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Identification and characterization of *o*-xylene-degrading *Rhodococcus* spp. which were dominant species in the remediation of *o*-xylene-contaminated soils

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Abstract

Soils contaminated with *o*-xylene were more difficult to bioremediate than those contaminated with other BTEX hydrocarbons (benzene, toluene, ethylbenzene, *m*-xylene and *p*-xylene). In order to identify microorganisms responsible for *o*-xylene degradation in soil, microbial community structure analyses were carried out with two soil samples in the presence of *o*-xylene and mineral nutrients. In two different soil samples, *Rhodococcus opacus* became abundant. We were also able to isolate *o*-xylene degrading *Rhodococcus* species from these soil samples. A primer set was developed to specifically detect a cluster of this *Rhodococcus* group including isolated *Rhodococcus* strains, *Rhodococcus opacus* and *Rhodococcus koreensis*. The growth of this bacterial group in an *o*-xylene-contaminated soil was followed by competitive PCR (cPCR). The decrease in *o*-xylene clearly paralleled the growth of the *Rhodococcus* group.

Introduction

Petroleum fuels are toxic compounds, and their toxicity is mainly caused by highly soluble and volatile BTEX component. These BTEX are common contaminants in groundwater and in soil associated with petroleum product releases (Cavalca et al. 2000; Mattison et al. 2005; Nicholson & Fathepure 2004). EPA reported that many polluted sites were contaminated by BTEX (U.S. EPA 1997). It is thus important to develop methods to accelerate the removal of these volatile components. Bioremediation is one useful technique to reduce the level of pollution from these compounds in soil.

Although bacteria capable of degrading *m*-xylene or *p*-xylene under aerobic conditions are common, and the catabolic pathways for these compounds have been well documented (Galli et al. 1992; Harayama et al. 1992; Williams & Sayers 1994), bacteria capable of degrading *o*-xylene were less frequently reported (Kim et al. 2002). Only a few bacteria that metabolize *o*-xylene have been isolated so far, and include *Nocardia* sp. (Gibson & Subramanian 1984), *Rhodococcus* (*Corynebacterium*) sp. C125 (Schraa et al. 1987; van der Meer et al. 1992), *Rhodococcus* sp. B3 (Bickerdike et al. 1997), *Rhodococcus opacus* R7 (Di Gennaro et al. 2001), *Rhodococcus* sp. DK17 (Kim et al. 2002, 2004) and *Pseudomonas stutzeri* OX1 (Baggi et al. 1987; Vardar & Wood 2004). However, it is unclear if these strains are important primarily for the *in situ* biodegradation of *o*-xylene in natural environments.

We were thus interested in identifying major *o*-xylene degraders in soil. Studies to identify and

characterize microorganisms that take an active part in the biodegradation of pollutants in natural environments have already been conducted. Hanson et al. (1999) have reported that either *Rhodococcus* sp. YT-2 or related high G + C gram-positive strains, but not well-characterized toluene degraders such as *Pseudomonas putida*, are mainly involved in the degradation of toluene in soil. *Alcanivorax* spp. and *Cycloclasticus* spp., respectively, have been shown to be major players in the degradation of alkanes and aromatic hydrocarbons in oil-contaminated marine environments (Kasai et al. 2002a, b; Syutsubo et al. 2001). The competitive ability of these organisms may enable them to persist and survive in such polluted environments.

In this study, we identified bacteria that are responsible to the *in situ* activity to degrade *o*-xylene. Furthermore, we isolated such bacteria, and demonstrated that these were closely related to *Rhodococcus opacus*.

Materials and methods

Soils

Sandy soil (called KN soil) was collected from the top 50 cm of a riverside field (Kamaishi, Iwate, Japan). Trichloroethylene (TCE) -contaminated soil (called CH soil) was obtained from a TCE-contaminated sandy aquifer at a depth between 2.0 and 2.5 m (Kimitsu, Chiba, Japan). The TCE concentration in the aquifer has been reported to be between 100 and 500 $\mu g\ l^{-1}$, though TCE was not detected when experiments were conducted with the CH soil. There is no evidence that both soils have a history of BTEX contamination. Soils were passed through a 2-mm sieve, and stored in the dark at 4 °C. The total contents of organic matter of the KN and CH soils were 2.7% and 3.0%, respectively, while the moisture of those soils expressed as percent water by weight was 7.0% and 19.2%, respectively.

