Change of bacterial communities in sediments along Songhua River in Northeastern China after a nitrobenzene pollution event

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Abstract

More than 100 tons of nitrobenzene and related compounds were released into Songhua River due to the explosion of an aniline production factory in November, 2005. Sediment samples were taken from the heavily polluted drainage canal, one upstream and three downstream river sites. The change of bacterial community structures along the river was studied by denaturing gradient gel electrophoresis (DGGE) and cloning and sequencing of 16S rRNA genes with five clone libraries constructed and 101 sequences acquired representing 172 clones. Both DGGE profiles and sequences of 16S rRNA genes from clone libraries demonstrated that the contaminated drainage canal and three downstream river sites were similar in that all had Betaproteobacteria, mainly grouped into Comamonadaceae, as the dominant group of bacteria, and all had Firmicutes, primarily as Clostridium spp. These results suggest that these latter two groups of bacteria may play potential roles in degradation and detoxification of nitrobenzene in the present contaminated river environments.

Introduction

Nitrobenzene is mainly used in the manufacture of aniline as the primary starting material. It is frequently released into the environment from effluent of plants of explosives, organic chemicals and plastics. Owing to its toxicity, nitrobenzene has been listed as a priority pollutant by the United States Environmental Protection Agency (Padda et al., 2003; US EPA, 2006). Numerous bacteria able to degrade nitrobenzene have been isolated. These include Acidovorax sp. strain JS42, Comamonas sp. strains JS765 and CNB-1 and Pseudomonas spp. from nitrobenzene-contaminated facility or waste treatment plants. Mechanisms of degradation of nitrobenzene and other nitroaromatic compounds with isolated strains have been investigated extensively (Nishino & Spain, 1995; Zhao & Ward, 2001; Parales et al., 2005; Wu et al., 2006). It has been found that the removal of nitro groups from nitroaromatic compounds by microorganisms may take place via oxidative pathways with mono-oxygenases or dioxygenases (Nishino & Spain, 1993, 1995; Lessner et al., 2003), or reductive pathways with nitroreductases, yielding nitroso, hydroxylamino or amino derivatives (Marvin-Sikkema & de Bont, 1994; Somerville et al., 1995; Spain, 1995). Biodegradation of nitrobenzene in a lab-scale reactor has also been investigated (Dickel et al., 1993); however, to our knowledge, few studies have been performed focusing on bacterial communities in nitrobenzene-polluted environments.

Songhua River is located in the northeast part of China. With a length of about 1840 km, it flows generally north- and eastward, crossing Jilin and Heilongjiang provinces, to Amur River and to the Sea of Okhotsk. The river is the main source of water for cities and villages along the river. On 13 November 2005, more than 100 tons of nitrobenzene and related compounds were spilled into Songhua River through a drainage canal due to the explosion of a chemical plant located in Jilin City, Jilin Province. The concentration of nitrobenzene in river water reached c. 0.5 mg L⁻¹ at c. 400 km downstream from the discharging point. Concern has been expressed about comprehensive environmental impacts of the toxic spill on the region’s ecosystem (World Health Organization Department for Health Action, 2005). Considering the important role of bacteria in the degradation of chemical compounds in the environment, determination of bacterial communities in sediments along the...
Bacterial communities change after pollution

severely contaminated river should provide some clues about the response of bacterial communities to the nitrobenzene contamination and potential functionally important bacterial groups in a nitrobenzene-polluted environment.

DQGE fingerprinting and cloning and sequencing of PCR-amplified 16S rRNA gene fragments have been successfully applied for the analysis of bacterial community structures in a wide range of environmental samples (Lipson & Schmidt, 2004; Webster et al., 2004; Hobel et al., 2005). In this study, these two approaches were used to determine the change of bacterial community in the sediments sampled along nearly 400 km of the contaminated river to gain an insight into the environmental influence of the toxic spill.

Materials and methods

Study site and sampling

The main pollutant spilled into the river was nitrobenzene. Some related compounds like benzene, aniline and xylol were also discharged in small amounts. To determine the environmental influence of nitrobenzene contaminations, sampling campaigns were performed on 24 December 2005, about 40 days after the explosion. The sampling site A was located upstream of the discharging point as a reference, the contaminated site B was at the drainage canal and sites C–E were at downstream of the river. Sites C–E were all chosen at the turnings of the river where pollutants might accumulate. Between these sites, there were no cities or towns by the side of the river, as well as other pollution sources. The velocity of river flow ranged from 0.5 to 1 m s⁻¹ during the sampling period. Sediment samples of each site were taken in triplicate and pooled, using a grab bucket after breaking the ice layer covering the river. Then sediments were transported on ice to the laboratory and maintained at −20 °C immediately. About 4 L of water samples were also taken from each sampling location for nitrobenzene analysis using liquid chromatography MS. The HPLC system consisted of an Alliance liquid chromatograph 2695 (Waters, Milford, MA) and MS was carried out with a single-quadruple mass spectrometer Q1000 (Micromass, Manchester, UK). The details of the location of sampling sites and nitrobenzene concentrations in sediments and water samples are listed in Table 1.

