v), adjusted to pH 3 using H_3PO_4, delivered at a flow rate of 1 mL/min through an Allsphere column (Alltech ODS-2 3u, USA; 4.6×150 mm, particle size 3 μm). The detection limit of each phthalate was 0.5 mg/L.

Nitrite and nitrate were analyzed using an ion chromatograph (Shimadzu HIC-10A, Japan) with an anion exchange column (Alltech Anion/R, USA; 250×4.1 mm, pore size 10 μm) and a conductivity detector (Shimadzu CDD-6A, Japan). The mobile phase was 5 mM *p*-hydroxy benzoate, adjusted to pH 8.5 using LiOH, and delivered at a flow rate of 2 mL/min. The detection limit for NO_3^-/NO_2^- was 1 mg/L.

Microbial diversity analysis based on 16S rDNA

The enriched denitrifying sludge used to seed all the batch tests was analyzed for its 16S rDNA. Genomic DNA of sludge was extracted, and then amplified by polymerase chain reaction (PCR) using the primer set of 8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1392R (5′-ACGGGCGGTGTGTRC-3′). Details of the PCR solution were as reported previously (Liu et al. 1997). The PCR amplification method of Muyzer et al. (1995) was slightly modified as follows: an initial denaturation at 94°C for 7 min; 35 cycles of denaturation (30 s at 92°C), annealing (30 s at 54°C) and extension (1 min at 72°C); and a final extension at 72°C for 10 min. The PCR-amplified product, after purification with the Wizard® PCR Preps DNA purification system (Promega Corp. USA), was then used to build a clone library with the TA Cloning Kit (Invitrogen Corp., USA), following the protocol in the manual. The inserts in a total of 43 clones were recovered by whole cell PCR using the primer set of M13F (5′-GTT TGA TCC TGG CTC AG) and M13R (5′-CAG GAA ACA GCT ATG AC), and then sequenced using an auto sequencer (Perkin Elmer, ABI model 377A, USA) and dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer, USA). The obtained 16S rDNA sequences were compared with the GenBank database by BLAST search. The sequences of clones and their closest references retrieved from GenBank were aligned and checked manually using BioEdit. A phylogenetic tree was then constructed using the neighbor-jointing method with MEGA 2.1. Bootstrap re-sampling analysis for 500 replicates was performed to estimate the confidence of tree topologies.

Microbial diversity analysis based on *nirK* gene

A primer set was designed for the analysis of the enriched denitrifying sludge based on its functional gene, *nirK*, which encodes the nitrite reductase. A pair of primers targeting *nirK* were designed as follows: Sequences of *nirK* gene were first retrieved from the nucleotide database for nine known species, including *Sinorhizobium meliloti* 1021 (AE007256), *Ochrobactrum anthropi* (AY916794), *Alcaligenes xylosoxidans* (AF051831), *Alcaligenes* sp. STC1 (AB046603), *Bradyrhizobium japonicum* (AJ002516), *Rhizobium hedysari* (U65658), *Rhodobacter sphaeroides* (U62291), *Azospirillum doebereinerae* (AY072263) and *Pseudomonas aureofacie* (Z21945). By aligning these sequences using the BioEdit, two specific conserved regions were identified and used as the forward primer, *nirK*-F (5′-TTCGTCTACCACTGCGC-3′, T_m=57.9°C), and reversen primer, *nirK*-R (5′-TCATCAGATCGTCGTTCCA-3′, T_m=57.0°C) for the amplification of the *nirK* gene fragment by PCR from the extracted genomic DNA. The positions of two primers in the *nirK* gene of *S. meliloti* 1021 (AE007256) are 502–518 and 1084–1102, respectively.

The PCR amplification of the *nirK* gene followed the same procedures for 16S rDNA amplification, except using an annealing temperature of 62°C. The amplified fragments were about 600 bp according to gel electrophoresis, comparable to the 601 bp between the two primers in the *nirK* gene. The PCR-amplified products were then used to build a clone library following the aforementioned protocol. A total of 18 clones were selected to recover the insert in the plasmids by whole cell PCR. The *nirK* gene fragments amplified by PCR were sequenced and then translated into amino acids for phylogenetic analysis.