Media

Mineral salts (MS) medium used for *o*-xylene degrading experiment contained ($g\ l^{-1}$): KH_2PO_4 (0.8), Na_2HPO_4 (5.58), $(NH_4)_2SO_4$ (1.8), $MgSO_4{\cdot}7H_2O$ (0.123), and ($mg\ l^{-1}$): $FeSO_4{\cdot}7H_2O$ (0.5), $MnSO_4{\cdot}5H_2O$ (1.54), H_3BO_3 (2.86), $CuSO_4{\cdot}5H_2O$ (0.039), $ZnCl_2$ (0.021), $CoCl_2{\cdot}6H_2O$ (0.041), $Na_2MoO_4{\cdot}2H_2O$ (0.025), $CaCl_2{\cdot}2H_2O$ (11.6). Tryptic soy broth (TSB: Difco, MD, USA) was used for isolation of soil bacteria.

Gas chromatography

Analysis of volatile aromatic compounds (BTEX) by headspace gas chromatography was performed using GC17A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and fitted with a capillary column (DB-624, 30 m×0.53 mm, J&W Scientific, CA, USA). The injector and detector temperatures were 120 °C and 160 °C, respectively. The temperature program gave a 3-min hold at 90 °C, an increase to 100 °C at 10 °C /min, and a 1 min hold at 100 °C.

Biodegradation of BTEX in soil

All experiments were conducted in 120-ml glass vials sealed with Teflon-coated butyl rubber septa and aluminum caps. Vials were dispensed with 7.0 g of the KN soil (6.5 g of dry weight) and 2.0 ml of MS medium, or 8.0 g of the CH soil (6.5 g of dry weight) and 1.0 ml of MS medium. An appropriate amount of volatile compounds (200 μM of benzene, toluene, ethylbenzene, *m*-xylene and *p*-xylene; or 50, 200 and 1000 μM of *o*-xylene; the concentration of BTEX was calculated based on the water content in each vial: 2.5 ml) was also supplemented in the vials. Sterilized soils (121 °C, 20 min) were used in control experiments. Vials were rotary shaken (20 °C, 90 rpm) and the concentration of BTEX was measured with 50 μl of gas sample. All biodegradation experiments were made in duplicates.

Extraction of DNA from soil samples

Five hundred milligrams of each soil sample were mixed with 50 μl of 0.1 M phosphate buffer (93.2 mM Na_2HPO_4, 6.8 mM NaH_2PO_4) containing yeast RNA ($40\ gl^{-1}$; Roche diagnostics, Basel, Switzerland). Six hundred μl of 0.05 M CH_3COONH_4 (pH 7.5) and 400 μl of 0.4% skim milk (Wako Pure Chemical Industries, Osaka, Japan) were then added, and the mixture was treated by gentle ultrasonic dispersion. After addition of 40 mg of lysozyme, the sample was

incubated for 30 min at 37 °C with shaking (100 strokes min^{-1}), then 150 μl of 20% SDS was added. The mixture was again vortexed and incubated for 30 min at 65 °C. After three cycles of freeze-thaw treatment, the suspension was centrifuged at 15,000×*g* for 10 min, and the aqueous solution was recovered. To the solution, 250 μl of 7.5 M CH_3COONH_4 (pH 7.5) was added on ice, and after 5 min, the suspension was centrifuged at 15,000×*g* for 10 min to recover an aqueous solution. Subsequently, 0.8 volume of 2-propanol was added to the aqueous solution, and after gentle mixing, the solution was incubated at room temperature for 2 h. Nucleic acids were precipitated by centrifugation at 15,000×*g* for 10 min, and resuspended in 250 μl of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Next, the suspension was extracted one or two times with phenol-chloroform solution (Sambrook et al. 1989) before 2.5 volumes of ethanol and 0.04 volume of 5 M NaCl were added. The solution was incubated at −80 °C for 30 min, and nucleic acids were precipitated by centrifugation at 15,000×*g* for 20 min. The preparation was washed with 1 ml of a 70% ethanol solution, and the nucleic acids were dissolved in 30 μl of TE buffer containing 100 μg ml^{-1} of RNase to incubate for 1 h at 40 °C. DNA was precipitated by centrifugation after adding 0.8 volume of isopropanol, washed in 70% ethanol, and dissolved in 30 μl of TE.

DGGE

Primers P2 and P3 (containing a 40-bp GC clamp; Muyzer et al. 1993) were used to amplify the variable V3 region of bacterial 16S rDNA (corresponding to positions 341 to 534 in the *Eschrichia coli* sequence). PCR was performed as described previously (Watanabe et al. 1998). DGGE was performed with a Dcode™ instrument (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions. Gels were made using a gradient of denaturants between 35% (containing 2.45 M urea and 14% formamide) and 55% (containing 3.85 M urea and 22% formamide). Then, 10 μl of the PCR-amplified mixture was subjected to electrophoresis in 10% (w/v) polyacrylamide gel at 200 V for 3.5 h at a running temperature of 58 °C. After the electrophoresis, the gel was stained with SYBR Green I (FMC Bioproducts, PA, USA) for 30 min according to the manufacturer's instructions. To determine a DNA sequence, a gel slice containing a DNA band was excised, and processed as described previously (Watanabe et al. 1998). A search of the GenBank nucleotide library for sequences similar to the sequences obtained was performed by using BLAST (Altschul et al. 1990) through the website of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Isolation of bacteria from soil

