Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 5 (10): 999-1010, 2010
ISSN 1816-4935 / DOI: 10.3923/jm.2006.307.318

Isolation and Molecular Characterization of Phenol-degrading Gram-positive Bacteria from Oil-contaminated Soil*

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Abstract: One of the aspects of bioremediation of contaminated site is the isolation and identification of bacterial isolates from polluted soil in order to choose the most active ones. Three phenol-degrading bacteria were isolated from contaminated soil samples using direct isolation method on mineral salt medium supplemented with 2.0 mM phenol as the sole carbon source. In this study, three phenol-degrading bacteria were characterized by morphological, biochemical properties and differentiated by DNA fingerprinting technique using Random amplified polymorphic DNA (RAPD) and specific and random amplification based on PCR (SARA-PCR) analyses. Phenol-degrading bacteria are designated *Bacillus* sp. PD-02, PD-06 and PD-08. The aerobic isolates grew on phenol at concentrations ranging from 2-16 mM. These isolates are a strictly aerobic, gram-positive, endospore-forming, rod-shaped, catalase positive, oxidase negative and grew at salinities ranging from 0.28 to 10.0% and temperatures ranging from 15 to 41°C. PCR application for detection of catabolic genes involved in phenol degradation showed the presence of catechol 1,2 Dioxygenase (C12DO) and phenol hydroxylase but the fragments of catechol 2,3 Dioxygenase (C23DO), salicylate-1-hydroxylaswe are not detected. The ability of the isolates to degrade phenol make them a potential candidate for use in bioremediation of environments contaminated by such or related compounds.

Key words: Phenol, biodegradation, RAPD, SARA-PCR, catabolic genes

Introduction

The basic idea behind bioremediation is to find bacteria that are capable of using the contaminant as carbon and energy source. Microbes able to grow on low concentrations of aromatic hydrocarbons as the sole source of carbon and energy were first identified (Gibson and Subramanian, 1984), however, bacteria able to thrive and grow in the presence of high concentrations of aromatic compounds have been reported (Inoue and Horikoshi, 1989; Inoue *et al*., 1991; Cruden *et al*., 1992; Weber *et al*., 1993). In many cases, the bacterial aerobic catabolism of (alkyl)phenol is initiated by a multicomponent phenol hydroxylase (mPH) that hydroxylases phenolic substrates to the corresponding catechols in the presence of O_2 and NAD(P)H. The mPH was first identified in phenol and (di)methylphenol (*dmp*) degradation pathways in *Pseudomonas* sp. CF600 by Shingler *et al*. (1992). In general, most aromatic compounds are aerobically degraded through a common intermediate, catechol or protocatechuate depending on the chemical structure of the starting compound (Harwood and Parales, 1996). The catechol is further degraded either by cleavage between two hydroxyl groups by catechol 1,2-dioxygenase (C12O) via an *ortho*-pathway or by cleavage adjacent to the hydroxyl groups by catechol 2,3-dioxygenase via a *meta*-pathway (Hamzah and Al-Baharna, 1994; Harwood and Parales,

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*Originally Published in Research Journal of Microbiology, 2006

1996). The enzyme activities of phenol hydroxyl;ase and catechol 2,3 dioxygenase in cell free extracts of Arthrobacter citreus were indicative of operation of a meta-cleavage pathway for phenol degradation (Karigar *et al*., 2006).

Phenolic compounds constitute one of the largest groups of natural products. They are predominantly found in plants, where they occur in a great variety of structures and functions. During the last century, the natural pool of phenolic compounds has been increased with products of industrial origin. Many of these synthetic compounds cause environmental pollution and human health problems as a result of their persistence, toxicity and transformation into hazardous intermediates (Timmis and Pieper, 1999; Gupta *et al*., 2002). Polymerase Chain Reaction (PCR) can be used to amplify genes allowing more sensitive detection than direct probing (Erb and Wagner-Dobber, 1993). Aromatic compound-degrading bacteria were monitored by PCR using primer sets for the general detection of the genes encoding catechol 1, 2-dioxygenase (C12DO) and catechol 2,3-dioxygenase (C23DO), which are the key enzymes for aromatic compound degradation (Picard *et al*., 1992). In this study, the first aim was to describe isolation and molecular characterization of three phenol-degrading bacteria obtained from oil-contaminated soil, the second aim was to detect catabolic genes related to degradation of phenol.