Batch anaerobic incubation experiment

For each sampling site, 4-g sediment samples in dry weight were added to a 22-mL airtight glass bottle, and filled with sterile mineral media spiked with 11 mg L⁻¹ of nitrobenzene. The bottle was then sealed with a Teflon–Silica gel stopper and covered with a parafilm. Two parallel bottles and one blank sterilized with 1% HgCl₂ (w/w) were set up in a double-layer airtight plastic bag with one anaerobic package (AnaeroPack, Mitsubishi Gas Chemical Co., Japan). The bottles were incubated at 10 °C without shaking. Residual nitrobenzene concentrations were determined at a given time interval.

DNA extraction and PCR amplification

DNA was extracted from 500 mg of sediments using the method of Tsai & Olson (1991). About 15 μg DNA g⁻¹ of wet sediment was obtained using electrophoresis on 1% (w/v) agarose gel and comparison with a molecular mass ladder visually. To acquire suitable amplicons, 10–100-fold dilutions of crude DNA were used as templates for subsequent PCRs.

For DGGE analysis, the V3 region of 16S rRNA gene was amplified using touchdown PCR methods described by Muyzer et al. (1993). The standard 50-μL PCR mixture (Takara, Dalian, China) included 1 × PCR buffer containing 1.5 mM MgCl₂, 200 μM of each deoxynucleotide triphosphate, 10 pmol of each primer, 1.25 U of TaKaRa Taq polymerase and about 40 ng of template DNA. Amplification products were confirmed by electrophoresis in 1.5% (w/v) agarose gel. Meanwhile, almost full-length 16S rRNA gene sequences were amplified using universal primers 27F (5’-AGAGTTTGATCCTGGCTCAG) and 1392R (5’-GACG GCGGGGTGTGAC) (Brought et al., 2002). The amplification reaction mixture was the same as above, and the conditions were: 95 °C for 10 min, 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min 30 s, and a final extension at 72 °C for 30 min. Amplicons were purified with the Qiaquick PCR cleanup kit (Qiagen Inc., Chatsworth, CA). To minimize PCR bias, three separate reactions were run for each sample and pooled.

DGGE analysis

DGGE was performed on a D-Code apparatus (Bio-Rad, Hercules, CA) under the same conditions as described

Table 1. Detailed locations of sampling sites and concentrations of nitrobenzene in water and sediments samples from Songhua River

| Sampling site | Distance (km) | Concentration (mg kg⁻¹) | Description |
|---------------|---------------|-------------------------|-------------|
| A             | −30*          | ND¹, ND                 | Reference site |
| B             | 0             | 17.4, 6.7               | Contaminated site |
| C             | 9             | 1.29, 0.4               | Downstream site |
| D             | 87            | ND                       | Downstream site |
| E             | 388           | ND                       | Downstream site |

¹Reference site A was located upstream of the discharge site. ND, not determined. The detection limits of the nitrobenzene concentration were 0.2 μg L⁻¹ for water samples and 0.05 mg kg⁻¹ for sediment samples.
were retrieved and aligned with clone sequences using PCR products were digested (3 h, 37°C) with HaeIII (Takara) and separated by electrophoresis in 2% agarose gels, and then grouped according to restriction fragment length polymorphism (RFLP) patterns. Representative clones were sequenced using an ABI 3730 automated sequencer (Invitrogen).

Cloning and sequencing of 16S rRNA genes

The cloning of amplified 16S rRNA gene fragments into the TOPO TA cloning vector pCR2.1 and the selection of TOP10 Escherichia coli transformants were all performed following the manufacturer’s instructions (Invitrogen, Shanghai, China). Cloned inserts were amplified from lysed colonies with vector-specific primers M13F (5’-GTAAAACGACGGCCAG) and M13R (5’-CAGGAAACGCTATGAC). PCR products were digested (3 h, 37°C) with HaeIII (Takara) and separated by electrophoresis in 2% agarose gels, and then grouped according to restriction fragment length polymorphism (RFLP) patterns. Representative clones were sequenced using the software CHIMERA_CHECK with bootstrap analyses for 1000 replicates (Kumar et al., 2001). Possible chimeras were checked using the software DOTUR (Schloss & Handelsman, 2005). The most similar reference sequences were retrieved and aligned with clone sequences using CLUSTALX (Thompson et al., 1997). Phylogenetic trees were constructed using MEGA version 3.1 by the neighbor-joining algorithm and the Jukes–Cantor distance estimation method with bootstrap analyses for 1000 replicates (Kumar et al., 2004). Possible chimeras were checked using the software CHIMERA_CHECK in RDP II and the software BELLEROPHON (Huber et al., 2004).