One gram of soil exhibiting active *o*-xylene biodegradation was suspended in 10 ml of sterilized water, and after the dispersion of bacteria in soil by a vortex mixing and a gentle ultrasonic treatment, it was appropriately diluted with sterilized distilled water. The diluted soil suspensions described above were spread on agar plates containing one-tenth-strength tryptic soy broth medium (1/10 TSB). After the plates have been incubated at 25 °C for 14 days, colonies developed on the plates were picked and purified by restreaking. The *o*-xylene-degradability of isolates has been determined as follows. Vials were dispensed with 10 ml of MS medium and 50 μM of *o*-xylene. For inoculation, individual strains were grown on 1/10 TSB plates and transferred to the vials using a sterile loop. Vials were sealed with Teflon-coated butyl rubber septa and aluminum cap. Vials were shaken (20 °C, 90 rpm) for 7 days and residual concentration of *o*-xylene was measured as described above. Isolates were also spread on agar plates containing only MS medium with vapor of *o*-xylene. After the plates have been incubated at 25 °C for 7 days, colonies developed on the plates were observed.

Sequencing of 16S rDNA and gyrB *gene of isolated bacteria and phylogenetic analysis*

Nucleotide sequences of 16S rDNA of isolated strains were amplified and determined by the methods described previously (Syutsubo et al. 2001). The nucleotide sequences obtained were aligned by using CLUSTAL W, version 1.7 (Thompson et al. 1994), with several reference sequences of members of the genus *Rhodococcus*. A phylogenetic tree was constructed from the evolutionary distance data (Kimura 1980) by the neighbor-joining method (Saitou & Nei 1987).

The bootstrap resampling method of Felsenstein (Felsenstein 1985) was used with 1000 replicates to evaluate the robustness of the branches of the inferred tree.

Fragments of the gene for the DNA gyrase β subunit (*gyrB*) of isolates were amplified, purified, and sequenced by the methods described previously (Kasai et al. 2002a; Yamamoto & Harayama 1995). The *gyrB* sequences were aligned by using CLUSTAL W, version 1.7, with several reference sequences of members of the genus *Rhodococcus* compiled in the Identification and Classification of Bacteria database (Kasai et al. 1998). A phylogenetic tree based on *gyrB* was constructed as described above for the 16S rDNA-based tree.

Competitive PCR for enumeration of o*-xylene degrading* Rhodococcus *in soil*

Twenty vials were dispensed with 7.0 g of the KN soil, 2.0 ml of MS medium and 200 μM of *o*-xylene. Vials were shaken (20 °C, 90 rpm) and the concentration of BTEX was measured with 50 μl of gas sample. One or two vials were taken periodically to estimate the population size of *Rhodococcus* bacteria in soil samples using competitive PCR (cPCR) methods described below.

The primer RB6R (5′-GTACTTGTTGACCG TCGCC−3′) used for cPCR was designed from the sequence conserved among the *o*-xylene-degrading strains isolated in this study (TKN14, TKN45, TKN46, TCH4, and TCH14), *R. opacus* DSM 43205^T and *R. koreensis* JCM 10743^T in highly divergent *gyrB* sequences (Figure 1). *gyrB* gene is more suitable for design of the specific primers than 16S rRNA. Because, *gyrB*'s molecular evolution rate is higher than that of 16S rRNA. Furthermore, quantification of copy number of *gyrB* gene is useful for the analysis of bacterial population, since it may be a single copy on each genome (Yamamoto & Harayama 1996). The primer set, RB2F (5′-GAGATGGCCTTCCTC AAC-3′; non-specific primer) and RB6R, was used to specifically detect the *R. opacus* and *R. koreensis* group that included all the *o*-xylene-degrading isolates. Competitor fragments were produced by using a competitive DNA construction kit (Takara Shuzo). The sizes of the target and competitor fragments were 414 bp and 336 bp, respectively. Amplification was performed with a Promega thermal cycler (Techne, Staffordshire, UK) by using a 50-μl mixture containing 1.25 U of *Taq* DNA polymerase (Amplitaq Gold; Applied Biosystems, CA, USA), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, each deoxynucleoside triphosphate at a concentration of 200 μM, 50 pmol of each primer, 2 μg of bovine serum albumin, 1 μl of appropriate concentration of the template DNA extracted from soil and 1 μl of appropriate concentration of the competitor fragment. The PCR conditions were 10 min for activating the polymerase at 94 °C and then 40 cycles of 1 min at 94 °C, 1 min at 61 °C, and 2 min at 72 °C, and finally 10 min of