Materials and Methods

Growth Medium and Culture Conditions

Bacterial isolates used in this study were isolated from oil-contaminated soil, Bu-Hasa seep pit of ADCO (Abu-Dhabi onshore oil operation, ADCO), Abu-Dhabi, United Arab Emirates in July 2004. This study was conducted in United Arab Emirates University. Mineral salts medium KMM1 (Ngugi *et al*., 2005) was used for isolation of naturally occurring phenol-degrading bacteria. Basal medium KMM1 contained per liter, NaCl (2.8 g), KCl (6.5 g), $MgCl_2.6H_2O$ (0.50 g), $CaCl_2.2H_2O$ (0.10 g), NH_4Cl (5.6 g), $NaSO_4$ (1.0 g) and KH_2PO_4 (1.0 g). Phenol was added to the media from a 2.0 M stock solution to a final concentration of 2.0 mM. Cultures were incubated on a shaker (100 rpm) at 30°C and monitored for the turbidity increase due to bacterial growth.

Isolation of Phenol-degrading Bacteria

The direct isolation method was used as previously described (Zhuang *et al*., 2002). One gram of contaminated soil was added to 9.0 mL of sterile 50 mM sodium tripolyphosphate and overtaxing vigorously for 1 min. Appropriate dilutions from each series were spread onto agar plates of KMM1 media supplemented with 2.0 mM phenol as a carbon and energy source, the plates were incubated at 30°C for 7 days and all the colonies that appeared on one plate were picked and purified by restreaking. Isolates were screened to select for bacteria that can grow rapidly on KMM1 plates with phenol as sole carbon source. Individual colonies, which, grew on the plates, were then re-inoculated in fresh KMM1 liquid media to ascertain their ability to degrade phenol. Three isolates designated as *Bacillus* sp. PD-02, PD-06 and PD-08 exhibited relatively faster growth rates than the rest were picked and chosen for further study.

Morphological, Biochemical and Physiological Characterizations

Gram-stain was performed as previously described (Smibert and Krieg, 1994). Growth of isolates at different temperatures was monitored as previously described (Zhuang *et al*., 2002). Enzyme profiles and carbon substrate utilization characteristics were determined using the API 20E assays according to the manufacturer's instructions (BioMérieux, Marcy, France). Highest concentration of phenol at which the isolates) could initiate growth was determined by monitoring the optical density of the culture growing at initial phenol concentrations ranging from 2-16 mM. Experiments were carried

Table 1: Properties of RAPD PCR primer sequences

Primers	Sequence	G+C (%)	Length (mers)
RAPD Primer 1	5'-GGTGCGGGAA-3'	70	10
RAPD Primer 2	5'-GTTTCGCTCC-3'	60	10
RAPD Primer 3	5'-GTAGACCCGT-3'	60	10
RAPD Primer 4	5'-AAGAGCCCGT-3'	60	10
RAPD Primer 5	5'-AACGCGCAAC-3'	60	10
RAPD Primer 6	5'-CCCGTCAGCA-3'	70	10

out in 250 mL flasks containing 50 mL of KMM1 and the test media was inoculated with 100 µL of a 24 h old culture grown in nutrient broth. Cultures were then incubated in a shaker (100 rpm) at 30°C for one week. Isolates were also inoculated into basal KMM1 without the substrates, which served as the control experiment. The tolerance of isolates to different concentrations of NaCl was tested as described by Sharma *et al*. (2000). The bacterial isolates were grown on KMM1 medium containing (0.28, 3, 5, 8 and 10 % (w/v) sodium chloride. The growth rate was determined with a Shimadzu model UV240 spectrophotometer at 600 nm in cuvettes with a 1 cm light path.