The sequences sharing 97% or greater similarity were grouped into the same operational taxonomic unit (OTU) using software DOTUR (Schloss & Handelsman, 2005). Coverage (C) was calculated as follows: \( C = 1 - (n_i/N) \), where \( n_i \) is the number of OTUs that occurred only once in the clone library and \( N \) is the total number of clones (Singleton et al., 2001). OTU richness \( S_{Chao1} \) and \( S_{ACE} \) were calculated using the software estimates version 8.0 with 100 random sample repetitions (Colwell, 2005). OTU diversity and distribution in the library was evaluated using Shannon diversity (\( H \)) and evenness (\( E \)) indices. Rarefaction curves were constructed using the software AREAREFACTWIN available at http://www.uga.edu/~strata/software.html. UniFrac computational analysis was performed to compare libraries from different sites following the software instructions (Lozupone & Knight, 2005). Bacterial communities from individual sites were clustered by application of the UPGMA method to the UniFrac metric matrix. Principal components analysis was performed with UniFrac metric matrix.

Nucleotide sequence accession numbers

The 101 16S rRNA gene sequences were submitted to the GenBank database under accession numbers EF589963–EF590063.

Results and discussion

Nitrobenzene residues and nitrobenzene degradation activity in the sediment

The concentrations of nitrobenzene residues in sediment and water samples about 40 days after the nitrobenzene pollution accident are shown in Table 1. Nitrobenzene was only detected in sediments and water samples from sites B and C, demonstrating that nitrobenzene might be degraded in sediments. This was further confirmed by the results of a batch anaerobic incubation experiment at 10°C (Zonglai et al., 2008). Nitrobenzene removal rates of contaminated site B and intermediate downstream site C were found to be higher (0.083–0.101 mg L⁻¹ h⁻¹) than those of downstream sites D and E (0.050–0.057 mg L⁻¹ h⁻¹). Nitrobenzene (11 mg L⁻¹) was almost completely removed in the sediments from sites B–E in 8 days with nitrosobenzene and aniline as the main degradation intermediates. In contrast, the removal rate in reference site A was remarkably low and complete removal needed more than 30 days. The enhanced nitrobenzene-removing abilities for the sediments of contaminated site B and downstream sites C–E could be due to the influence of nitrobenzene pollution.

DGGE profile of 16S rRNA gene fragments

In this study, the change of bacterial community along river sediments was elucidated by the DGGE phylotype. As shown in Fig. 1, DGGE bands 1–5 were detected in all profiles, and several band positions were unique for some sampling sites, such as bands 6 and 7 for A, bands 8 and 9 for B and bands 10 and 11 for C and D. Meanwhile, the intensities of several bands like 1 and 3 in B, C and D were stronger than those in A, indicating that the bacterial groups represented by these bands possibly played an important role in nitrobenzene biodegradation.

DGGE patterns were compared with one another by pairwise similarity coefficient of Dice (\( S \)), which ranged from 0.551 to 1.000. Cluster analysis demonstrated that band patterns of C, D and E were closely related to one another using the unweighted-pair group method using average linkages (UPGMA).
another, with $S$ values of 0.829–0.838 and the pattern of B was similar, to some extent, to C, D and E ($S$ values, 0.680–0.688) and significantly different from A ($S$ value, 0.551). These results indicate that, in comparison with reference site A, bacterial communities in contaminated site B and downstream sites C–E retained some common characteristics that were possibly due to the influence of nitrobenzene pollution.

16S rRNA gene clone libraries

Furthermore, to determine the detailed compositions of bacterial communities in sediments, five 16S rRNA gene clone libraries were constructed and c. 30–40 clones were processed by RFLP for each library; at least one clone of each pattern was sequenced. After discarding three possible chimeras and one sequence of poor quality, 101 sequences of c. 1400 nucleotides were acquired (23 for A, 15 for B, 24 for C, 23 for D and 16 for E) and 88 OTUs were determined.