Accession numbers

The nucleotide sequence data reported in this paper were submitted to the GenBank, EMBL and DDBJ databases, and were assigned the following accession numbers: DQ411040–DQ411063.

Results

Effect of temperature

In all degradation experiments, only two metabolites of DMP, i.e. MMP and phthalate, plus no more than 1 mg/L of benzoate, were found in the mixed liquor. There was no other metabolites, including VFA, detectable in the mixed liquor throughout the degradation. Figure 1 illustrates the concentration changes at pH 7 for temperatures ranging from 25 to 45°C for: (a) DMP, (b) MMP, (c) phthalate, and (d) nitrate. Figure 1a shows that over 90% of DMP was de-esterified in 15 h, and complete de-esterification was achieved in 50 h for all temperatures. Figure 1b shows the corresponding increase of MMP, which is the de-esterified metabolite of DMP, and its subsequent degradation. Results show that MMP, after 15–20 h, was further de-esterified into phthalate, as shown in Fig. 1c, and was completed mineralized at 30°C and 35°C after 135 h. Residual phthalate was found after 135 h at other temperatures: 0.17 mM at 25°C, 0.72 mM at 40°C and 0.75 mM at 45°C. Figure 1a–c clearly illustrate the sequential degradation of

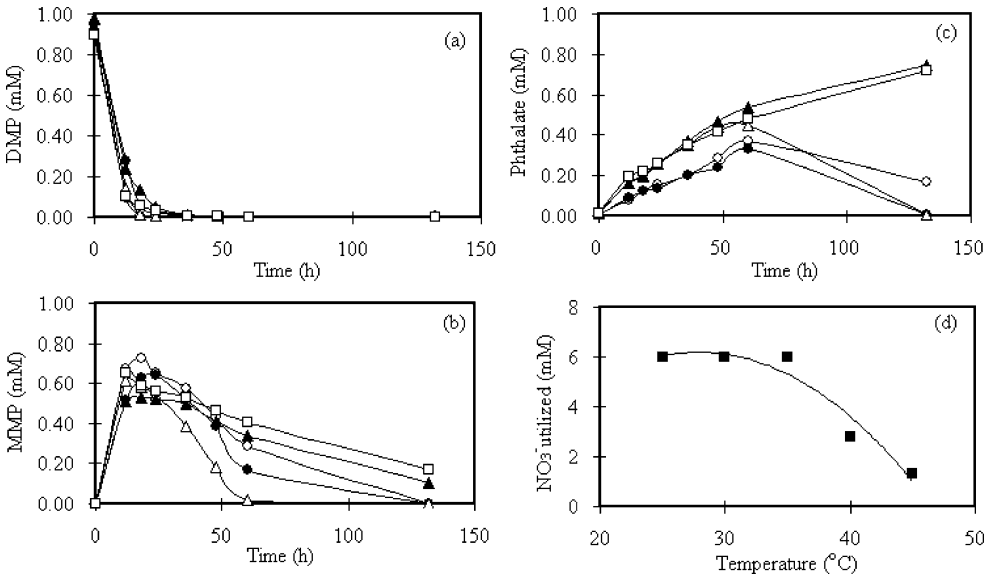

Fig. 1 Time profiles of DMP and its metabolites during denitrifying degradation at various temperatures at pH 7: (**a**) DMP, (**b**) MMP, and (**c**) phthalate (○ 25°C; ● 30°C; △ 35°C; ▲ 40°C; □ 45°C); and (**d**) NO_3^- utilization at various temperatures (■)

DMP into MMP and then phthalate, which was subsequently mineralized completely. Denitrification of phthalate was inhibited at 40°C and 45°C. This is evidenced in Fig. 1c showing increased phthalate residues during DMP degradation, and in Fig. 1d showing reduced nitrate consumption from about 6 mM for 25–35°C to below 3 mM for 40–45°C. Based on the results on degradation rates of DMP and its by-products, 35°C was identified as the optimum temperature for DMP denitrification.