Molecular Characterization of Phenol Degrading Bacteria

Isolation of Bacterial Genomic DNA

DNA extractions were carried out using the Genomic Prep cells and tissue DNA isolation Kit (Amersham Pharmacia, USA), following the instructions of the manufacturer. The DNA was resuspended in Tris-EDTA (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6). The quality and quantity of the extracted DNA were checked by measuring the UV absorption spectrum (Sambrook *et al*., 1989). The pure DNA was used as a template in PCR reactions for genetic diversity of bacterial isolates and detection of catabolic genes.

Random Amplified Polymorphic DNA (RAPD) Assay

For this RAPD analysis (Williams *et al*., 1990) the random primer set was RAPD Ready-To-Go analysis kit, obtained from Amersham Pharmacia (USA). The information for these primers set is depicted in Table 1. The Ready-to-go RAPD analysis kit is supplied as microfuge tubes containing a dried bead which includes premixed dNTPs, buffer and a mixture of Ampli Taq and Stoffel fragment. Before PCR, beads were rehydrated with sterile deionized water. Final concentration in a total of 25 µL reaction mix: 1.5 units Taq, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 uM of each dNTPs and stabilizers including BSA, 25 pM primers. DNA concentration was 20 ng. All the RAPD amplifications proceeded according to the following scheme 5 min at 95°C for the first cycle; 1 min at 95°C, 1 min at 36°C and 2 min at 72°C for the next 45 cycles and 5 min at 72°C for the last cycle. PCR was performed in thermal cycler (Techne, Genius).

Specific and Random Amplification (SARA-PCR) Assay

The oligonucleotide primers Cat A138 and catA742 (Table 2) were employed in the amplification of SARA-PCR (Knijff *et al*., 2001) assays. PCR using Ready to go PCR beads, was performed in 25 µL reaction mixtures, containing 30 pM of primer Cat A138, 1.5 pM of primer CatA742 and 20 ng of genomic DNA. Amplification was programmed as follows: Five cycles at 94°C 30 sec, at 32°C, 35 sec and 2 min at 72°C followed by 30 cycles at 30 sec 94°C, 30 sec, 45°C and 1 min at 72°C, there was a 7 min extension at 72°C.

Detection of Catabolic Genes of the Phenol Degradation

PCR Primers for Detection of Catabolic Genes

PCR primers were synthesized with an Applied Biosystems DNA synthesizer (Invetrogen) and were purified by high-performance liquid chromatography. Catechol 2,3 Dioxygenase was amplified

Table 2: Phenotypic characteristic of phenol-degrading isolates

Test		PD-02	PD-06	PD-08
Cell morphology		Rod	Rod	Rod
Gram stain		Positive	Positive	Positive
Catalase test		+	+	+
Oxidase test		-	-	-
Starch hydrolysis		+	+	+
beta-galactosidase	ONPG	-	-	-
Arginine dehydrogenase	ADH	+	+	+
Lysine decarboxylase	LDC	-	-	+
Ornithinse decarboxylase	ODC	-	-	+
Citrate utilization	CIT	+	+	+
H_2S production	H2S	-	-	-
Urease	URE	-	-	-
Tryptophane deaminase	TDA	-	-	-
Indole production	IND	-	-	-
Acetoin production	VP	+	+	+
gelatinase	GEL	+	+	+
Glucose	GLU	-	-	-
Manitol	MAN	-	-	-
Inositol	INO	-	-	-
Sorbitol	SOR	-	-	-
Rhamnose	RHA	-	-	-
Saccharose	SAC	-	-	-
Melibiose	MEL	-	-	-
Amygdalin	AMY	-	-	-
Arabinose	ARA	-	-	-
Nitrate reduction	NO_2	+	+	-
Growth at°C				
15		+	+	+
25		+	+	+
30		+	+	+
41		+	+	+
Growth in percentage NaCl (%)				
0.28		+	+	+
3		+	+	+
5		+	+	+
8		+	-	-
10		+	-	-

