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16S rDNA-based characterization of BTX-catabolizing microbial associations isolated from a South African sandy soil

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Abstract

In the presence of different selection pressures, particularly pH and electron donor concentration, indigenous microbial associations which catabolize selected petroleum hydrocarbon components (benzene, toluene and *o*-, *m*- and *p*-xylene (BTX)) were enriched and isolated from a petroleum hydrocarbon-contaminated KwaZulu-Natal sandy soil. Electron microscopy revealed that, numerically, rods constituted the majority of the populations responsible for BTX catabolism. Molecular techniques (polymerase chain reaction (PCR) and 16S rDNA fingerprinting by denaturing-gradient gel electrophoresis (DGGE)) were employed to explore the diversities and analyze the structures of the isolated microbial associations. Pearson product-moment correlation indicated that the different, but chemically similar, petroleum hydrocarbon molecules, effected the isolation of different associations. However, some similar numerically-dominant bands characterized the associations. A 30% similarity was evident between the *m*- and *o*-xylene-catabolizing associations regardless of the molecule concentration and the enrichment pH. PCR-DGGE was also used to complement conventional culture-based microbiological procedures for environmental parameter optimization. Band pattern differences indicated profile variations of the isolated associations which possibly accounted for the growth rate changes recorded in response to pH and temperature perturbations.

Introduction

Although biosynthetic sources of aromatic hydrocarbons have been implicated, their elevated concentrations in the environment originate principally from the widespread accidental release from leaking oil storage tanks and fractured pipelines (Corseuil & Alvarez 1996). Petroleum hydrocarbon contamination of soils and water can enter biological food webs and, thus, threaten indigenous organisms and the human population. The most soluble and mobile components (benzene, toluene and xylene (BTX)) pose particular threats and their acute toxicities and genotoxicities have, therefore, mandated their classification as priority pollutants (Tsao et al. 1998; Solano-Serena et al. 1999).

Through the interactions of microbial associations even complex xenobiotic compounds can be mineralized (Slater & Lovatt 1995) with the numbers and types of microorganisms present in the local environment determining the biodegradative capacity. To facilitate rapid and efficient bioremediation of a polluted site by bioaugmentation, enrichment of key indigenous catabolic associations is, therefore, considered. Environmental conditions play pivotal roles in determining biological activity during bioremediation (Vogel 1996). Therefore, elucidation of the optimal ranges of specific environmental parameters is funda-

mental for the promotion of microbiological activity for contaminant biodegradation.

Although the applications of indigenous microbial populations in bioremediations have been realized, understanding the molecular microbial ecology of the key catabolizing associations has received little attention. Additionally, the distributions of the associations in response to environmental perturbations need to be understood. Mason et al. (1998) postulated that the successful implementation of *in situ* bioremediation requires, in particular, a detailed characterization of the contaminated site in relation to the pollutant, hydrogeochemistry and microbiology. Furthermore, microorganisms need to be considered in relation to their catabolic potentials and key environmental factors which limit the rate of pollutant degradation.

We have, therefore, adopted the 16S rDNA-based polymerase chain reaction (PCR) and denaturing-gradient gel electrophoresis (DGGE) to address, in part, the microbiological considerations. Thus, we explored the diversities and analyzed the structures of isolated BTX-catabolizing soil microbial associations and monitored their profile variations which were effected by changes in environmental pH and temperature.