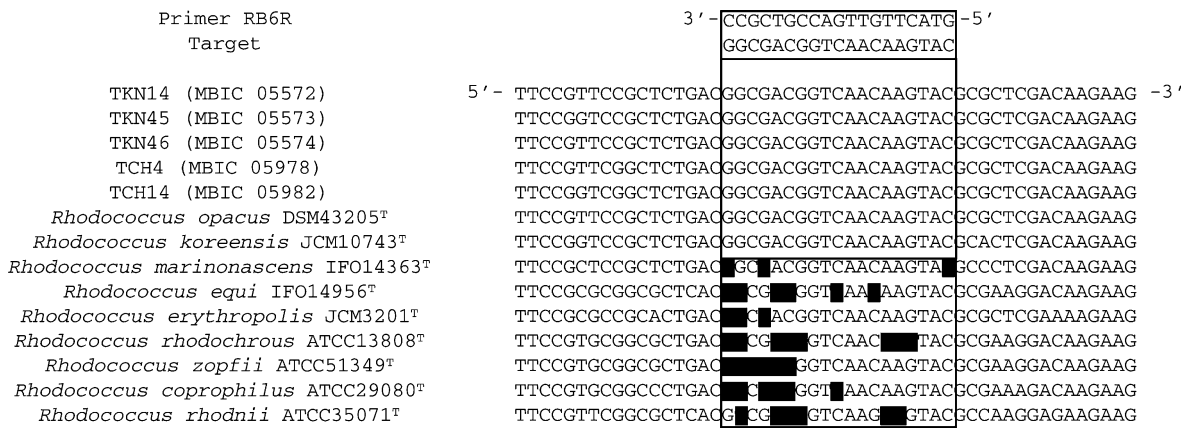

Figure 1. Sequence alignment of *gyrB* in the vicinity of the primer (RB6R) sequence. The primer- and target-site sequences are displayed in the uppermost box, the target-site sequence was conserved in all the *o*-xylene-degrading *Rhodococcus* in the second box, while mismatches were found in other *Rhodococcus* strains shown in the third box.

extension at 72 °C. The PCR products were separated by electrophoresis through 2.0% agarose gel, and stained with ethidium bromide. The band intensity was quantified by using image-processing software (NIH image, version 1.60; National Institutes of Health, MD, USA), and the copy number of a target sequence in the PCR mixture was determined by comparing the band intensity of the target fragment with that of the competitor. The number of bacterial cells was considered to be equal to the copy number of the *gyrB* sequence (Kasai et al. 2002a).

Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been deposited in DDBJ/EMBL/GenBank databases under the accession numbers AB183436 to AB183450.

Results and discussion

Biodegradation of BTEX in soil

Biodegradability of BTEX in soil was investigated using two different soil samples (KN soil, CH soil). The soil water content of the samples was adjusted to 38.5% (mass of liquid/mass of dry soil) by adding appropriate amount of MS medium to which 200 μM of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene or *p*-xylene were added. These soil samples were incubated at 20 °C with shaking, and the concentration of BTEX was measured using a headspace gas chromatography procedure (Figure 2). In general, the biodegradation of BTEX started after a lag period of several days. The lag periods were shortest for the degradation of toluene and *m*-xylene, followed by the degradation of benzene, ethylbenzene and *p*-xylene. The biodegradation of benzene, toluene, ethylbenzene, *m*-xylene and *p*-xylene was completed within 5 days in both soils, while it took a longer time to degrade *o*-xylene, namely 11 and 8 days in the KN and CH soils, respectively.

The biodegradation of *o*-xylene at different initial concentrations was also investigated (Figure 3). In the KN soil, it took 9 or 11 days for the complete biodegradation when the initial concentration of *o*-xylene was set at 50 μM or 200 μM, respectively, while it took 30 days when the initial concentration of *o*-xylene was 1000 μM. A similar tendency in the biodegradation of *o*-xylene was observed in the CH soil. From these results, we concluded that *o*-xylene is most recalcitrant among the BTEX compounds in both the KN and CH soils.