Table 3: Primer sequence, expected PCR products and amplified internal fragments of catabolic genes in phenol-degrading isolates

Gene	Primer	Sequence 5'-3'	Products (bp)	PD-02	PD-06	PD-08
Catechol 1,2 Dioxygenase	Cat A138	AGACCTGGAAATCACCGAAGACG	605	+	+	+
	CatA742	GGGTGGCGTAGGCAAAGTCGTC				
Catechol 2,3 Dioxygenase	23 Cat-F	CGACCTGATCTCCATGACCGA	238	-	-	-
	23 Cat-R	TCAGGTCAGCACGGTCA				
Salicyalte 1-hydroxylase	NahG1001	CGAGCAGCAGGGTGGCGAAGTG	615	-	-	-
	NahG408	AACAGGCGGGCGAGGAAGTAGG				
Phenol hydoxylase	pheUf	CCAGG(C/G)(C/G/T)GA(G/A)AA(				
		A/G)GAGA(A/G)GAA(G/A)CT	444	+	+	+
	PheUr	CGG(A/T)A(G/A)CCGCGCCAGAACCA				

+; Positive for PCR amplification, -; Negative for PCR product

by the PCR using primer sequences described by Mesarch *et al*. (2000), while the primer sequences to amplify salicylate-1-hydroxylase and catechol 1,2 dioxygenase were the a same as described by Civilini *et al*. (1999). Primer sequences to generate the internal fragment of multicomponent phenol hydroxylase were the same as described by Futamata *et al*. (2001). All primer sequences and expected PCR products were shown in Table 3.

PCR Reaction and Cycling Conditions

The pure Taq Ready To-Go PCR beads were applied according to the manufacturer's specifications. Final concentrations are in a total of 25 μL reaction mix including 1.5 units Taq, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTPs and stabilizers including BSA, 25 pM of each primers. DNA amounts were 30 ng. For salicylate -1-hydroxylase, Catechol 1,2 dioxygenase (Civilini *et al*., 1999) and catchol 2,3 dioxygenase (Mesarch *et al*., 2000), the cycling conditions were : Five cycles at 30 sec, 94°C, 30 sec, at 55°C and 1 min at 72°C followed by 30 cycles at 30 sec 94°C, 30 sec, 65°C and 1 min at 72°C, there was a 7 min extension at 72°C. The PCR conditions used for the phenol hydoxylase (Futamata *et al*., 2001) were as follows: step 1, 10 min of activation at 94°C; step 2, five cycles consisting of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C; step 3, five cycles consisting of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C; step 4, 25 cycles consisting of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C; step 5, 10 min of extension at 72°C.

Electrophoresis of PCR Products

After amplification, electrophoresis of all PCR products were performed in 1.5% (w/v) agarose gel pre-stained with ethidium bromide (Sambrook *et al*., 1989) using a Tris-Borate-EDTA buffer system (1×TBE = 90 mM Tris-base, 90.0 mM Boric acid and 2.0 mM EDTA). Gels were observed under UV illumination.

