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Phenol degradation and genotypic analysis of dioxygenase genes in bacteria isolated from sediments

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ABSTRACT

The aerobic degradation of aromatic compounds by bacteria is performed by dioxygenases. To show some characteristic patterns of the dioxygenase genotype and its degradation specificities, twenty-nine gram-negative bacterial cultures were obtained from sediment contaminated with phenolic compounds in Wuhan, China. The isolates were phylogenetically diverse and belonged to 10 genera. All 29 gram-negative bacteria were able to utilize phenol, m-dihydroxybenzene and 2-hydroxybenzoic acid as the sole carbon sources, and members of the three primary genera *Pseudomonas*, *Acinetobacter* and *Alcaligenes* were able to grow in the presence of multiple monoaromatic compounds. PCR and DNA sequence analysis were used to detect dioxygenase genes coding for catechol 1,2-dioxygenase, catechol 2,3-dioxygenase and protocatechuate 3,4-dioxygenase. The results showed that there are 4 genotypes; most strains are either PNP (catechol 1,2-dioxygenase gene is positive, catechol 2,3-dioxygenase gene is negative, protocatechuate 3,4-dioxygenase gene is positive) or PNN (catechol 1,2-dioxygenase gene is positive, catechol 2,3-dioxygenase gene is negative, protocatechuate 3,4-dioxygenase gene is negative). The strains with two dioxygenase genes can usually grow on many more aromatic compounds than strains with one dioxygenase gene. Degradation experiments using a mixed culture representing four bacterial genotypes resulted in the rapid degradation of phenol. Determinations of substrate utilization and phenol degradation revealed their affiliations through dioxygenase genotype data.

Introduction

Phenol and phenolic compounds are important for many industries. They are found in the waste of many industrial processes, such as oil refineries, cooking plants, industrial resin manufacturing, petroleum-based processing plants, pharmaceuticals, plastic manufacturing, and varnish manufacturing industries.[1] Their extensive use has led to the widespread contamination of soils, rivers, industrial effluents,

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and landfill runoff waters. Phenolic compounds have adverse effects on aquatic life, plants and many other organisms, and they can act as substrate inhibitors during the biotransformation process. Thus, it is necessary to eliminate phenolic compounds effectively to protect the environment and to safeguard the health of human beings.[2]

For the removal of phenolic compounds, biological methods have attracted more attention than physical and chemical methods because many different bacteria are known to utilize phenolic compounds as their sole carbon and energy sources.[3] The biodegradation of phenol and its derivatives by bacteria has been extensively studied. A large number of different bacterial species including gram-positive bacteria, such as *Bacillus*[4] and *Rhodococcus*, and gram-negative bacteria, such as *Pseudomonas*,[5,6] *Klebsiella*, *Ochrobactrum*, *Bordetella*, *Achromobacter*, *Halomonas*,[7] *Ralstonia*[8] and *Alcaligenes*, have been reported to degrade phenolic compounds. Among these genera, the *Pseudomonas* genus is known to be an efficient degrader of phenolic compounds, and its presence is very well-established in contaminated sites. *Pseudomonas* sp. CP4 was shown to degrade more than 90% of the initial 500 mg L^{-1} phenol in 24 h, and it was an efficient partner in a mixed culture with *Pseudomonas aeruginosa* 3 mT for the degradation of 3-chlorobenzoate (3-CBA) and phenol/cresol mixtures.[9]

The aerobic degradation pathway of phenolic compounds by bacteria is well-known.[10] Despite the vast changes that occur in phenolic compounds in aquatic and terrestrial environments, the degradation of different phenolic compounds usually proceeds through a limited number of metabolic pathways. Most phenolic compounds are first converted to catechol or protocatechuate.[11] In the α-ketoacid and β-ketoadipate pathways, catechol or protocatechuate is further oxidized by catechol 2,3-dioxygenase, catechol 1,2-dioxygenase or protocatechuate 3,4-dioxygenase to β-ketoadipate.[12,13] This β-ketoadipate is then further converted, with two additional steps, into Krebs cycle intermediates.

To obtain further insight about environmental bacteria that are capable of degrading aromatic compounds, we attempted to collect bacteria that use multiple aromatic compounds and analyze their affiliations from dioxygenase genotype data. This study also includes an analysis of the phenol degradation capability of pure cultures containing both mixed and different genotypes.