Analysis of bacterial community structure in soil before and after o-*xylene biodegradation, and the isolation of* o-*xylene degraders*

Changes in microbial community structure that accompanied the *o*-xylene biodegradation in soil samples were analyzed by using PCR-DGGE analysis. KN-soil samples before and after *o*-xylene degrading experiments at the different initial concentrations *o*-xylene were analyzed by DGGE. As a result, two major populations, bands A and B, became dominant due to the incubation with *o*-xylene and inorganic nutrients (Figure 4). Band A also appeared in KN-soil samples devoid

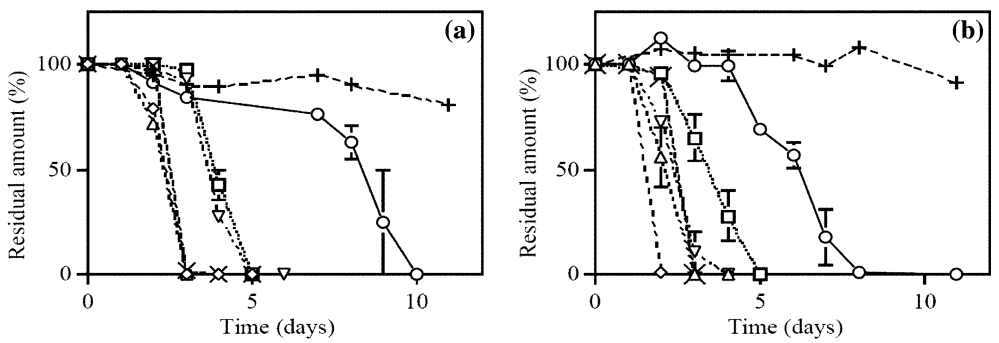

Figure 2. Biodegradation of BTEX hydrocarbons in KN soil and CH soil. Vials containing MS medium, KN soil (a) or CH soil (b), and 200 μM of benzene (□), toluene (Δ), ethylbenzene (×), *o*-xylene (○), *m*-xylene (◇), or *p*-xylene (▽), were incubated at 20 °C with shaking. Vials containing sterilized soil supplemented with 200 μM of *o*-xylene and MS was used as a control (+).

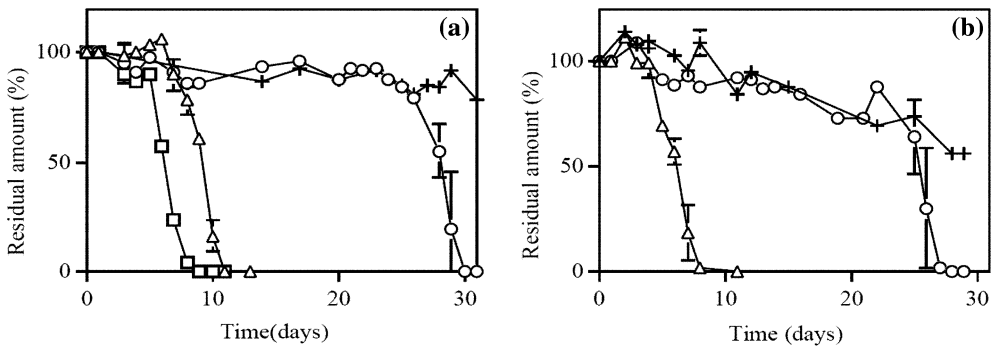

Figure 3. Biodegradation of *o*-xylene in KN soil and CH soil. Vials containing MS medium, KN soil (a) or CH soil (b), and 50 μM (□), 200 μM (Δ) or 1000 μM (○) of *o*-xylene, were incubated at 20 °C with shaking. Vials containing sterilized soil supplemented with 1000 μM of *o*-xylene and MS medium was used as a control (+).

of the *o*-xylene biodegradation activity indicating that this population represents a major microbiota endogenous to the KN soil. On the contrary, band B was only detected in samples exhibiting the *o*-xylene biodegradation activity indicating that this population is involved in the biodegradation of *o*-xylene.

The bands A and B were excised and sequenced. DGGE bands A detected in different samples were identical in their DNA sequences which were identical to the 16S rDNA sequences of *Pseudomonas* strains. DGGE bands B detected in different samples were also identical to the 16S rDNA sequence of *Rhodococcus opacus* DSM 43205^T (Klatte et al. 1994; accession no. X80630).

From KN-soil samples in which biodegradation of *o*-xylene has proceeded, bacteria capable of growing on agar plates containing 1/10 TSB (without *o*-xylene) were isolated after the incubation at 25 °C for 14 days. Among tens of isolates, TKN14, TKN45, and TKN46 were able to use *o*-xylene as sole carbon and energy sources. Furthermore, the PCR-amplified fragment of 16S rDNA from TKN14 (Lane 5, Figure 4), TKN45 and TKN46 (data not shown) migrated at a position equivalent to band B.