Materials and methods

Association enrichment and isolation

Petroleum-contaminated soil (500 g), collected near a leaking (>2 y) oil storage tank on a construction site in a Pietermaritzburg industrial area, in the KwaZulu-Natal Province, South Africa, was mixed with 1 l distilled water and shaken vigorously by hand to displace the microorganisms. The suspension was then filtered through a series of 3 #2 Whatman filter papers into clean flasks. A basic mineral salts solution, which contained (gl^{-1}): K_2HPO_4, 1.5; KH_2PO_4, 0.5; $(NH_4)_2SO_4$, 0.5; and $MgSO_4{\cdot}7H_2O$, 0.2 (pH 7.3), was prepared and sterilized (121 °C 15 min). Aliquots (50 ml) of the mineral salts, held in 250 ml Erlenmeyer flasks, were then inoculated with 5 ml of the filtrate/microbial suspension. Small open bottles, which contained 10 ml of the BTX molecule of interest (100%), were inserted in each enrichment flask. Thus, each molecule was made available to the microbial populations via volatilization. The flasks were incubated in the dark at 30 °C for 7 days. A series of subcultures were made from the primary enrichments and the isolated associations were then cultured (30 °C) in the presence of specific BTX concentrations (50, 5, 0.5 and 0.05 mM). The flasks were divided into two groups based on pH which was poised initially at 5.5 or 7.5 with 1N HCl or 1N NaOH, respectively.

Optimization of growth parameters

The pH 5.5- and 7.5-poised BTX (0.5 mM)-catabolizing cultures were mixed to ensure that all the catabolic components of the soil were present. Combined growth- and substrate catabolism rates were determined for each mixed association in batch culture at (citrate and phosphate buffered) pH values of 4, 5, 6, 7 and 8 and temperatures of 15, 20, 25 and 30 °C. The pH and temperature values were chosen as representative of the soil ranges recorded in KwaZulu-Natal, South Africa. The protocol adopted was to determine the optimum pH for each association and then use this as a fixed variable in the temperature study. For each batch culture, aliquots (1.5 ml) were taken during the log phase and characterized by DGGE.

DNA isolation and purification

Genomic DNA of the isolated associations and soil DNA were extracted according to the protocol described by Duarte et al. (1998) but with the buffers poised at pH 8. Additional modifications were made to the procedure described by Duarte et al. (1998) for the indirect soil DNA extraction. Soil (20 g) and 100 ml of 0.1% (m/v) sodium pyrophosphate were used. Purification of soil DNA was made as outlined by Röling et al. (2000).

PCR conditions and denaturing-gradient gel electrophoresis (DGGE)

PCR was made on part of the 16S rDNA region using the primer set 357f and 518r (Muyzer et al. 1993). The PCR and DGGE reaction mixtures and conditions were followed as described by Rölling et al. (2000) with the exception that a 50–65% (v/v) denaturing-gradient was used. Analysis of gels was made by GelCompar 4.0 as described by the same researchers.

Sequencing of some randomly-selected clones

PCR primers 8f and 1512r were used to amplify 16S rDNA sequences. The products were cloned in pGEM-T linear plasmid vector and *Escherichia coli*