Effect of initial pH

After identifying the optimum DMP degradation temperature, a second series batch experiments were conducted at the optimum temperature of 35°C at initial pH ranging from pH 5 to 9. Similar to Fig. 1a–c, Fig. 2a–c illustrates the sequential degradation of DMP into MMP and then phthalate, which was subsequently mineralized. The pH was lowered during denitrification of DMP. Results in Fig. 2a–c also show that DMP degradation preferred the initial pH of 7–9, at which DMP was completely mineralized at about the same rate with a final pH of 6.9–7.2. For the experiments conducted at the initial pH of 6 and 5, the final pH became pH 5.5 and 4.8, respectively, resulting in the incomplete de-esterification of DMP and phthalate degradation even after 200 h. Figure 2d illustrates that substantially lower quantities of nitrate were utilized at pH 6 and pH 5, reflecting the incomplete degradation of DMP and its metabolites.

Based on results of these first two series, a third series of degradation experiments were conducted at 35°C and pH 7 for various NO_3^-/DMP ratios.

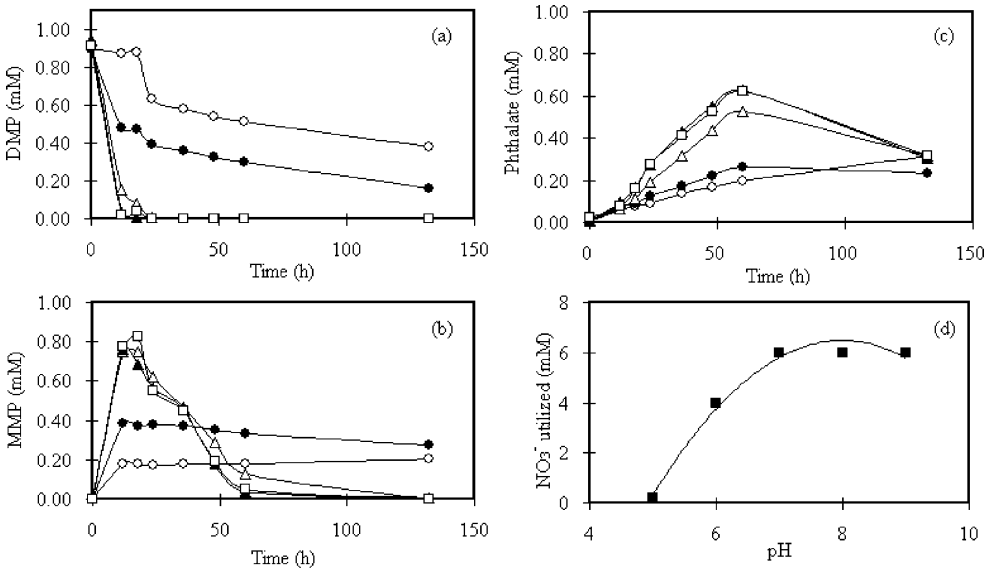

Fig. 2 Time profiles of DMP and its metabolites during denitrifying degradation at different pH at 35°C: (**a**) DMP, (**b**) MMP, and (**c**) phthalate (○ pH 5; ● pH 6; △ pH 7; ▲ pH 8; □ pH 9); and (**d**) NO_3^- utilization at various pH

Effect of NO_3^-/DMP ratio

Figure 3 illustrates variations of $\Delta NO_3^-/\Delta DMP$, i.e. the ratio between the utilized NO_3^- and the removed DMP, in treating wastewaters of various initial NO_3^-/DMP ratios. Results show that the molar ratio of $\Delta NO_3^-/\Delta DMP$ ranged 6.7–7.6, with an average of 7.2, when NO_3^- was in excess in the wastewaters in which the initial NO_3^-/DMP ratio was above 9. For wastewaters with NO_3^-/DMP ratio below 8, the $\Delta NO_3^-/\Delta DMP$ ratio was proportional to the initial NO_3^-/DMP due to the incomplete mineralization of DMP.