Using UniFrac metric analysis, clones of downstream sites C–E as well as contaminated site B were found to be significantly different from those clones of reference site A, consistent with the results of DGGE cluster analysis (Fig. 2). Coverage values ($C$) and Shannon diversity indexes ($H$) were calculated for five bacterial clone libraries. Both results indicated that the sequence populations from reference site A and intermediate downstream site C were more diverse than those from contaminated site B and downstream sites D and E. This was further confirmed by higher richness ($S_{\text{Chao1}}$ and $S_{\text{ACE}}$) and evenness of sites A and C (Table 2) and was consistent with the DGGE band profile as shown in Fig. 1. However, as rarefaction curves did not reach saturation (data not shown), the clone number for each sample was not sufficient and may affect the index values. Considering that intermediate downstream site C was adjacent to reference site A, these statistical indexes, together with the DGGE and UniFrac metric analysis, demonstrated that the

![Fig. 1. DGGE profiles of PCR-amplified 16S rRNA gene segments from DNA extracted from Songhua River A–E sediments shown on the left, with a dendrogram generated by UPGMA cluster analysis comparison of DGGE patterns on the right.](image)

![Fig. 2. Principal components analysis performed using UniFrac metric based on all 16S rRNA gene sequences from Songhua River A–E sediments shown on the left (Components 1, representing 16S rRNA gene sequences; Components 2, representing sampling sites), with bacterial community (A–E) clustering also using the UPGMA method to UniFrac metric shown on the right.](image)

| Sample | No. of clones screened | No. of OTUs | $S_{\text{Chao1}}$ | $S_{\text{ACE}}$ | Shannon index | Evenness index | % Coverage |
|--------|------------------------|-------------|------------------|------------------|----------------|----------------|------------|
| A      | 36                     | 23          | 21               | 23               | 2.66           | 0.85           | 61         |
| B      | 33                     | 14          | 13               | 14               | 2.22           | 0.84           | 78         |
| C      | 37                     | 22          | 22               | 23               | 2.57           | 0.83           | 59         |
| D      | 34                     | 14          | 13               | 14               | 2.10           | 0.80           | 76         |
| E      | 32                     | 15          | 15               | 15               | 2.16           | 0.80           | 69         |

*Coverage and diversity indexes were calculated based on 97% similarity cutoff for OTU.*

Table 2. Coverage and diversity indexes of bacterial 16S rRNA gene clone libraries for sediments of Songhua River

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nitrobenzene spill leads to a decrease in bacterial diversity in polluted river sediments.

**Phylogeny of bacteria in sediments**

All bacteria from the cloned libraries were classified into the phyla *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Gemmatacomonadetes* and *Verrucomicrobia* (Figs 3 and 4), all of which were commonly detected in freshwater sediment environments as reported previously (Tamaki et al., 2005; Wilms et al., 2006; Winter et al., 2007). Clones belonging to *Proteobacteria*, *Bacteroidetes*, and *Acidobacteria* were present in all river sites. These phyla have been usually identified as...
dominant groups in sediment environments and were generally important contributors to biogeochemical processes (Barns et al., 1999; Spring et al., 2000).

The clones from reference site A were mainly grouped into the classes *Acidobacteria*, *Alphaproteobacteria* and *Actinobacteria*. The most notable characteristics for clones of contaminated and downstream sites B–E were the predominance of *Betaproteobacteria* and *Firmicutes*. Until now, many reported nitrobenzene-degrading bacteria are *Comamonadaceae* of the class *Betaproteobacteria* (Groenewegen & de Bont, 1992; Schenzle et al., 1999; Zhao & Ward, 1999). The clones belonging to *Comamonadaceae* were indeed similar to the reported nitroaromatic compounds-utilizing strains. As listed in Table 3, clone OTU B24 from contaminated site B was 99.6–99.8% similar to the nitrobenzene-degrading *Acidovorax* sp. strain JS42 (Lessner et al., 2003) and *p*-chloroaniline-degrading *Diaphorobacter* sp. strain PCA039. Another OTU B02 showed 97–98% similarities to several strains of *Comamonas* sp. including phenol or benzoic acid-degrading PND-3, PP3-1 (accession no. EU276094) and

**Fig. 4.** Phylogenetic relationships of the other representative bacterial 16S rRNA gene sequences from clone libraries A–E as determined by the neighbor-joining method. Bootstrap values of >50% (obtained with 1000 resamplings) are shown at the nodes. The scale bar indicates 0.05 nucleotide substitution per site.