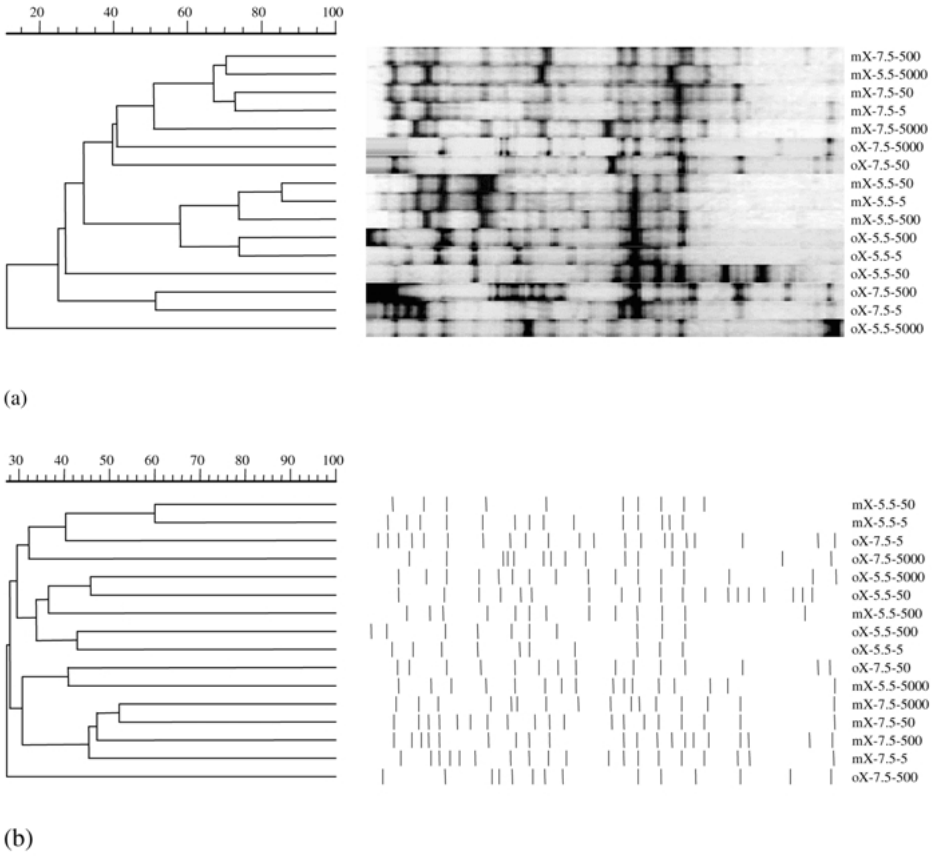

Figure 1. Pearson product-moment (a) and Jaccard coefficient (b) clustering of normalized DGGE gels showing the sequence patterns of the *o*- and *m*-xylene-catabolizing (oX and mX) associations at different pH values (5.5 and 7.5) and component concentrations (0.05, 0.5, 5 and 50 mM indicated by 5, 50, 500 and 5000, respectively).

JM109 competent cells as specified by the manufacturer (Promega, Madison, WI, USA). All clones were reamplified using the primer set F357-GC/R518 and products were compared on DGGE gels with the original association profiles. For obtaining the complete sequences of the 16S rDNA reamplification of all 8f-1512r clones was made using the T7/sP6 primer set. Sequencing PCR was made with ABI PRISM™ Dye Terminator Cycle Sequencing Core Kit (Perkin Elmer) and the purified products were run in SEQUAGEL-6 sequence gel (National Diagnostics, USA) in a 373A/DNA Sequencer (Applied Biosystems, USA). Both strands of the 16S rDNA gene were sequenced. BLAST (Basic Logical Alignment Search Tool) network service was used to compare these sequences to available databases for determination of their approximate phylogenetic affiliations.

Results

Enrichment of indigenous soil microbial associations

In this study, batch culture enrichments were used to isolate aerobic catabolic associations from a petroleum hydrocarbon-contaminated KwaZulu-Natal (South Africa) sandy soil. Following primary enrichment, the principal selection pressures were pH and electron donor concentration. After the 16-week primary enrichment, subculturing and isolation period the microbial associations were examined by light and scanning electron microscopy. The associations were characterized by mixtures of Gram-positive and Gram-negative rods and cocci. Scanning electron microscopy revealed that, numerically, the rods constituted the majority of the populations responsible for BTX catabolism. Also, the results suggested that the associations consisted of only three types of rod-shaped members, depending on size and shape.

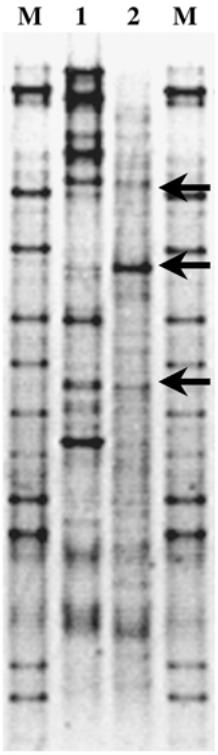

Figure 2. 16S rDNA profiles of petroleum hydrocarbon-contaminated (Lane 1) and uncontaminated soils (Lane 2). Dominant members present in the pristine soil also occur in the presence of petroleum (Arrows).