Specific degradation rates of DMP and its intermediates

Figure 4 illustrates that the specific degradation rates of DMP and its intermediates, MMP and phthalate, increased with the increase of initial DMP concentration. The maximum degradation rates were 0.32, 0.19, 0.14 mM/(gVSS·h) for DMP, MMP and phthalate, respectively.

16S rDNA analysis of microbial community

Based on 16S rDNA sequences, 43 clones were classified into 14 OTUs, between any two of which the similarity was less than 98.5%. Table 1 summarizes each OTU's population, sequence length, affiliation, and the closest species with similarity. Among the 14 OTUs, only 7 (15 out of 43 clones) were closely related to known species with a similarity of over 97%. This indicates that a large amount of population in the sludge is unrelated to any known species. Figure 5 illustrates the phylogenetic relationship of OTUs in the DMP-degrading sludge and their close relatives. Of the 14 OTUs, 12 belong to either α-subdivision or β-subdivision of *Proteobacteria*, and the remaining two belong to the *Acidobacteria* division.

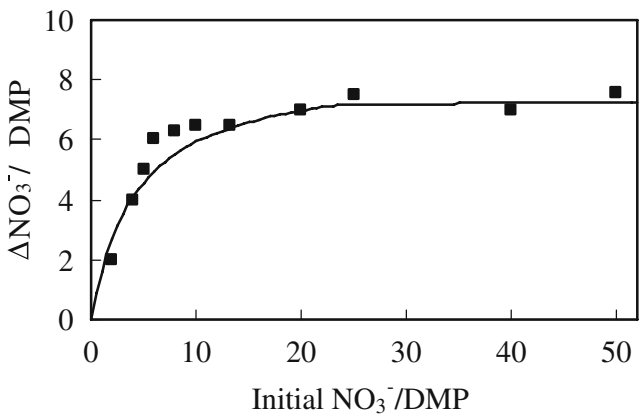

Fig. 3 $\Delta NO_3^-/\Delta DMP$ at various initial NO_3^-/DMP ratios

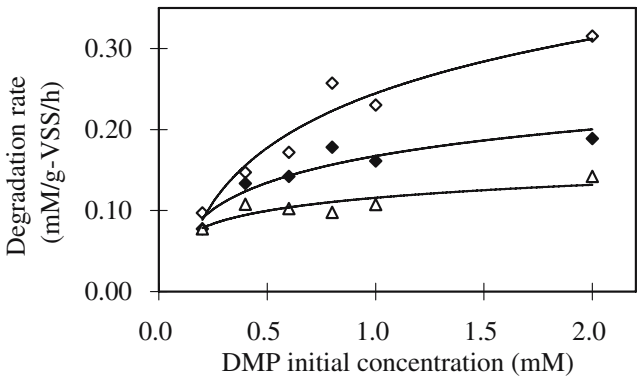

Fig. 4 Specific degradation rates of DMP, MMP and phthalate at 10 mM NO_3^-, pH 7 and 35°C. (◊ DMP; ♦ MMP; ▵ phthalate)

nirK gene diversity analysis

The primer set of *nirK*-F and *nirK*-R was used to amplify the *nirK* gene from the extracted genomic DNA of the sludge. The amplified *nirK* gene fragments were cloned, sequenced, and then translated into amino acids for phylogenetic analysis. A total of 18 clones were retrieved and classified into 10 OTUs, the similarity between any two of which was less than 98.5%. These 10 OTUs were clustered into a group closely related to *B. japonicum* USDA110 (AJ002516), as shown in Fig. 6. Among them, gene *nirK*-24 (8 out of the 18 clones) was the most dominant, followed by *nirK*-21 (2/18). The remaining 8 OTUs had only 1 clone each.

Discussion

Partial denitrification may produce nitrite, which is often toxic to biomass (Yarbrough et al. 1980). However, in this study, nitrite concentration in the mixed liquor was mostly below the detection limit of 0.01 mM and never exceeded 0.2 mM, a level which was insufficient to inhibit the activity of denitrifiers (Glass et al. 1997).