Results

Isolation and Characterization of Phenol-degrading Bacteria

Three bacterial isolates from direct plate method on mineral salt medium supplemented with phenol as a carbon source showed phenol degradation, namely PD-02, PD-06 and PD-08. Gram staining technique indicated that these phenol degrading isolates are rod in shape, Gram-positive, spore forming bacilli. Additional characterization of phenol-degrading isolates by classical microbiological tests indicated that these isolates are catalase-positive and oxidase-negative and starch hydrolysis-positive. Biochemical analyses were performed to characterize these isolates using API20E test, the results of API 20 E are summarized in Table 2. As the characteristics listed in Table 2 show, isolate PD-02 is quite similar to isolate PD-06 in all biochemical tests, except the isolate PD-02 is more tolerance to higher concentrations of sodium chloride (upto 10 %), while isolate PD-08 can be distinguished from the other isolates by a number of characteristics. For example, isolate, PD-08 does not reduce nitrate (nitrate reduction test, negative), lysine and ornithine decarboxylase are positive. Qualitative test for these bacterial isolates was performed on a KMM1 medium containing phenol as a carbon and energy source. Growth rate of isolate PD-02 on phenol at 4, 8, 10, 14 and 16 mM is very slow at the first three days. After the third day growth rate increase markedly at 14 mM and also decline rapidly after the fourth day. At the highest concentration (16 mM) growth initiated immediately after the third day and begins to decrease after fourth day. At the lowest concentration used (2 mM) growth rate of PD-02 initiated from the first day and continue to increase until the third day, after that, growth rate begin to decline as shown in Fig. 1A. In case isolate PD-06, growth rate is very slow at concentration 10 mM, but the growth rate increase with the phenol concentration decrease as show in Fig. 1B. Growth rate of isolate PD-08 starts from the first day on all phenol concentration ranging from 2.0 to 10 mM. When the growth rate starts to decline after the fourth day at 4 mM of phenol concentration, simultaneously, the growth rate starts to increase markedly at 10 mM. The growth rate in the first three days was rapid at lower concentration (4 mM), but is slower at the highest concentration (10 mM), as shown in Fig. 1C.

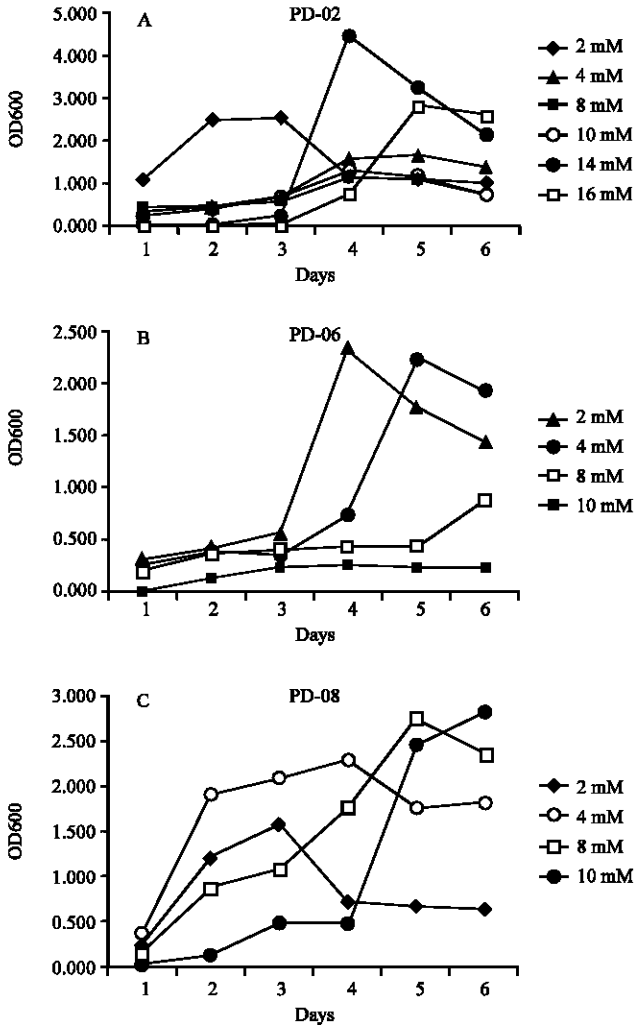

Fig. 1: Time course characteristics for growth of the three isolates on phenol concentrations (2-16 mM). Growth was determined by measuring the turbidity at 600 nm (OD600). (A) Effect of phenol concentrations on the growth of isolate-PD-02, (B) Effect of phenol concentrations on the growth of isolate-PD-06. (C) Effect of phenol concentrations on the growth of isolate-PD-08

Molecular Characterization of Phenol-degrading Isolates

RAPD-PCR

Genomic DNA of phenol-degrading bacteria was subjected to a RAPD-PCR analysis to differentiate identical isolates revealed by biochemical tests. Five random oligonucleotide primers out of six tested primers showed identical DNA fingerprint among the three phenol-degrading isolates, but the RAPD profile with random primer-2 allowed isolate PD-02 to be grouped together with isolate PD-06 by the bands of 300 bp, while isolate PD-08, had no bands at 300 bp as shown in Fig. 2A.