Materials and methods

Media

Yeast extract and peptone were purchased from Oxoid Ltd (Basingstoke, England). The minimal medium (MM) was composed of the following (in g L^{-1} of deionized water): KH_2PO_4 1 g, Na_2HPO_4 1.3 g, $(NH_4)_2SO_4$ 1 g, $MgSO_4$ 0.2 g, $MnCl_2{\cdot}4H_2O$ 0.005 g, $NaMoO_4{\cdot}2H_2O$ 0.001 g, and $CuCl_2$ 0.0005 g. The pH was adjusted to 7.0. After autoclaving the media at 120 °C for 20 min, it was supplemented with filter-sterilized solutions of 0.05 g L^{-1} $FeSO_4{\cdot}7H_2O$, 0.02 g L^{-1} $CaCl_2$, and 0.005 g L^{-1} $ZnSO_4{\cdot}7H_2O$ 0.005 g. Different aromatic compounds were used as the sole carbon and energy sources, respectively. Solid MM plates contained 15 g L^{-1} agar. The LB medium was composed of the following (in g L^{-1} of deionized water): 10 g NaCl, 10 g peptone, and 5 g yeast extract, pH 7.0.

Isolation of phenol-degrading bacteria

Sediment samples were collected at a site near the primary pollutant-emission outlet of a chemical plant located in Wuhan, China. Pollutants have been released into the environment from this site without any control for many years; these pollutants include phenolic compounds, primarily phenol, chlorophenols, and methylaminophenol. The sediment contained approximately 457 mg kg^{-1} phenol, pH 6.36. Sediment samples were collected and then stored in closed containers at 4 °C before use. Enrichment cultures were prepared from the sediment slurry using liquid MM medium. Ten grams of slurry was added to 90 mL of MM medium in a 250-mL Erlenmeyer flask. Phenol was added at a concentration of 500 mg/L. The flasks were incubated at 30 °C with shaking (200 rpm) for 2 days. The culture suspension was serially diluted and plated onto MM agar medium containing 500 mg L^{-1} phenol.[14] The cultures that were capable of forming clear zones were checked for purity by plating them on LB agar. Isolated colonies were gram-stained and examined microscopically. In total, 29 of the 50 pure isolated cultures were stored at −20 °C in LB broth containing 20% glycerine.

Growth on monoaromatic compounds

Analytical-grade monoaromatic compounds (phenol, m-dihydroxybenzene, benzene-1,2,3-triol, 3,5-dinitrosalicylic acid, 4-dimethylaminobenzaldehyde 1,2-diaminobenzene, 2-hydroxybenzoic acid, 2,4,6-trinitrophenol, o-aminobenzoic acid, 4-nitrobenzoic acid, and potassium 2-carboxybenzoate) were prepared as stock solutions at 10 g L^{-1}. Each stock solution was filter-sterilized through a 0.2 μm filter and added to liquid MM medium at a final concentration of 100 mg L^{-1}. The solid culture method was used to determine the carbon source in use; this approach has been accepted and used in many studies.[14] The cultures were grown overnight in LB broth (tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; and NaCl, 10 g L^{-1}), followed by two washes with 50 mM phosphate buffer and resuspension in the same volume of liquid MM medium, and then 2 μL of each of the cultures was spotted onto monoaromatic compound MM plates. In this way, 8 cultures per plate were conveniently tested. Duplicate plates were prepared for each monoaromatic compound, and then they were incubated at 30 °C. Each plate was checked for growth after 4 days of culture. MM agar plates without monoaromatic compound were used as controls.

16S rRNA gene isolation and sequencing

Genomic DNA was isolated from the bacterial strains that were capable of degrading one or more of the monoaromatic compounds tested using the method developed by Yoon et al.[15] Purified DNA was then subjected to PCR amplification. Universal primers were used, with fD1 for positions 7–26 in the *Escherichia coli* 16S rRNA gene and rD1 for positions 1541–1525 (Table 1). Fifty microliters of each PCR mixture consisted of 3 μL of extracted DNA, 2 μL of dNTPs (2.5 mM), 2 μL of primers

Table 1 – Names and sequences of primers used in this study.