DMP degradation at various NO_3^-/DMP ratios

Mineralization of DMP under denitrifying condition may be expressed as follows:

$$C_{10}H_{10}O_4 + 8.4H^+ + 8.4NO_3^- \rightarrow 4.2N_2 + 10CO_2 + 9.2H_2O \quad (1)$$

Thus, completely mineralizing each mole of DMP would consume 8.4 mol of nitrate. Figure 3 illustrates that for wastewaters with an initial NO_3^-/DMP ratio above 9, removing each mole of DMP consumed 6.7–7.6 mol (averaging 7.2 mol) of nitrate. For wastewaters with

Table 1 Abundance, affiliation, closest species, similarity, and population of OTUs

No.	Phylum	OTU	Sequence length	Closest species (accession number)	Similarity (%)	Population (clone ratios)
1	α-*Proteobacteria*	Den-9	788	Uncultured bacterium 1700-8 (AY425768)	96	1/43
2	α-*Proteobacteria*	Den-45	749	*Hyphomicrobium denitrificans* DSM 1869 (Y14308)	99	2/43
3	α-*Proteobacteria*	Den-74	782	*Bradyrhizobium* sp. Shinshu-th2 (AB121773)	96	3/43
4	β-*Proteobacteria*	Den-95	787	Uncultured bacterium 87 (AJ412674)	99	18/43
5	β-*Proteobacteria*	Den-71	789	*Acidovorax avenae* C1 (AF508114)	99	7/43
6	β-*Proteobacteria*	Den-29	574	*Acidovorax avenae* C1 (AF508114)	98	1/43
7	β-*Proteobacteria*	Den-77	786	*Rubrivivax gelatinosus* OK3O3 (AF487435)	97	2/43
8	β-*Proteobacteria*	Den-7	631	*Rubrivivax gelatinosus* OK3O3 (AF487435)	96	1/43
9	β-*Proteobacteria*	Den-127	786	Uncultured bacterium LO13.11 (AF358003)	99	1/43
10	β-*Proteobacteria*	Den-134	717	*Hylemonella* sp. WQH1 (AJ565430)	98	1/43
11	β-*Proteobacteria*	Den-30	769	Uncultured bacterium CCU24 (AY221080)	97	1/43
12	β-*Proteobacteria*	Den-72	661	*Azoarcus* sp. mXyN1 (X83533)	99	1/43
13	*Acidobacteria*	Den-81	787	Uncultured bacterium SJA-36 (AJ009461)	99	1/43
14	*Acidobacteria*	Den-16	774	Uncultured bacterium SJA-36 (AJ009461)	97	3/43

insufficient NO_3^-, some of the DMP could only be partially mineralized to MMP and phthalate, instead. As a result, the $\Delta NO_3^-/\Delta$DMP ratio in wastewaters with an initial NO_3^-/DMP ratio below 8 was reduced significantly.

In this study, removing each mole of DMP consumed only 7.2 mol of nitrate. This suggests that only 86% of free electrons in DMP was utilized for denitrification. The remaining 14% of the free electrons in DMP were presumably conserved in the newly produced biomass. Assuming that the dry biomass contained 53% carbon (based on the composition of $C_5H_7NO_2$), the biomass yield was estimated as 0.17 mg/mg DMP. On the other hand, based on N balance, 70–100% of NO_3^--N was converted to N_2 with an average of 83%; the remaining 17% of NO_3^--N was presumably converted to biomass, similar to the conclusion based on free electron flow.

Rate-limiting step for DMP degradation

It was thus evidenced from Figs. 1a–c and 2a–c that under denitrifying condition, DMP was first de-esterified sequentially into MMP and then phthalate, before further de-aromatization followed by complete mineralization. A similar pathway of DMP mineralization was observed under aerobic (Wang et al. 2003) and anaerobic conditions (Kleerebezem et al. 1999b). Figure 4 shows the plots of the maximum degradation rates of DMP, MMP and phthalate in wastewaters containing 10 mM of nitrate and various concentrations of DMP. It clearly shows that under all conditions, the degradation rate of DMP was higher than MMP, which was in turn higher than phthalate. Subsequently, phthalate was completely demineralized because no residual VFA was detected in the mixed liquor. Based on these observations, de-aromatization of phthalate appeared to be the rate-limiting step of DMP degradation. Similar observations were reported for the anaerobic degradation of phthalate esters (Bauer et al. 1998).