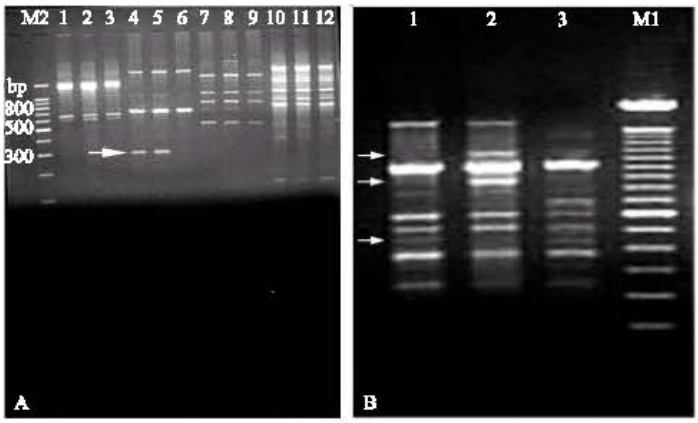

Fig. 2: Molecular characterizations of phenol-degrading bacteria using RAPD-PCR technique (Gel-A) and SARA-PCR assay (Gel-B). In RAPD-PCR, Lane M2: 100 bp DNA ladder (Promega); Lanes: 1-3 with primer-1; Lanes: 4-6 with primer-2; Lanes 7-9 with primer-3; and lanes 10-12 with primer-5 for isolates PD-02, PD-06 and PD-08, respectively. In SARA-PCR, Lane 1: isolate PD-02; Lane 2: isolate PD-06 and Lane 3: isolate PD-08 and Lane M1: 100 bp DNA ladder (*In vitrogen*)

SARA-PCR

In the present study we developed SARA-PCR assay, in order to identify identical isolates (PD-02 and PD-06). In SARA-PCR reaction the primer pair catA138/catA742 was used in this reaction However, the amplification reaction was performed at peculiar operative conditions different from the original protocol, in order to gain only one advantage of a SARA-PCR assay (generation of a fingerprint). Several amplification conditions, DNA concentrations and annealing temperature were tested to achieve reproducible PCR products. The most useful DNA fingerprints were obtained at an annealing temperature of 45°C and a 20 times higher concentration of upstream primer catA138 (30 pM) than downstream primer catA742 (1.5 pM). The SARA-PCR patterns obtained for the three selected isolates are shown in Fig. 2B. The size of amplification products ranged from 250 to 1600 bp.

Molecular Analysis of Catabolic Genes

To confirm that these isolate, PD-02, PD-06 and PD-08 containing the catabolic gene sequences. Genomic DNA from these isolates was used as a template in PCR reaction using specific oligonucleotide primers. PCR product of predicted size of catechol 1,2 dioxygenase was determined with positive amplification of 605 bp fragment as shown in Fig. 3A. The first step of phenol degradation is determined by the phenol hydroxylase, (multicomponent phenol hydroxylase). The multicomponent phenol hydroxylase is possibly involved in phenol oxidation. In order to prove this possibility, we used the PCR method to try to amplify a DNA segment of the three phenol-degrading isolates by using primers, pheUf and pheUr complementary to the gene of the largest and the most conserved subunit of the multicomponent phenol hydroxylase. PCR amplification for the partial fragment resulted in positive amplification of specific fragment as was expected (441 bp) as shown in Fig. 3B. On the other hand, catabolic genes encode for catechol 2,3 dioxygenase and salicylate-1-hydroxylase were detected with negative PCR amplification.