The morphology and color of colonies of TKN14, TKN45 and TKN46 on 1/10 TSB plates were rough and pink. This type of colonies were predominant when soil samples exhibiting active *o*-xylene biodegradation were spread on 1/10 TSB plates. Moreover, during the degradation of *o*-xylene by strain TKN14 in MS medium, *o*-methylbenzylalcohol, *o*-tolualdehyde, and *o*-toluic acid were detected as intermediates. The genes which mediate oxygenation of *o*-xylene to *o*-methylbenzylalcohol were isolated from strain TKN14, and these genes were induced by addition of *o*-xylene (Maruyama et al. 2005).

DGGE profiles of 16S rDNA amplified from CH soil samples were presented in Figure 5. Since major bands C appeared in all soil samples, it was considered that the corresponding populations have a less important role in biodegradation. Bands D and E appeared after the biodegradation of 200 μM and 1000 μM *o*-xylene, respectively. The sequence of band D was identical to the 16S rDNA sequence of *Hydrogenophaga*. There is a possibility that *Hydrogenophaga* also have an important role of *o*-xylene degradation in soil. However we cannot isolate the strain, which has the sequence closely related to band D.

While the sequence of band E was identical to that of band B (*Rhodococcus*) from KN-soil (Figure 4). Among isolates from CH soil samples that have been subjected to *o*-xylene biodegradation, some of isolated strains, TCH4 and TCH14 were found to use *o*-xylene as sole carbon and energy sources. The PCR-amplified fragment of 16S rDNA from TCH4 (Lane 4, Figure 5) and TCH14 (data not shown) migrated at positions equivalent to band E.

Phylogenetic analysis of o-*xylene degraders in soil*

16S rDNA fragments of strains TKN14, TKN45, TKN46, TCH4, and TCH14 were sequenced and the neighbor-joining trees were constructed (Figure 6a). All isolates formed a cluster with *R. opacus* DSM 43205^T. Sequences of *gyrB* fragments of five isolates were also determined and phylogenetic positions of isolates were presented

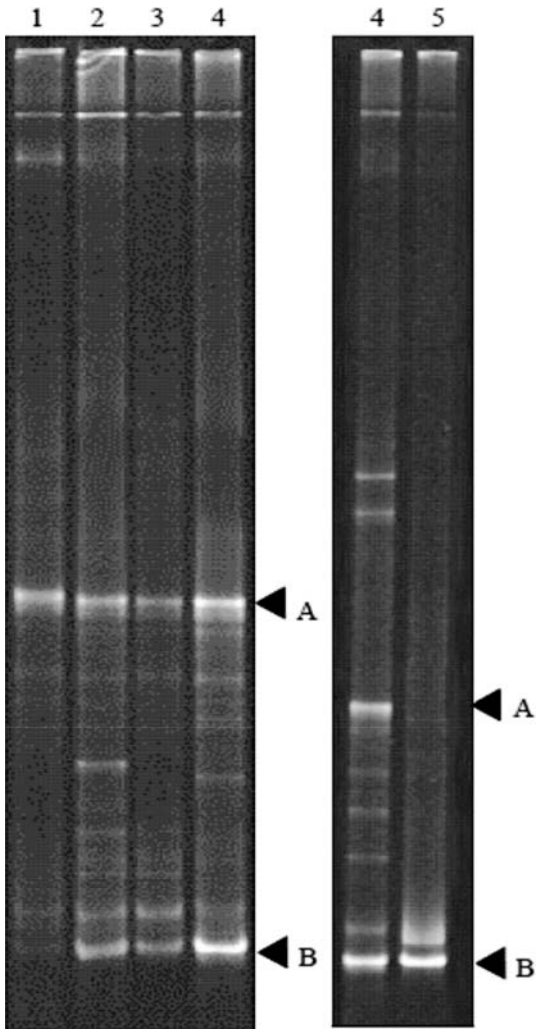

Figure 4. DGGE analysis of PCR-amplified 16S rDNA fragments isolated from KN soil before and after degradation of *o*-xylene. DNA was isolated from KN soil before *o*-xylene biodegradation (lane 1, day 0), KN soil supplemented with mineral nutrients and 50 μM *o*-xylene (lane 2, day 11), KN soil supplemented with mineral nutrients and 200 μM *o*-xylene (lane 3, day 13), KN soil supplemented with mineral nutrients and 1000 μM *o*-xylene (lane 4, day 31), and strain TKN14 (lane 5).

in Figure 6b. All isolates formed a cluster that included two type strains, *R. opacus* DSM 43205^T and *Rhodococcus koreensis* JCM 10743^T.