In this study, the maximum degradation rates of DMP, MMP and phthalate were 0.32, 0.19, 0.14 mM/(gVSS·h), respectively. Aerobic volumetric degradation rates of DMP varied from 0.08 mM/(L·h) (Wang et al. 2004) to 3.4 mM/(L·h) (Juneson et al. 2002) were reported. An anaerobic degradation rate of 0.14 mM/(gVSS·h) was reported for phthalate (Tur and Huang 1997). There was no denitrifying degradation rate of DMP, MMP and phthalate in literature for comparison.

16S rDNA analysis of microbial community

Of the 14 OTUs, 9 belong to β-*Proteobacteria*, 3 belong to α-*Proteobacteria,* and the remaining 2 belong to *Acidobacteria*. Among the 9 OTUs of β-*Proteobacteria* (33 out of 43 clones) in the sludge, Den-95 (18/43) was the most detected species, which is closely related (99% similarity) to an uncultured clone 87 (AJ412674) found abundant in a denitrifying sludge treating landfill leachate (Etchebehere et al. 2002). Den-95 is, accordingly, likely the major denitrifiers in this study. Den-71 (7/43), the second most detected OTU, and Den-29 (1/43) are most closely affiliated with *Acidovorax avenae* C1 (AF508114) with similarities of over 99% and 98%, respectively. Because *A. avenae* C1 is a denitrifying phenol-degrader (Baek et al. 2003), both Den-71 and Den-29 may be the uncultured members of *Acidovorax* and may also be able to degrade aromatics under denitrifying conditions. Den-72 (1/43) is closely related to two aromatics-degrading denitrifiers in β-*Proteobacteria*, i.e. *Azoarcus* sp. mXyN1 (X83533) and