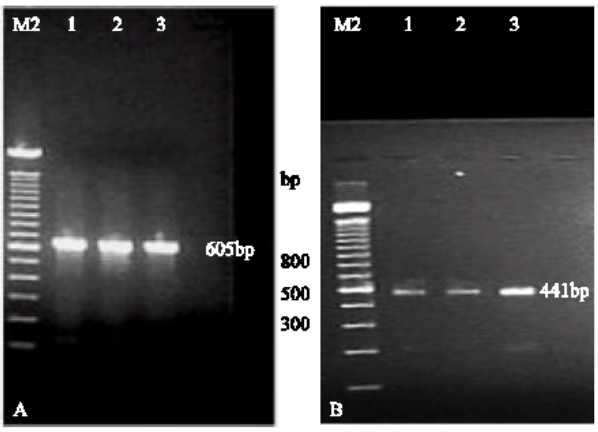

Fig. 3: Detection of catabolic genes in phenol-degrading isolates using specific PCR reaction; Gel A: PCR products of catechol 1,2 Dioxygenase (C12DO); Gel B: PCR Products of multicomponent phenol hydroxylase (mpH). Lane M1: 100 bp DNA ladder (promega), Lane M2: 100 bp DNA ladder (*In vitrogen*); Lane 1: PCR product of isolate PD-02, Lane 2: PCR product of isolate PD-06 and Lane 3: isolate PD-08

Discussion

Phenol is a man-made as well as a naturally occurring aromatic compound and an important intermediate in the biodegradation of natural and industrial aromatic compounds. Environmental contamination can be viewed as an ecological malaise and bioremediation can be prescribed as medicine. Bioremediation is a low-cost treatment alternative for the cleanup of petroleum-contaminated soils and groundwater (Mesarch *et al*., 2000). Microbial isolates for site remediation may be obtained from a variety of sources. The obvious sources are from the contaminated soil itself, where it can be assumed that such species will have adapted to the prevailing ecological conditions.

Three Gram-positive phenol-degrading bacteria were isolated from oil-contaminated soil by direct isolation using phenol as the sole source of carbon and energy. The direct isolation method involves growing isolates directly from environmental samples and is often used to isolate the dominant members in a microbial community. These results indicate the use of the direct isolation method to discover environmentally important bacteria. In this investigation, three bacterial isolates that have capability to degrade phenol were isolated from contaminated soil and designated as *Bacillus* sp. PD-02, PD-06 and PD-08. Due to its simplicity, the direct isolation method is most commonly used to isolate microbes that are capable of degrading a variety of hydrocarbons. However, it has been pointed out that this direct method is highly selective, resulting in the isolation of a few microbial species from diverse natural microbial populations (Dunbar *et al*., 1997). PAH-degrading bacteria belonging to the Gram-positive nocardioforms and spore-forming *Paenibacillus* groups have recently been isolated from the rhizosphere of salt marsh plants (Daane *et al*., 2001). This study suggests that Gram-positive bacteria may play a key role in phenol degradation on contaminated soil. Screening for relatively fast-growing phenol-degrading bacteria resulted in the recovery of three isolates PD-02, PD-06 and PD-08. Although the isolation methods were unbiased and could select for both Gram-positive and Gram-negative bacteria, all three candidate isolates were Gram-positive. The dominance of Gram-positive bacteria should not be unexpected. Gram-positive bacteria have a stronger cell envelope than Gram-negative bacteria and this allows them to thrive in the highly variable

environmental conditions. Francis and Tebo (2002) reported that the ability of *Bacillus* sp. to produce endospores is an adaptation mechanism that may allow strain MN-003 to survive in the highly variable environmental conditions of the intertidal marine sediments from which it was isolated. In this study, the three gram positive isolates were shown to be different from each other based on physiological, biochemical and molecular analyses.

The growth rate of three isolates on different concentrations of phenol was PD-02>PD-08> PD-06. Isolate PD-02 had the fastest maximal specific growth rate, while the growth rates of strains PD-08 and PD-06 were slower than that of isolate PD-02 (Fig. 1). In step before adding the bacteria to contaminated site, it was important to measure the capability of bacterial isolates to grow on different salt concentrations, our results demonstrated that the phenol-degrading bacteria were able to survive in the presence of NaCl concentrations up to 5% in case isolates PD-06 and isolate PD-08 and up to 10% in case isolate PD-02 (Table 2). In previous study, Venosa and Zhu (2003) reported that the rates of hydrocarbon biodegradation decreased with increasing salinity in the range of 3.3-28.4%.