Enumeration of o-*xylene-degrading* Rhodococcus *in soil*

In order to estimate population sizes of *o*-xylene-degrading *Rhodococcus* bacteria, we designed

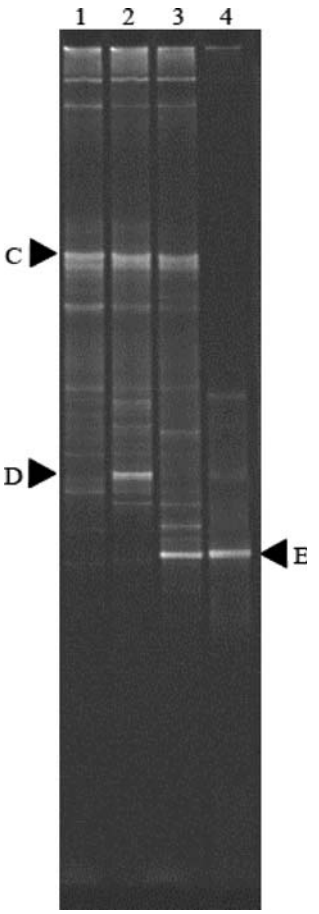

Figure 5. DGGE analysis of PCR-amplified 16S rDNA fragments obtained from CH soil before and after degradation of *o*-xylene. DNA was extracted from CH soil before *o*-xylene biodegradation (lane 1, day 0), CH soil supplemented with mineral nutrients and 200 μM *o*-xylene (lane 2, day 11), CH soil supplemented with mineral nutrients and 1000 μM *o*-xylene (lane 3, day 29), and strain TCH4 (lane 4).

PCR primers that specifically amplify genes of *Rhodococcus* strains within the *R. opacus* and *R. koreensis* cluster. As shown in Figure 1, primer RB6R can only recognize *gyrB* from *o*-xylene-degrading isolates, *R. opacus* DSM 43205^T and *R. koreensis* JCM 10743^T, but cannot recognize *gyrB* from other related *Rhodococcus* strains, and sequences of PCR-amplified fragments with a set of primers RB2F and RB6R were closely related to *gyrB* sequences from *Rhodococcus opacus* and *R. koreensis* groups. Thus, the combination of primers RB6R (specific primer) and RB2F (non-specific primer) allowed the specific amplification of *gyrB* from the *R. opacus* and *R. koreensis* group (data not shown).