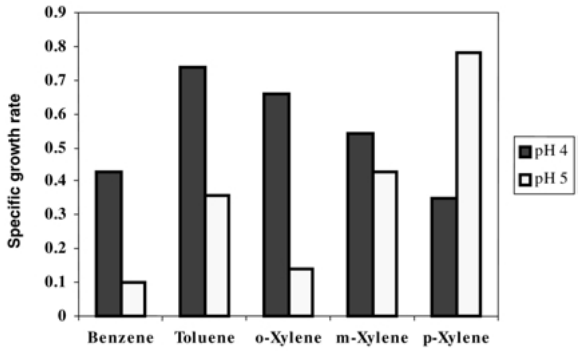

Figure 3. Growth rates (μ (h^{-1})) of the BTX (0.5 mM)-catabolizing associations at 30 °C and two different pH values.

Figure 1 shows the 16S rDNA sequence patterns of the enriched *o*- and *m*-xylene-catabolizing associations at different pH values and molecule concentrations after Pearson product-moment (Figure 1a) and Jaccard coefficient (Figure 1b) clustering. Pearson product-moment analysis characterizes DGGE association profiles on the basis of the normalized densitogram of the individual lanes. This analysis highlighted the differences and similarities of the enriched associations. The Jaccard coefficient clustering was used to account for the presence or absence of individual bands in the original gels. These bands corresponded to specific association members and identified their locations within the respective association profiles. Both analyses showed that the BTX-catabolizing associations were characterized by complex DGGE banding patterns. According to the Jaccard coefficient, each association consisted of between 10 (0.05 mM *o*-xylene at pH 5.5) and 20 (0.05 mM *m*-xylene at pH 7.5) members. Some similar members constituted the different associations as indicated by the 30% clustering after the analysis of band presence and position.

Analysis of soil DNA

Samples of petroleum hydrocarbon-contaminated and uncontaminated soils were taken from the same area. The different samples were then homogenized by hand and analyzed by DGGE. The analysis indicated complex sequence patterns in both the chemically-compromised and pristine soils. Although not direct evidence for banding pattern changes in response to pollutant molecules, the bands appeared to show that contamination effected differences in the dominance of the microbial members between the polluted (Figure 2, Lane 1) and the pristine soils (Lane 2). Soil contamination did not necessarily change the complexity of the sequence patterns but effected a shift in dominance to the section of the gradient with a reduced concentration of the denaturant. Thus, occurrence of the species with easily-denatured melting domains was increased in the presence of the petroleum hydrocarbons. Despite the change in the expression of dominant members, as indicated by band intensities, some similarities were recorded between the contaminated and pristine soils. Furthermore, some of the numerically-dominant species in the latter were retained in the presence of the pollutants (arrows).

Optimization of environmental parameters

Figure 3 shows the calculated specific growth rates of the combined BTX-degrading associations. Increased cellular growth rates, as determined by optical density, were recorded at pH 4 for the BTX degraders. The one exception was *p*-xylene where a higher growth rate occurred at pH 5. The growth rates at pH 4 and 5 in the presence of *m*-xylene were comparable while marked

differences in rates were recorded for the benzene-, toluene-, *o*- and *p*-xylene degrading associations at these two pH values. Although the results for the other temperatures are not included, the isolated associations exhibited generally highest growth rates at 30 °C.

The results of the DGGE analyses of the associations during the log phases of the environmental parameter optimization studies are shown in Figure 4. Some similar dominant bands were present in the associations which catabolize a common petroleum hydrocarbon component despite the changes in environmental temperature (Arrow 1). A few dominant bands common to the different BTX-catabolizing associations were also evident (Arrow 2). However, Pearson product-moment comparisons indicated that perturbations in environmental temperature effected a 65% difference in the BTX-catabolizing associations.

Sequencing of 16S rDNA of randomly selected clones

Complete 16S rDNA (8f-1512r) analysis was made with the primer set T7 and sP6 to identify some clones from two isolated toluene-degrading associations (Table 1). Some of the sequenced members, also present in the combined associations are identified in Figure 4. Cloning highlighted the numerical dominance of some associations members (Y14-5, Y17-2 and Y17-8) while also revealing some non-numerically dominant components, hardly detectable in the respective association profiles. Clones Y14-2 and Y17-8 showed a 99% similarity to the same species from the Gamma Proteobacteria family, *Rhodanobacter lindanoclasticus*. These two clones also exhibited bands in the same region along the denaturing gradient (data not shown), exemplifying that molecule concentration and enrichment pH effected some similarities in the profiles of the catabolic microbial associations. Thus, cloning and sequencing reflected the limitation and fidelity of DGGE.