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| Sampling site | Type clone* | Other representative(s)1 | No. of clones1 | Phylogenetic cluster | Related neighbor (% similarity) | Accession no. | Comments |
|---------------|-------------|--------------------------|----------------|----------------------|--------------------------------|---------------|----------|
| A             | A17         | A25, A16                 | 6              | Alphaproteobacteria  | Sphingomonas sp. Amb_16S_1187 (98.5) | EF018731     |          |
|               | A22         | 1                        | 1              | Betaproteobacteria   | Aquabacterium sp. B1_52R (97.2) | AM157286     |          |
|               | A21         | A01, A11, A02            | 6              | Actinobacteria       | Pseudonocardia yunnanensis (98.3) | D85472       |          |
|               | A23         | A09, A10                 | 5              | Bacteroidetes        | Flavobacterium bacterium FJS32 (98.6) | AM157286     |          |
|               | A13         | A07, A06, A03, A08, A15  | 10             | Acidobacteria        | Uncultured Acidobacteriaceae bacterium FCPS449 (97.7) | EF516744     |          |
|               | A12         | 2                        | 2              | Chloroflexi          | Anaerolinea sp. AKYH1185 (93.2) | AY921706     |          |
|               | A19         | 1                        | 1              | Gemmatimonadetes     | Gemmatimonas sp. JG34-KF-418 (98.8) | AJ352278     |          |
|               | A18         | 1                        | 1              | Planctomycetes       | Uncultured Planctomycetes Elev._16S_938 (90.3) | EF019757     |          |
| B             | B02         | B20, B06                 | 11             | Betaproteobacteria   | Comamonas sp. PND-3 (98.0) | DQ301784     |          |
|               | B24         | 3                        | 3              | Betaproteobacteria   | Acidovorax sp. JS42 (99.6) | CP000539     |          |
|               | B07         | 1                        | 1              | Gammaproteobacteria  | Pseudomonas stutzeri (98.0) | EF530571     |          |
|               | B01         | B11                      | 2              | Deltaproteobacteria  | Uncultured bacterium clone ZZ14C2 (98.1) | AY214190     |          |
|               | B03         | B14                      | 5              | Firmicutes           | Soehngenia saccharolytica (96.2) | AY353956     |          |
|               | B22         | B04                      | 2              | Actinobacteria       | Corexibacter sp. AKYH1301 (98.2) | AY921795     |          |
|               | B15         | B19                      | 7              | Bacteroidetes        | Uncultured Rikenellaceae bacterium (98.3) | AJ249109     |          |
|               | B23         | 2                        | 2              | Chloroflexi          | Anaerolinea sp. 23e08 (89.1) | EF515373     |          |
| C             | C19         | 1                        | 1              | Alphaproteobacteria  | Uncultured Hypomicrobiaceae clone D10_29 (95.0) | EU266798     |          |
|               | C02         | 3                        | 3              | Betaproteobacteria   | Rhodocyclusaceae clone PYR10d2 (94.0) | DQ123667     |          |
|               | C03         | C12, C14, C22            | 9              | Betaproteobacteria   | Rhodofex sp. 50 (98.6) | AY250098     |          |
|               | C18         | 1                        | 1              | Gammaproteobacteria  | Acinetobacter sp. PHD-4 (99.0) | DQ227342     |          |
|               | C01         | C16                      | 4              | Firmicutes           | Clostridium sp. M09_Pitesti (99.5) | DQ378230     |          |
|               | C04         | 1                        | 1              | Actinobacteria       | Uncultured bacterium clone ANTLV2_D07 (99.2) | DQ521512     |          |
|               | C09         | C11, C06, C17, C10       | 9              | Bacteroidetes        | Flavobacterium sp. R-7550 (98.6) | AJ440979     |          |
|               | C24         | 2                        | 2              | Acidobacteria        | Acidobacteriaceae bacterium Elev._16S_476 (96.5) | EF019289     |          |
|               | C05         | C21, C15, C23, C25       | 6              | Chloroflexi          | Anaerolinea sp. 23e08 (95.1) | EF515373     |          |
| D             | D10         | 2                        | 2              | Betaproteobacteria   | Rhodobacter sp. ARK10036 (91.3) | AF468358     |          |
|               | D24         | D04, D05, D22            | 11             | Betaproteobacteria   | Propionibivrio limicola (96.7) | AJ307983     |          |

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Several sequences in the class Gammaproteobacteria from contaminated site B and downstream sites C–E also showed similarities to the strains able to utilize nitroaromatic compounds. For example, the sequence C18 from intermediate downstream site C was related to phenol-degrading Acinetobacter sp. strain PHD-4 and B07 from contaminated site B was similar to 1,2,4-trichlorobenzene-degrading Pseudomonas stutzeri strain T7 (98%). In addition to Comamonadaceae of the class Betaproteobacteria, numerous Pseudomonas spp. strains have also been reported to be capable of degrading nitrobenzene (Nishino & Spain, 1993). However, in this study, only one sequence B07 from contaminated site B was related to Pseudomonas spp.