Primer	Sequence (5′–3′)[a]	Fragment length (bp)	References
fD1	AGAGTT TGATCCTGGCTCAG	1519	Winker and Woese (1991)[40]
rD1	AAGGAGGTGATCCAGCC		
CAT2-3f	TGATCGAGATGGACCGTGACG	821	Alquati et al. (2005)[41]
CAT2-3r	TCAGGTCAGCACGGTCATGAA		
Cat1-2f	AAACCCGCGCTTCAAGCAGAT	650	Marta et al. (2006)[27]
Cat1-2r	AAGTGGATCTGCGTGGTCAGG		
PRO3-4f	CTAYAARACCWSCGTSSYGCGC	490	This study
PRO3-4r	GATCAYCGGRTCGCCYTSG		

[a] Y = C or T, R = A or G, S = C or G, and W = T or A.

fD1 and rD1 (10 mM), 5 units of *Taq* DNA polymerase, 5 μL of 10 × PCR buffer and ddH_2O up to 50 μL. The thermocycling conditions were as follows: an initial denaturation step at 94 °C for 1 min, followed by 35 cycles of 56 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 10 min using a Personal Biometra Thermal Cycler DNA engine tetrad (Gottingen, Germany). The resulting PCR products were subjected to electrophoresis through a 1.0% (w/v) agarose gel, which was stained with ethidium bromide and visualized under UV light. An approximately 1500 bp PCR product was purified with an Omega Bio-Tek E.Z.N.A Gel Extraction Kit, and the purified DNA was cloned into plasmid vector pMD-18 T (Takara, DaLian, China). The clones were checked with PCR to be sure they contained the correct insert size. Sequencing was then performed using M13 universal primers on an ABI® 3730 automated DNA sequencer.[16]

Sequence analysis

The sequences were edited to remove vector contaminants and primer sequences. To identify the sequences, the cloned sequences were compared with the 16S rRNA gene sequences of existing bacteria in the NCBI database. Related sequences were obtained from the GenBank Nucleotide database using the BLAST search program. All the sequences were edited to a common length and aligned using the ClustalW program. A phylogenetic tree was constructed by neighbor joining method. To test the stability of the groups, a bootstrap analysis of 10,000 replications was performed with a MEGA version 4.1 program.[17,18]

Amplification of dioxygenase genes

The templates for PCR amplification were the genomic DNA that was isolated from the gram-negative bacteria that were used previously for 16S rRNA gene amplification. The catechol 2,3-dioxygenase gene and catechol 1,2-dioxygenase gene were amplified with CAT2-3f/CAT2-3r primers and Cat1-2f/Cat1-2r primers, respectively (Table 1). The degenerate PCR primers PRO3-4f and PRO3-4r were used to amplify a 490-bp fragment of the protocatechuate 3,4-dioxygenase gene (Table 1). Primer pairs PRO3-4f and PRO3-4r are based on the coding sequence for the beta subunits of protocatechuate 3,4-dioxygenase found in *P. aeruginosa* (X60740), *Pseudomonas putida* (D37783) and *Ralstonia pickettii* (CP001068). These strains were used as positive controls to determine whether one or more of the dioxygenase genes are present in the 29 isolates used in this study. Each PCR mixture with a final volume of 25 μL contained 0.2 mM dNTP, 20 pmols of each primer, 1 μL of extracted DNA and 2 units of *Taq* DNA polymerase with 1× *Taq* polymerase buffer. The PCR touchdown thermocycling conditions were as follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles with 94 °C for 30 s, annealing temperature step-downs of 0.3 °C (from 60.2 °C to 50 °C) for 1 min, and 72 °C for 2 min, with a final extension of 7 min at 72 °C. Product formation was confirmed by 1% (w/v) agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light. The dioxygenase genes were cloned, sequenced and analyzed as described in sections "16S rRNA gene isolation and sequencing" and "Sequence analysis".

Degradation of phenol

Phenol degradation experiments were performed in liquid MM medium containing 500 mg/L phenol in triplicate flasks. A culture was grown (50 mL) in LB liquid medium overnight at 30 °C with shaking at 200 rpm, and it was harvested and rinsed twice with 50 mM phosphate buffer. For each single culture experiment involving only one bacterial species per flask, a freshly prepared 2% (v/v) inoculum of *Pseudomonas* sp. PH11, *Pseudomonas* sp. PH7, or *Ralstonia* sp. PH19 was used. For the mixed culture experiments, which involved a combination of all three bacteria per flask, 0.67% (v/v) inoculums of *Pseudomonas* sp. PH11, *Pseudomonas* sp. PH7 and *Ralstonia* sp. PH19) were used. The flasks were then incubated at 30 °C with shaking at 200 rpm. Samples were taken at 6 h intervals and analyzed for phenol content. The abiotic controls consisted of preparations that were incubated under the same conditions using autoclave-killed bacteria. To determine the quantity of phenol present in the liquid medium, the colorimetric assay developed by Martin was performed.[19] Phenol reacts with 4-aminoantipyrin and forms a red indophenol dye under alkaline conditions. The absorbance of this dye was measured at 460 nm in a Beckman Coulter DU800 UV/Vis Spectrophotometer. The phenol concentration was determined by comparing the absorbance value with that of a standard curve for phenol (0–500 mg L^{-1}). All the tests in this study were performed over three independent experiments.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were deposited in the GenBank Nucleotide database. The accession