Discussion

Recognition has been made that, although important, the molecular ecology and distribution of key pollutant-catabolizing associations have received little attention (Mason et al. 1998). Molecular techniques are ideal tools to address this paucity (Heuer & Smalla 1997). To fully appreciate sequence diversity and structure of BTX-catabolizing associations we, therefore, adopted a combination of PCR and DGGE. The same approach was used to study association dynamics in response to selected conditions and the source soil and its pristine equivalent. For these two soils considerable band similarities were observed. Spatial and temporal differences may have accounted for the differences in the profiles. Common numerically-dominant bands also characterized the contaminated soil and the enriched associations. In general, van Verseveld et al. (1999) found by DGGE profiling that isolated communities, particularly the dominant members, from the same site were very stable. From the enrichment/isolation studies it was clear that BTX biodegradation was operative at the different pH values and electron donor concentrations.

The isolated associations were characterized by complex banding patterns indicative of high numbers of equally abundant species (Heuer & Smalla 1997). Although differences were recorded in response to each molecule, the common selection pressures of molecule concentration and pH, of the primary enrichments, effected the recorded 30% similarities which, although relatively low, suggested some metabolic versatility. Numerical dominance does not necessarily reflect catabolic activity dominance. If, however, the numerically-dominant members (intense bands) were the predominant active catabolic species, the associations could be exploited in different situations. Thus, for example, the toluene-catabolizing associations could facilitate sub-optimal enhanced (bioaugmented) remediation of petroleum hydrocarbon-contaminated soils in the absence of other petroleum component-catabolizing associations. Also, a robust/versatile microbial association could be constructed by selecting the components with the highest activities.

To explore key rate-limiting factors exemplary to South African soils combined associations were used. Growth rate changes, in response to environmental variable perturbations, were accompanied by DGGE band pattern changes although for temperature some common patterns were apparent. The results, therefore, exemplified the importance of site-specific determinants.

Denaturant gradient band intensity was interpreted as indicative of numerical dominance of particular sequences or association members but not activity dominance. Further work is, therefore, required with 16S rRNA. Muyzer (1998) stated that a DGGE comparative analysis of PCR-amplified DNA and mRNA of functional genes enabled the discrimination of active and inactive members in a microbial association and, thus, the link between structure and function.