It is interesting that many clone sequences were also similar to those obtained from cold environments. For example, the OTU A23 in the Bacteroidetes showed similarity to the bacterium FJS32 (96.8%) isolated from subglacial sediments in Southern Hemisphere glaciers (Foght et al., 2004), two OTU sequences C09 and D15 in this phylum were closely related to Antarctic bacterium R-7550 (97.3–98.5%), and two OTU sequences C03 and C12 in the

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**Table 3. Continued.**

| Sampling site | Type clone* | Other representative(s)† | No. of clones‡ | Phylum cluster | Related neighbor (% similarity) | Accession no. | Comments |
|---------------|-------------|--------------------------|----------------|---------------|-------------------------------|----------------|----------|
| D17           | 1           | Gammaproteobacteria      |                | Xanthomonadaceae bacterium CYCU-0215 (94.2) | DQ232379 | Derived from oil polluted soil |
| D25           | D19, D08, D02 | 17                       | Firmicutes     | Clostridium sp. M09_Pitesti (97.1) | DQ378230 |
| D12           | 5           | Firmicutes               |                | Trichococcus sp. PL-189 (99.3) | AY570584 | Derived from a low-temperature biodegraded oil reservoir |
| D15           | D09         | 2                        | Bacteroidetes  | Flavobacterium bacterium (98.5) | AJ440979 | Isolated from Antarctic lake |
| D16           | 1           | Acidobacteria            |                | Uncultured Acidobacteria bacterium (92.7) | AY510254 |
| E             | E12         | 11                       | Betaproteobacteria | Betaproteobacteria bacterium GOUTA12 (97.9) | AY050584 | Derived from monochlorobenzene contaminated groundwater |
| E10           | E04         | 6                        | Deltaproteobacteria | Geobacter sp. M20_Pitesti (95.4) | DQ378240 | Derived from oil polluted soil |
| E07           | 1           | Firmicutes               |                | Acetivibrio sp. RA13C3 (94.9) | AF407403 | Derived from monochlorobenzene contaminated groundwater |
| E24           | 1           | Bacteroidetes            |                | Bacteroidetes clone SB-5 (91.2) | AF029041 |
| E09           | E16,        | 4                        | Acidobacteria  | Acidobacteriaceae bacterium BS073 (98.3) | AB240251 |
| E01           | E02, E14    | 7                        | Chloroflexi    | Uncultured bacterium clone M30_Pitesti (97.2) | DQ378250 | Derived from oil polluted soil |
| E23           | 1           | Verrucomicrobia          |                | Verrucomicrobius sp. Ellin511 (96.4) | AY960774 |

*The type clone was mainly used in phylogenetic analysis.
†Related OTU sequences.
‡Clones represented by type clone and additional related OTUs sequences.

BJS-Z-2 (accession no. EF061933). Two OTUs (C03 and C12) from immediate downstream site C were related (95–97%) to Rhodoferax sp. strain 50 (Jeon et al., 2003) and Polaromonas sp. strain CJ2 responsible for naphthalene biodegradation. A high similarity has been observed between nitrobenzene and naphthalene degradation gene clusters (Lessner et al., 2002). These results indicated that the genera Comamonas, Acidovorax, Diaphorobacter, Rhodoferax and Polaromonas of the family Comamonadaceae possibly played an essential role in nitrobenzene degradation of this study.

Sequences of the phylum Firmicutes were mostly classified into Clostridium spp. and showed a relationship with Clostridium acetobutylicum and Clostridium thermoacetica. It has been reported that C. acetobutylicum and C. thermoacetica could reduce the nitro group of nitroaromatics to the corresponding amines (Rafii et al., 1991; Gorontzy et al., 1993; Khan et al., 1997; Huang et al., 2000). These results suggest that Clostridium may play a role in reduction of the nitro group of nitrobenzene in polluted sediments of this study.
class Betaproteobacteria were related to Rhodoferax spp. clones BFM 9H and KAR67 derived beneath a high Arctic glacier (Van Trappen et al., 2002; Cheng & Foght, 2007; Hansen et al., 2007). These results might be related to the fact that the river is covered by ice for nearly 5 months annually and the sediment temperature was below 4 °C at the time of sampling, suggesting that cold environments favor the growth and survival of specific organisms on a worldwide basis.

The results of this study indicated that the nitrobenzene spill has significantly influenced the bacterial communities in the river sediments. Strains belonging to Comamonadaceae and Clostridium spp. may play a role in degradation and detoxification of nitrobenzene in contaminated river environments.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389–3402.