numbers of the 16S rRNA genes from strains PH1 to PH29 are JN171666 to JN171694, respectively. The accession numbers of the catechol 1,2-dioxygenase gene from *Pseudomonas* sp. PH11, the catechol 2,3-dioxygenase gene from *Pseudomonas* sp. PH7, and the protocatechuate 3,4-dioxygenase gene from *Ralstonia* sp. PH19 are JN171696, JN171695 and JN171697, respectively.

Results

Isolation of phenol-degrading gram-negative bacteria

The twenty-nine gram-negative isolates that were used as part of this study all formed isolated colonies when plated on solid MM media. Each isolate was identified on the basis of their morphology and 16S rDNA gene sequence analysis. The isolated strains included 8 (27.5%) *Pseudomonas* spp., 6 (20.5%) *Acinetobacter* spp., 6 (20.5%) *Alcaligenes* spp., 2 *Ralstonia* (7%) spp., 2 (7%) *Bordetella* spp., 1 (3.5%) *Burkholderia* sp., 1 (3.5%) *Azospirillum* sp., 1 (3.5%) *Plesiomonas* sp., 1 (3.5%) *Sphingomonas* sp., and 1 (3.5%) *Achromobacter* sp.

Taxonomic identification of the isolates

On the basis of the consensus sequences for the 16S rRNA gene, a phylogenetic tree was constructed using sequences from the 29 strain isolates and representative gram-negative bacteria (Fig. 1). The phylogenetic analysis showed that the 29 aromatic compound-degrading bacteria shared high 16S rDNA gene sequence similarities with one another and belonged to two clusters. Members of the genera *Azospirillum* and *Sphingomonas* were supported by a >99% bootstrap value and were well-established within Cluster 2. Members of Cluster 1 possessed much broader specificity and could be divided into two diverse sub-clusters. The *Acinetobacter*, *Plesiomonas*, and *Pseudomonas* genera showed closer relations, supporting a >95% bootstrap value to confirm their positions within Subcluster 1. A close relation between the *Ralstonia*, *Bordetella*, *Achromobacter*, and *Alcaligenes* genera were supported by a high bootstrap value, and these strains were well-established within Subcluster 2.

Carbon-source utilization

The ability of the gram-negative bacteria to grow on a variety of different carbon sources was tested in liquid media containing one of 11 monoaromatic compounds as the sole carbon source. Among the 29 isolated species, *Pseudomonas* spp., *Acinetobacter* spp., *Alcaligenes* spp., *Ralstonia* spp., *Bordetella* sp. PH21 and *Achromobacter* sp. PH23 were able to utilize at least six of the monoaromatic compounds tested (Table 2). *Pseudomonas* spp., *Acinetobacter* spp., *Alcaligenes* spp., and *Ralstonia* spp. showed much greater metabolic versatility than the other strains. All the isolates grew well on phenol, m-dihydroxybenzene and 2-hydroxybenzoic acid. These compounds are considered intermediates, which are produced by aromatic substrate degradation via the salicylate pathway.[20] O-aminobenzoic acid was also a good growth substrate for 28 isolates. The only exception was *Sphingomonas* sp. PH20. The *Pseudomonas* sp. PH7 and PH9 strains were able to utilize