RAPD profiles of phenol degrading isolates used in this work formed two groups with distinctive patterns. Two phenol-degrading bacteria PD-02 and PD-06 were identical as determined by RAPD analysis giving identical RAPD profiles with all primers except primer-2 differentiated the three isolates into two groups; first group includes isolates PD-02 and isolate PD06 and second group include the third isolate PD-08, although DNA fingerprint using RAPD analysis did not result in identification of the isolates, the RAPD profiles were found to be distinct for the isolates, PD-08. Enciso-Moreno *et al*. (2004) used RAPD PCR technique to identify *Serratia marcescens* at the strain level. SARA-PCR was applied to separate identical isolates, PD-02 from isolate PD-06, in SARA-PCR several species-specific patterns could be observed that allowed the visual distinction of the three isolates tested. Knijff *et al*. (2001) reported that SARA-PCR has the potential to give taxonomic information at strain level. In this study, SARA-PCR assay is proved to be more discriminative than RAPD-PCR assay.

Polymerase Chain Reaction (PCR) has been particularly useful for detecting genes involved in the degradation of hydrocarbon compounds. Bacteria that aerobically degrade aromatic hydrocarbons use dioxygenase enzymes to activate and cleave the aromatic ring; therefore, the corresponding genes are excellent targets for PCR assay (Joshi and Walia, 1996). In the present study, the application of the specific PCR primers for C12DO, C23DO, salicylate-1-hydroxylase and phenol hydroxylase resulted in amplification of 605 bp of catechol 1,2 dioxygenase gene and 441 bp of phenol hydroxylase gene from phenol-degrading bacteria. However, the other catabolic genes showed no amplification products. PCR detection of phenol hydroxylase and catechool 1,2 dioxygenase in phenol-degarding isolates suggesting that phenol is degraded by otho pathway. Arai *et al*. (2000) reported that *Comamonas testosteroni* TA441 degraded phenol via meta-pathway because phenol hydoxylase and Catechol 2, 3 dioxygenase were expressed by phenol but catechol 1,2 dioxygenase not detected in *C. testosteroni* TA441 strain.

There several reports on the degradation mechanism of phenol and benzoate by pure culture of bacteria possessing both C12DO and C23DO genes (Ampe and Lindley, 1996; Heald and Jenkins, 1996; Heesche-Wagner *et al*., 1999; Muller and Babel, 1996). Recently, some Gram-positive soil bacteria including the *Arthrobacter* and *Rhodococcus* species were also isolated from various environments and characterized as containing a similar C12DO-dependent catechol-degrading *ortho*-pathway like the one found in the Gram-negative bacteria (Eck and Bettler, 1993; Eulberg *et al*., 1997; Murakami *et al*., 1997; Strachan *et al*., 1998). The majority of bacteria use the *meta* pathway of catechol degradation, especially if the bacteria have multicomponent phenol hydroxylases, like *Pseudomonas* sp. strain CF600 (Powlowski and Shingler, 1990). However, there is also evidence that the multicomponent phenol hydroxylase and the *ortho* pathway of catechol degradation can coexist (Ehrt *et al*., 1995).

Conclusions

Gram-positive bacteria that are capable of using phenol as sole sources of carbon were isolated and characterized. SARA-PCR with the specific primers can be considered a reliable technique to identify phenol degrading isolates at the species level. On the basis of *meta* or *ortho* fission of the aromatic ring in the degradation of phenol, the main catabolic type was found in PD-02, PD-06, PD-08 isolates was ortho pathway through the detection of multicompoenet phenol hydroxylase and catechol 1,2 dioxygenase.

Acknowledgements

I gratefully acknowledge Dr. Ahsraf Montasser, Associate Professor of Entomology, Zoology Department, Ain Shams University for the excellent assistance in preparing the photographs.

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