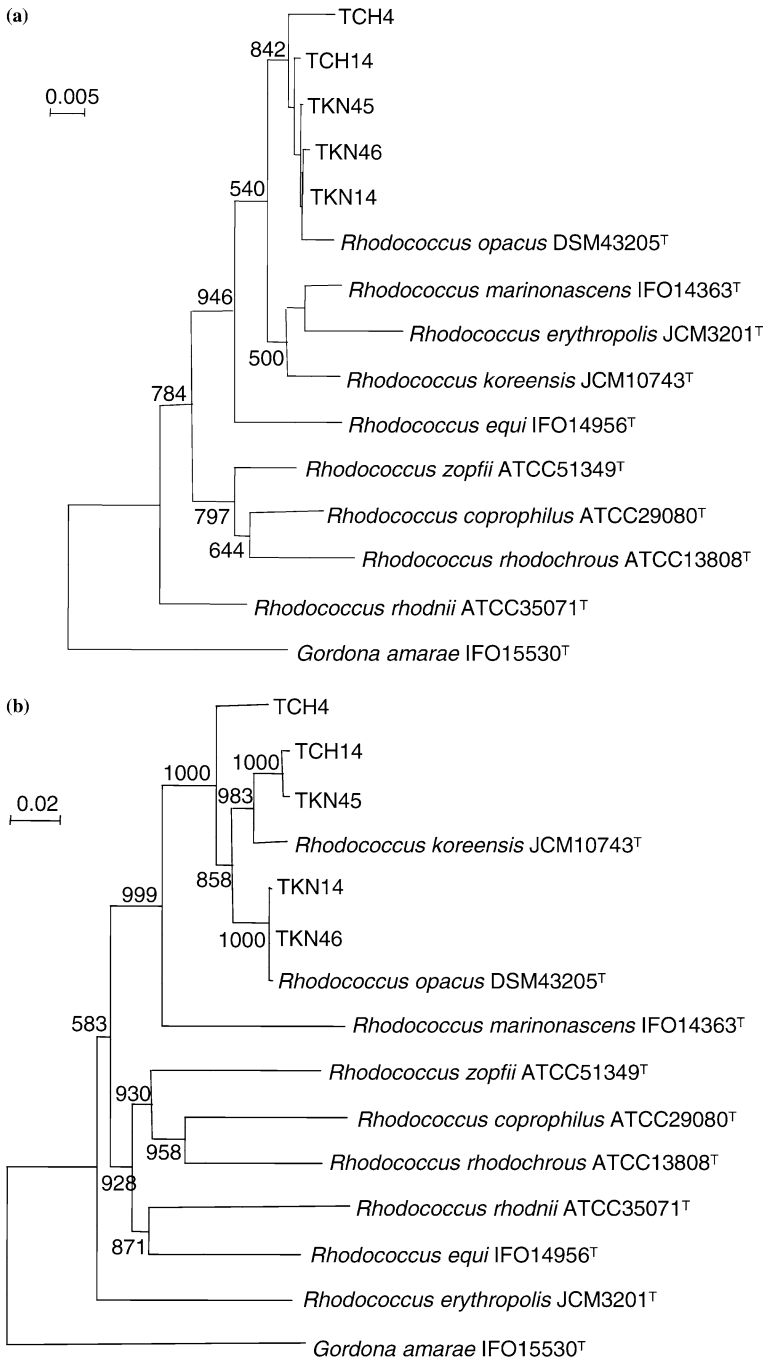

Figure 6. Phylogenetic tree of isolated *o*-xylene-degrading strains based on the sequences of 16S rRNA (a) and *gyrB* (b) reconstructed with the neighbor-joining method. The numbers at the branch nodes are bootstrap values (per 1000 trials); only values greater than 500 are shown.

Population changes of *o*-xylene degraders in KN soil samples during the degradation of 200 μM of *o*-xylene were analyzed by cPCR using the primer set RB2F and RB6R (Figure 7). Population of the *R. opacus* and *R. koreensis* group rapidly increased from 6.5×10^4 copies/g-soil to

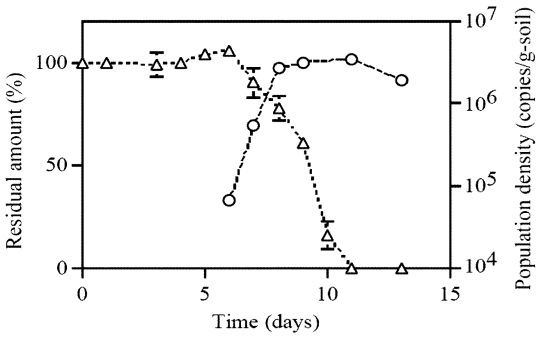

Figure 7. Change in the population density of *o*-xylene-degrading *Rhodococcus* in KN soil estimated by cPCR. Each 200 μM of *o*-xylene was added in 120-ml vials containing 7 g of KN soil and 2 ml of MS medium, and the vials were incubated at 20 °C with shaking. DNA was extracted from the soil samples for the enumeration of *o*-xylene-degrading *Rhodococcus* while residual amount of *o*-xylene was determined by headspace gas chromatography procedure. Symbols: Δ, residual amount of *o*-xylene; ○, estimated population density.

3.5×10^6 copies/g-soil between day 6 and day 11 though this bacterial group was not detected before day 5 (detection limit was 10^4 copies/g-soil). Degradation abilities of *o*-xylene by *R. opacus* DSM43205^T and *R. koreensis* JCM10743^T were clearly lower than our isolates. However, it was reported that *R. opacus* R7 are able to degrade *o*-xylene (Di Gennaro et al. 2001), and also, during this experiment, *o*-xylene was degraded according to the growth of the *R. opacus* and *R. koreensis* group. Furthermore, the same tendency was observed for different initial concentrations (50 and 1000 μM) of *o*-xylene (data not shown). Therefore, we think that these results suggested that isolated *o*-xylene-degrading strains belonging to the *R. opacus* and *R. koreensis* group are major players in the biodegradation of *o*-xylene in soil under the present experimental conditions.

Gasoline and diesel fuel have been shown to be successfully degraded by bioremediation procedures under aerobic conditions. BTEX in gasoline are, in general, relatively easy to be degraded. However, Solano-Serena et al. (2000) reported that *o*-xylene degradation in soil needed a longer period compared to other gasoline constituents. Our results were in agreement with their results.

However, once acclimated, *o*-xylene in soil was degraded at a speed comparable to those of the degradation of other BTEX compounds. Therefore, it is likely that the recalcitrance of *o*-xylene to biodegradation was due to insufficient number of *o*-xylene-degrading populations in non-acclimated soil, and that the growth of *o*-xylene-degrading *Rhodococcus* takes a long time until it reaches a sufficient number for efficient biodegradation. If this is the case, bioaugmentation can be applied to speed up the remedial process of *o*-xylene.

The effectiveness of bioaugmentation is a subject of controversy in bioremediation: many microorganisms have been used for bioaugmentation, however, their efficacy has often been unknown, or they have failed to produce expected performance (Vogel 1996). One of the major challenges to bioaugmentation is the *in situ* growth of introduced microorganisms, and therefore, the selection of correct microorganisms is critical for successful bioaugmentation. The *Rhodococcus* strains isolated in this study became major populations in two different soils when *o*-xylene was present, and may therefore be effective in bioaugmentation. Our preliminary results demonstrated that this was the case.

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