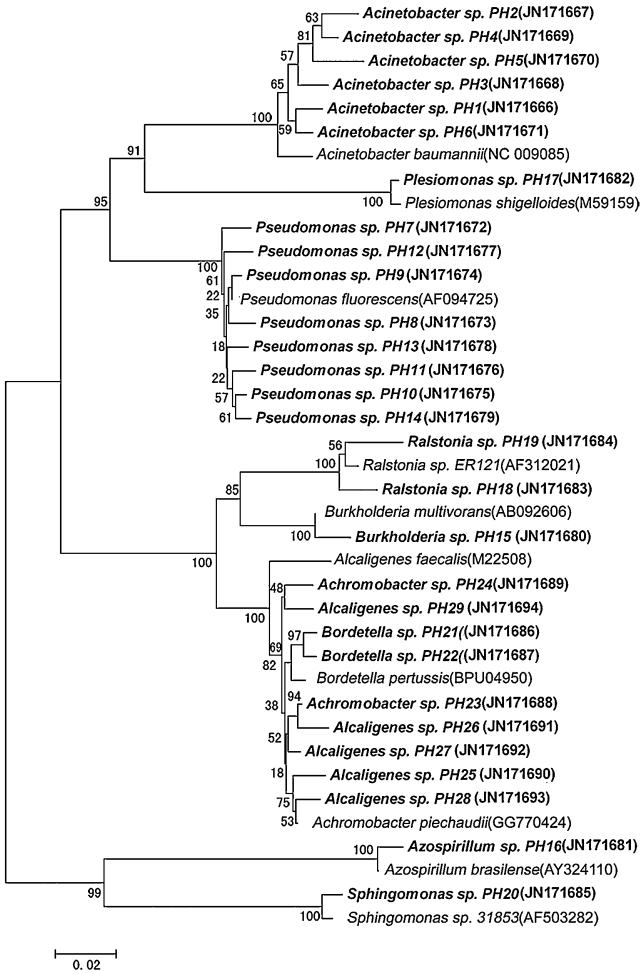

Fig. 1 – Phylogenetic tree of the 29 gram-negative strains isolated in this study and related species. The dendrogram was based on an approximately 800 bp segment of the 16S rRNA gene sequence and was constructed by neighbor-joining method. The sequences generated from this study are highlighted in bold text and are compared with other related species. The scale bar indicates a 2% sequence divergence. Bootstrap probabilities are shown near the nodes, and GenBank accession numbers are given in parentheses.

all the tested compounds. Seven other isolates, namely *Acinetobacter* sp. PH3, *Acinetobacter* sp. PH4, *Pseudomonas* sp. PH10, *Pseudomonas* sp. PH11, *Pseudomonas* sp. PH14, *Alcaligenes* sp. PH24 and *Alcaligenes* sp. PH28, were able to utilize 10 different monoaromatic compounds.

Amplification of dioxygenase genes

To determine whether catechol 1,2-dioxygenase was present, PCR amplifications were performed using Cat1-2f and Cat1-2r primers, which are specific for the *pheB* gene of *P. putida*. The expected 650 bp fragment was amplified from genomic DNA that was isolated from all the gram-negative bacteria except for *Burkholderia* sp. PH15, *Pseudomonas* sp. PH8 and *Pseudomonas sp.* PH13 (Table 3). To determine whether the

Table 2 – Growth on monoaromatic compounds by gram-negative bacterial strains.[a]

Organism	Monoaromatic compounds[b]									
	1	2	3	4	5	6	7	8	9	10
Acinetobacter sp. PH1	+	+	+	+	+	+	–	+	+	–
Acinetobacter sp. PH2	+	+	+	–	+	+	+	+	–	–
Acinetobacter sp. PH3	+	+	+	+	+	+	–	+	+	+
Acinetobacter sp. PH4	+	+	+	+	+	+	–	+	+	+
Acinetobacter sp. PH5	+	+	–	–	–	+	–	+	+	–
Acinetobacter sp. PH6	+	+	–	–	+	+	+	+	+	–
Pseudomonas sp. PH7	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp. PH8	+	+	+	+	–	+	–	+	+	–
Pseudomonas sp. PH9	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp. PH10	+	+	+	+	+	+	–	+	+	+
Pseudomonas sp. PH11	+	+	+	+	+	+	+	+	+	–
Pseudomonas sp. PH12	+	+	+	+	+	+	–	+	+	–
Pseudomonas sp. PH13	+	+	+	+	–	+	–	+	+	–
Pseudomonas sp. PH14	+	+	+	+	+	+	–	+	+	+
Burkholderia sp. PH15	+	+	–	–	+	+	–	+	–	–
Azospirillum sp. PH16	+	–	–	–	–	+	–	+	–	–
Plesiomonas sp. PH17	+	+	–	–	–	+	–	+	–	–
Ralstonia sp. PH18	+	+	+	+	+	+	–	+	+	–
Ralstonia sp. PH19	+	+	+	–	+	+	–	+	–	–
Sphingomonas sp. PH20	+	–	–	+	–	+	–	–	–	–
Bordetella sp. PH21	+	+	–	–	+	+	–	+	+	–
Bordetella sp. PH22	+	–	–	–	+	+	–	+	–	–
Achromobacter sp. PH23	+	+	–	–	+	+	–	+	–	–
Alcaligenes sp. PH24	+	+	+	+	+	+	+	+	–	+
Alcaligenes sp. PH25	+	+	–	–	+	+	–	+	+	–
Alcaligenes sp. PH26	+	+	–	+	+	+	–	+	–	–
Alcaligenes sp. PH27	+	+	–	–	+	+	+	+	–	–
Alcaligenes sp. PH28	+	+	+	+	+	+	+	+	–	+
Alcaligenes sp. PH29	+	+	–	–	+	+	–	+	–	–