Barns SM, Takala SL & Kuske CR (1999) Wide distribution and diversity of members of the bacterial kingdom Acidobacterium in the environment. *Appl Environ Microbiol* 65: 1731–1737.

Broff JE, McArthur JV & Shimkets LJ (2002) Recovery of novel bacterial diversity from a forested wetland impacted by reject coal. *Environ Microbiol* 4: 764–769.

Cheng SM & Foght JM (2007) Cultivation-independent and - dependent characterization of bacteria resident beneath John Evans Glacier. *FEMS Microbiol Ecol* 59: 318–330.

Cole JR, Chai B, Farris RJ, Wang Q, Kulam-Syed-Mohideen AS, McGarrell DM & Bandela AM (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res* 35: D169–D172.

Coell RW (2005) EstimateS: Statistical estimation of species richness and shared species from samples. Version 8.0. User’s Guide and application. http://purl.oclc.org/estimates.

Dickel O, Haug W & Knackmuss HJ (1993) Biodegradation of nitrobenzene by a sequential anaerobic-aerobic process. *Biodegradation* 4: 187–194.

Foght J, Aislalbie J, Turner S, Brown CE, Ryburn J, Saul DJ & Lawson W (2004) Culturable bacteria in subglacial sediments and ice from two Southern Hemisphere glaciers. *Microb Ecol* 47: 329–340.

Gorontzy T, Käver J & Blotevogel KH (1993) Microbial transformation of nitroaromatic compounds under anaerobic conditions. *J Gen Microbiol* 139: 1331–1336.

Groenewegen PEJ & de Bont JAM (1992) Degradation of 4-nitrobenzoate via 4-hydroxylaminobenzoate and 3,4-dihydroxybenzoate in *Comamonas acidovorans* NBA-10. *Arch Microbiol* 158: 381–386.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41: 95–98.

Hansen AA, Herbert RA, Mikkelsen K, Jensen LL, Kristoffersen T, Tiedje JM, Lomstein BA & Finster KW (2007) Viability, diversity and composition of the bacterial community in a high Arctic permafrost soil from Spitsbergen, Northern Norway. *Environ Microbiol* 9: 2870–2884.

Hobel CFV, Marteinsson VT, Hreggvidsson GÓ & Kristjánsson JK (2005) Investigation of the microbial ecology of intertidal hot springs by using diversity analysis of 16S rRNA and chitinase genes. *Appl Environ Microbiol* 71: 2771–2776.

Huang S, Lindahl PA, Wang C, Bennett GN, Rudolph FB & Hughes JB (2000) 2,4,6-Trinitrotoluene reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. *Appl Environ Microbiol* 66: 1474–1478.

Huber T, Faulkner G & Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* 20: 2317–2319.

Jeon CO, Park W, Padmanabhan P, DeRito C, Snape JR & Madsen EL (2003) Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ biodegradation in contaminated sediment. *Proc Natl Acad Sci USA* 100: 13591–13596.

Khan TA, Bhadra R & Hughes J (1997) Anaerobic transformation of 2,4,6-TNT and related nitroaromatic compounds by *Clostridium acetobutylicum*. *J Ind Microbiol Biotechnol* 18: 198–203.

Kumar S, Tamura K & Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics Analysis and sequence alignment. *Brief Bioinform* 5: 150–163.

Lessner DJ, Johnson GR, Parales RE, Spain JC & Gibson DT (2002) Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. *Appl Environ Microbiol* 68: 634–641.

Lessner DJ, Parales RE, Narayan S & Gibson DT (2003) Expression of the nitroaromatic dioxygenase genes in *Comamonas* sp. strain JS765 and *Acidovorax* sp. strain JS42 is induced by multiple aromatic compounds. *J Bacteriol* 185: 3895–3904.

Lipson DA & Schmidt SK (2004) Seasonal changes in an alpine soil bacterial community in the Colorado Rocky Mountains. *Appl Environ Microbiol* 70: 2867–2879.

Lozupone C & Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 71: 8228–8235.

Marvin-Sikkema FD & de Bont JAM (1994) Degradation of nitroaromatic compounds by microorganisms. *Appl Microbiol Biotechnol* 42: 499–507.
Muyzer G, Waal ECD & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59: 695–700.

Nishino SF & Spain JC (1993) Degradation of nitrobenzene by a Pseudomonas pseudoalcaligenes. Appl Environ Microbiol 59: 2520–2525.

Nishino SF & Spain JC (1995) Oxidative pathway for the biodegradation of nitrobenzene by Comamonas sp. strain JS765. Appl Environ Microbiol 61: 2308–2313.