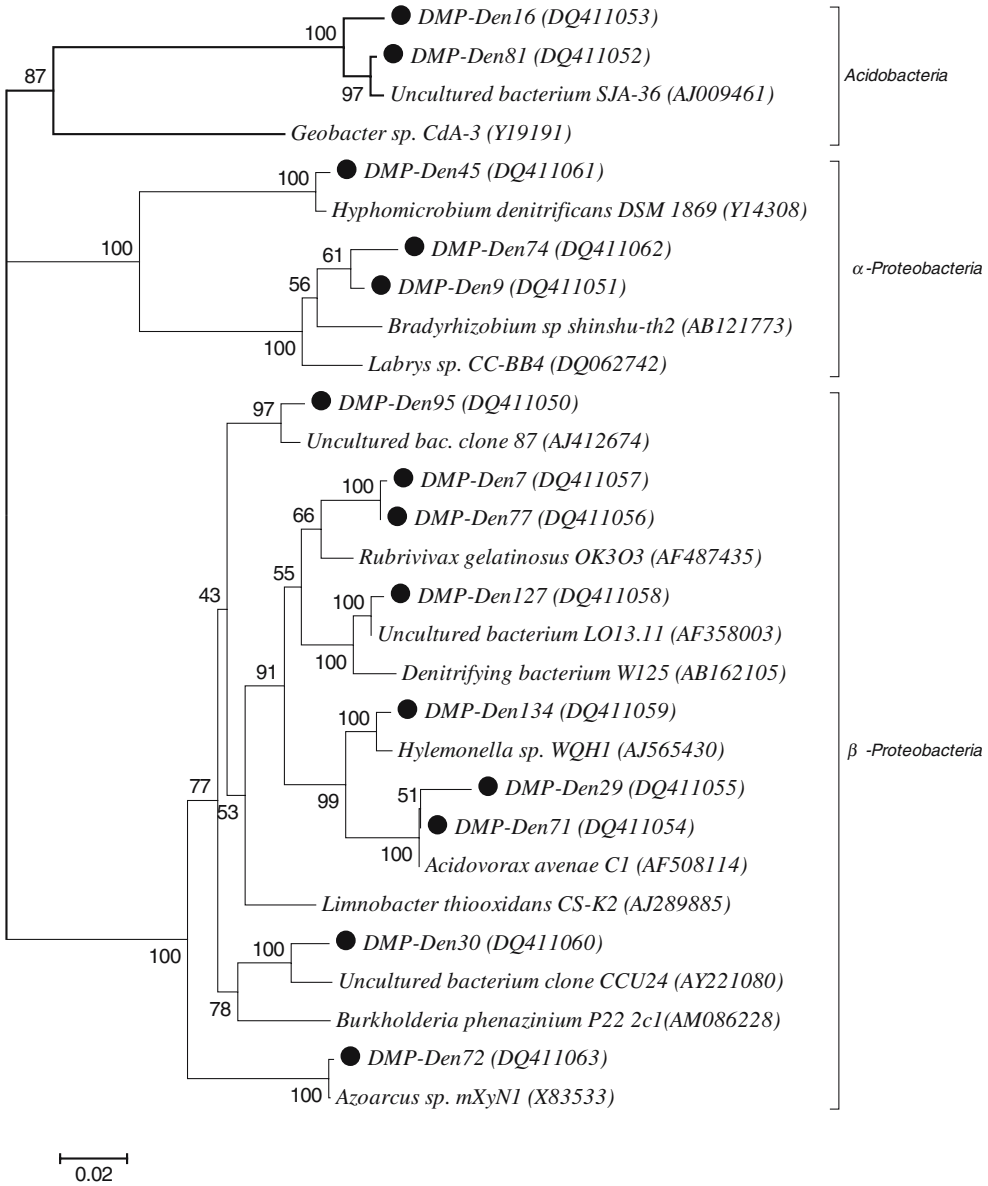

Fig. 5 Phylogenetic tree of OTUs in the DMP-degrading sludge and their close relatives based on 16S rDNA sequences. The tree was based on Jukes–Cantor distance and constructed using the neighbor-joining algorithm with 500 bootstrapping. The scale bar represents 0.02 substitution per nucleotide position. Numbers at the nodes are the bootstrap values

Thauera aromatica LG356 (AJ315680) with 99% similarity. *Azoarcus* sp. mXyN1 is capable of degrading toluene and *m*-xylene (Rabus and Widdel 1995), whereas *T. aromatica* is capable of degrading toluene and benzoate (Mechichi et al. 2002). Den-127 (1/43), having a 97% similarity with denitrifying bacterium W125 (AB162105), is also likely to have denitrifying ability. There is no indication on the functional properties of the remaining OTUs Den-7, Den-30, Den-77 and Den-134.

Two OTUs in the α-*Proteobacteria* subdivision, Den-45 (2/43) and Den-74 (3/43), are closely related to denitrifier *Hyphomicrobium denitrificans* (Y14308) and a *Bradyrhizobium* sp. (AB121773), respectively. Another OTU Den-9 (1/43) is closely related to an environmental clone 1700-8 (AY425768) with 96% similarity. However, its functionality is not clear.

The remaining two OTUs in *Acidobacteria*, i.e. Den-81 (1/43) and Den-16 (1/43), are closely related (with 99% and 97% respective similarities) to an uncultured bacterium SJA-36 (AJ009461) found dominant in anaerobic chlorobenzene-degrading consortia (von Wintzingerode et al. 1999).

nirK gene analysis of microbial community

As shown in Fig. 6, OTU *nirK*-17 and *nirK*-24 are distantly related to any known bacteria; instead, it is closely related to the *nirK* gene of several uncultured clones from different sources, including CK004 (AY072274) in an acid forest soil, uncultured bacterium AK2A (AY583381), KEP14 (DQ182205) and KRP39 (DQ182180) in activated sludge.