[a] Growth was checked on MM plates containing 100 mg/L growth substrate. The plates were incubated for 4 days at 30 °C.
[b] 1, m-dihydroxybenzene; 2, benzene-1,2,3-triol; 3, 3,5-dinitrosalicylic acid; 4, 4-dimethylaminobenzaldehyde; 5, 1,2-diaminobenzene; 6, 2-hydroxybenzoic acid; 7, 2,4,6-trinitrophenol; 8, o-aminobenzoic acid; 9, 4-nitrobenzoic acid; and 10, potassium 2-carboxybenzoate.

catechol 2,3-dioxygenase gene was present, the CAT2-3f and CAT2-3r primers were used, and they were specific for the *xylE* gene of *P. aeruginosa*. The expected 821 bp fragment was amplified from genomic DNA that was isolated from only *Pseudomonas* sp. PH9 and *Pseudomonas* sp. PH7 (Table 3). No PCR product was generated with the other 27 isolates. To determine whether the protocatechuate 3,4-dioxygenase gene was present, the degenerate primers PRO3-4f and PRO3-4r were used. The expected 490 bp fragment from the protocatechuate 3,4-dioxygenase gene was amplified from genomic DNA that was isolated from *Acinetobacter* sp. PH1, 3–4, and 6; *Pseudomonas* sp. PH8, 10, and 12–14; *Burkholderia* sp. PH15; *Azospirillum* sp. PH16; *Ralstonia* sp. PH18 and 19; *Bordetella* sp. PH21; and *Alcaligenes* sp. PH 24 and 28 (Table 3). No PCR product was generated from the other 13 strains.

Sequence analysis of dioxygenase genes

To verify the presence of the catabolic genes catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, and protocatechuate 3,4-dioxygenase, PCR-amplified fragments of these genes were sequenced from three different bacteria, namely *Pseudomonas* sp. PH11, *Pseudomonas* sp. PH7, and *Ralstonia* sp. PH19. These strains are capable of degrading multiple monoaromatic compounds (Table 2), and so they were chosen for further studies. The partial nucleotide sequence of the catechol 1,2-dioxygenase gene from *Pseudomonas* sp. PH11 was 99, 96 and 94% similar to the same gene found in *P. putida* KT24400, *P. arvilla* and *Stenotrophomonas maltophilia* KB2, respectively (Table 4). The partial nucleotide sequence of the catechol 2,3-dioxygenase gene from *Pseudomonas* sp. PH7 was 99, 99 and 92% similar to the *xylEJI104-1* gene present in *P. aeruginosa* JI104, the *nahH* gene present in *P. stutzeri* CLN100, and the catechol 2,3-dioxygenase gene present in *Achromobacter xylosoxidans* LHB21, respectively (Table 4). The partial nucleotide sequence of the protocatechuate 3,4-dioxygenase gene for *Ralstonia* sp. PH19 was 95 and 87% similar to the protocatechuate 3,4-dioxygenase gene found in *R. pickettii* 12J and the *pcaH* gene found in *R. solanacearum* strain IPO1609 (Table 4).