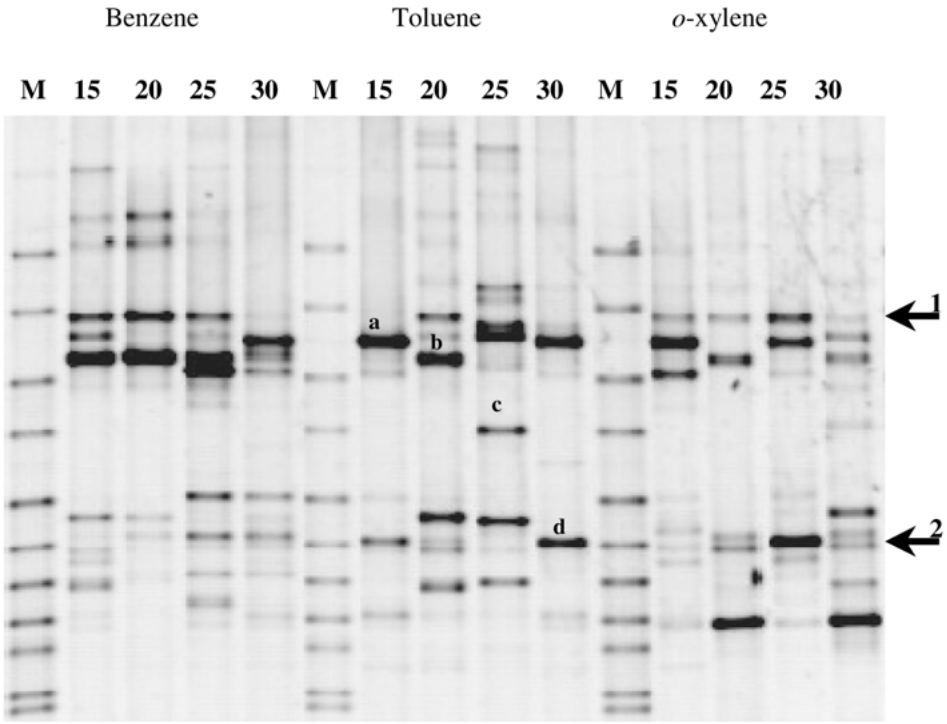

Figure 4. DGGE profile of the BTX (0.5 mM)-catabolizing associations buffered at pH4) and incubated at different temperatures (°C). Arrow 1 indicates a dominant band common to the benzene-catabolizing associations, in particular, but also common to the other catabolizing associations, Arrow 2 shows another band common to the associations. **a-d** indicate some of the sequenced members Y14-1, Y14-2, Y14-3 and Y17-2 (Table 1).

Table 1. Phylogenetic affiliations based on 16S rDNA genes of randomly-selected clones from toluene-degrading associations at two concentrations and pH values (Y14-1 to 6; 0.5 mM at pH 7.5 and Y71-1 & 2; 5 mM at pH 5.5). Sequencing (8f/1512r) was made with the primer set (T7/sP6) after reamplification.

Clone #	Phylogenetic affiliation	% similarity	Closest relative	Accession #
Y14-1	*Firmicutes*/Bacilli	92	*Bacillus* sp. GL1	AB024598
Y14-2	Gamma Proteobacteria	98	*Rhodanobacter lindanoclasticus*	AF039167
Y14-3	*Firmicutes*/Bacilli	94	*Bacillus* sp. GL1	AB024598
Y14-4	Alpha Proteobacteria*	91	Unid. Bacterium clone 1956	AF097800
Y14-5	*Proteobacteria*	94	Bacterial sp. 1165	Z95709
Y14-6	*Firmicutes*/Clostridia	88	Eubacterium env. OPS	AF018188
Y17-2	*Proteobacteria*/Rhizobiales	97	*Hyphomicrobium denitrificans*	Y14308
Y17-8	Gamma Proteobacteria	98	*Rhodanobacter lindanoclusticus*	AF039167

* Indicates the most probable phylogenetic affiliation.

Other researchers (Urbach et al. 1999) used bromodeoxyuridine (BrdU), a thymidine analogue, to demonstrate the isolation of DNA from metabolically-active bacteria.

Since molecular techniques based on 16S rDNA do not give information on physiological capabilities (Röling et al. 2000a), community-level physiological profiling (CLPP) must be used to elucidate carbon source utilization patterns (Garland 1997; Röling et al. 2000a,b). Thus, a combination of DGGE analyses of 16S rDNA, CLPP and detection of specific enzymes would be the best approach to implement and monitor augmented bioremediation.

Through cloning and sequencing, some of the numerically-dominant and non-dominant association members were identified. Banding pattern variations,

congruent with sequence variations, exemplified the potential use of sequencing in conjunction with DGGE prior to implementing enhanced bioremediation. DGGE could then be used, without the expense of sequencing, to monitor the catabolic species. The identification of some clones within our combined catabolic associations (Figure 4, Table 1) exemplifies this.

Environmental parameter optimization and association characterization should precede bioaugmentation to identify the optima for the dominant (active) species. In general, the contaminated soil and the enriched associations were characterized by similar dominant bands in the upper part of the denaturing gradient. Thus, if numerical dominance is proven to be correlated with activity dominance, the use of PCR-DGGE to assess catabolic potential and monitor intrinsic and bioaugmented remediation would be justified. Soil samples could be collected from a contaminated site and the resultant PCR-DGGE profile used to elucidate the potential for intrinsic bioremediation or the need to inoculate with a catabolic association(s). Awareness of the limitation of DGGE analysis for this application, where the numerically-dominant members of the association "overshadow" the non-dominant ones, would evidently be paramount.

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