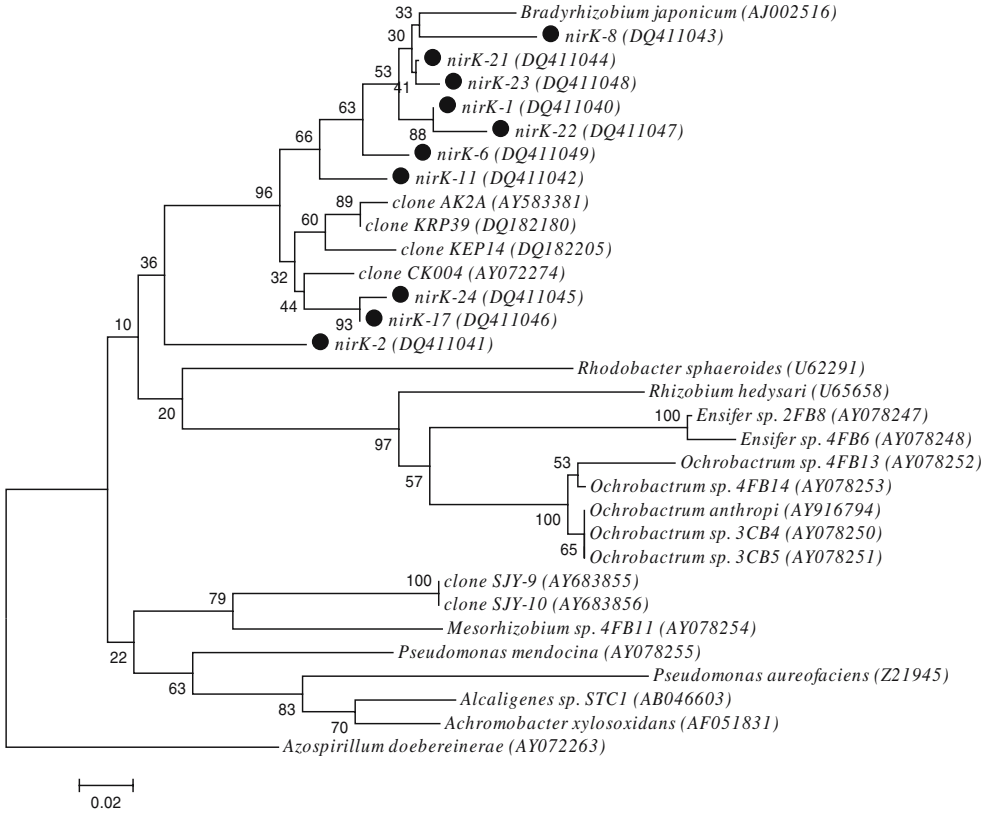

Fig. 6 Phylogenetic tree of *nirK* OTUs in the DMP-degrading sludge and their close relatives based on partial *nirK* gene sequences. 110 amino acids were used in each sequence for comparison. The tree was based on Jukes–Cantor distance and constructed using the neighbor-joining algorithm with 100 bootstrapping. The scale bar represents 0.02 substitution in amino acid. Numbers at the nodes are the bootstrap values greater than 50

The remote similarity with any known bacteria indicates that the *nirK* genes found in this study were not well-characterized so far. OTUs *nirK*-1, *nirK*-6, *nirK*-8 and *nirK*-11, *nirK*-21, *nirK*-22, *nirK*-23 grouped with *B. japonicum*, a bacterium which was extensively characterized for its denitrification enzymes (Velasco et al. 2001), whereas OTU *nirK*-2 was closely related to the *nirK* gene of two uncultured bacteria, i.e. SJY-9 (AY860744) and SJY-10 (AY860745).

Phylogenetic analysis of the 14 OTUs based on 16S rDNA (Fig. 5) show that many OTUs are closely related to known denitrifiers, suggesting that these OTUs, although scattered in various "groups", are likely capable of conducing denitrification as well. On the other hand, all the OTUs based on the *nirK* gene in this study are affiliated with the α-*Proteobacteria* only, similar to that reported in a previous study (Song and Ward 2003). This discrepancy is likely due to the fact that the *nirK*-specific primer set in this study was developed based on the *nirK* genes of a very limited number (9 in total) of known cultures. As a consequence, some *nirK* genes in the community were not retrieved by this primer set.

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