Padda RS, Wang C, Hughes JB, Kutty R & Bennett GN (2003) Mutagenicity of nitroaromatic degradation compounds. Environ Toxicol Chem 22: 2293–2297.

Parales RE, Huang R, Yu CL, Parales JV, Lee FKN & Lessner DJ (2005) Purification, characterization, and crystallization of the components of the nitrobenzene and 2-nitrotoluene dioxygenase enzyme systems. Appl Environ Microbiol 71: 3806–3814.

Rafii F, Franklin W, Heßlich RH & Cerniglia CE (1991) Reduction of nitro-aromatic compounds by anaerobic bacteria isolated from the human gastrointestinal tract. Appl Environ Microbiol 57: 962–968.

Schenzle A, Lenke H, Spain JC & Knackmuss HJ (1999) Chemoselective nitro group reduction and reductive dechlorination initiate degradation of 2-chloro-5-nitrophenol by Ralstonia eutropha JMP134. Appl Environ Microbiol 65: 2317–2323.

Schloss PD & Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71: 1501–1506.

Singleton DR, Furlong MA, Rathbun SL & Whitman WB (2001) Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl Environ Microbiol 67: 4374–4376.

Somerville CC, Nishino SF & Spain JC (1995) Purification and characterization of nitrobenzene nitroreductase from Pseudomonas pseudoalcaligenes JS45. J Bacteriol 177: 3837–3842.

Spain JC (1995) Biodegradation of nitroaromatic compounds. Annu Rev Microbiol 49: 523–555.

Spring S, Schulze R, Overmann J & Schleifer K-H (2000) Identification and characterization of ecologically significant prokaryotes in the sediment of freshwater lakes: molecular and cultivation studies. FEMS Microbiol Rev 24: 573–590.

Tamaki H, Sekiguchi Y, Hanada S, Nakamura K, Nomura N, Matsumura M & Kamagata Y (2005) Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. Appl Environ Microbiol 71: 2162–2169.

Thompson JD, Gibson TJ, Plewniak F, Jeanmoign F & Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882.

Tsai YL & Olson BH (1991) Rapid method for direct extraction of DNA from soil and sediments. Appl Environ Microbiol 57: 1070–1074.

U.S. Environmental Protection Agency (2006) Water Quality Standards Database, 20 January. http://oaspub.epa.gov/wqsdatabase/wqsi_epa_criteria.rep_parameter.

Van Trappen S, Mergaert J, Van Eygen S, Dawydnt P, Cnockaert MC & Swings J (2002) Diversity of 746 heterotrophic bacteria isolated from microbial mats from ten Antarctic lakes. Syst Appl Microbiol 25: 603–610.

Webster G, Parkes RJ, Fry JC & Weightman AJ (2004) Widespread occurrence of a novel division of bacteria identified by 16S rRNA gene sequences originally found in deep marine sediments. Appl Environ Microbiol 70: 5708–5713.

Wilms R, Sass H, Köpke B, Köster J, Cyponka H & Engelen B (2006) Specific bacterial, archaeal, and eukaryotic communities in Tidal-Flat sediments along a vertical profile of several meters. Appl Environ Microbiol 72: 2756–2764.

Winter C, Hein T, Kavga M, Mach RL & Farnleitner AH (2007) Longitudinal changes in the bacterial community composition of the Danube River: a whole-river approach. Appl Environ Microbiol 73: 421–431.

World Health Organization Department for Health Action (2005) Health Action in Crises Highlights No 84: 21, November to 4 December. www.who.int/entity/hac/donorinfo/highlights/2005/28Nov_4Dec05_weeklyhighlights.pdf.

Wu JF, Jiang CY, Wang BJ, Ma YF, Liu ZP & Liu SJ (2006) Novel partial reductive pathway for 4-chloronitrobenzene and nitrobenzene degradation in Comamonas sp. strain CNB-1. Appl Environ Microbiol 72: 1759–1765.

Zhao JS & Ward OP (1999) Microbial degradation of nitrobenzene and mono-nitrophenol by bacteria enriched from municipal activated sludge. Can J Microbiol 45: 427–432.

Zhao JS & Ward OP (2001) Substrate selectivity of a 3-nitrophenol-induced metabolic system in Pseudomonas putida 2NP8 transforming nitroaromatic compounds into ammonia under aerobic conditions. Appl Environ Microbiol 67: 1388–1391.

Zonglai L, Min Y, Dong L & Rong Q (2008) Simulation study about nitrobenzene biodegradation by microbes from multi-sites of Songhua River after the nitrobenzene spill accident. J Environ Sci 27: 778–786.