Phenol degradation by pure and mixed cultures

Pseudomonas sp. PH11, *Pseudomonas* sp. PH7, *Pseudomonas* sp. PH10, and *Pseudomonas* sp. PH8 represent the four different dioxygenase genotypes and can grow well in several monoaromatic compounds. To assess the importance of dioxygenase genes in phenol degradation, these strains were selected to study their abilities to degrade phenol. The degradation of phenol by pure and mixed cultures was studied at a phenol concentration of 500 mg L^{-1} (Fig. 2). A mixed culture of all

Table 3 – PCR amplification of the dioxygenase genes from gram-negative bacterial strains.[a]

Organism	Catechol 1,2-dioxygenase gene	Catechol 2,3-dioxygenase gene	Protocatechuate 3,4-dioxygenase gene	Genotype[b]
PH1	Positive	Negative	Positive	PNP
PH2	Positive	Negative	Negative	PNN
PH3	Positive	Negative	Positive	PNP
PH4	Positive	Negative	Positive	PNP
PH5	Positive	Negative	Negative	PNN
PH6	Positive	Negative	Positive	PNP
PH7	Positive	Positive	Negative	PPN
PH8	Negative	Negative	Positive	NNP
PH9	Positive	Positive	Negative	PPN
PH10	Positive	Negative	Positive	PNP
PH11	Positive	Negative	Negative	PNN
PH12	Positive	Negative	Positive	PNP
PH13	Negative	Negative	Positive	NNP
PH14	Positive	Negative	Positive	PNP
PH15	Negative	Negative	Positive	NNP
PH16	Positive	Negative	Positive	PNP
PH17	Positive	Negative	Negative	PNN
PH18	Positive	Negative	Positive	PNP
PH19	Positive	Negative	Positive	PNP
PH20	Positive	Negative	Negative	PNN
PH21	Positive	Negative	Positive	PNP
PH22	Positive	Negative	Negative	PNN
PH23	Positive	Negative	Negative	PNN
PH24	Positive	Negative	Positive	PNP
PH25	Positive	Negative	Negative	PNN
PH26	Positive	Negative	Negative	PNN
PH27	Positive	Negative	Negative	PNN
PH28	Positive	Negative	Positive	PNP
PH29	Positive	Negative	Negative	PNN

[a] The PCR-amplified products of the dioxygenase genes were analyzed by agarose gel electrophoresis. Positive, the strain has the expected fragment; negative, the strain does not have the expected fragment.

[b] The first letter represents the catechol 1,2-dioxygenase gene, the second letter represents the catechol 2,3-dioxygenase gene, and the last letter represents the protocatechuate 3,4-dioxygenase gene. P, the strain has the expected fragment; N, the strain does not have the expected fragment.

four bacteria representing 4 different dioxygenase genotypes degraded 15.8% of the initial phenol after 12 h of incubation. Within the next 24 h, 78.5% of the added phenol was degraded, and after 42 h, more than 99.5% of the phenol was degraded. The ability of the mixed culture to degrade phenol (in 42 h and 48 h) is of interest because the degradation was statistically significantly ($p < 0.001$) more quickly than in the other three pure cultures. Within 48 h, *Pseudomonas* sp. PH7 had a statistically significantly ($p < 0.001$) higher phenol degradation level (99.7%) than those of *Pseudomonas* sp. PH11 (93.4%), *Pseudomonas* sp. PH8 (86.3%) and *Pseudomonas* sp. PH10 (92.1%). During the initial 12 h lag phase, *Pseudomonas* sp. PH7 and *Pseudomonas* sp. PH11 had relatively higher phenol degradation levels. These levels reached 20.6 and 17.9%, respectively, in comparison with the mixed culture, in which only 15.8% was degraded.

Table 4 – Sequence homologies of the dioxygenase genes for phenol-degrading bacteria.

Organism	Dioxygenase gene (accession number)	Homology	Homologous gene (accession number)	Source
Pseudomonas sp. PH11	Catechol 1,2-dioxygenase gene (JN171696)	99% 96% 94%	Catechol 1,2-dioxygenase gene (AE015451) Catechol 1,2-dioxygenase (D37783) Catechol 1,2-dioxygenase gene (EU00039)	*P. putida* *P. arvilla* *Stenotrophomonas maltophilia*
Pseudomonas sp. PH7	Catechol 2,3-dioxygenase gene (JN171695)	99% 99% 92%	*xylEJI*104-1 gene (X60740) *nahH* gene (AJ539383) Catechol 2,3-dioxygenase gene (GU199432)	*P. aeruginosa* *P. stutzeri* *Achromobacter xylosoxidans*
Ralstonia sp. PH19	Protocatechuate 3,4-dioxygenase gene (JN171697)	95% 87%	*pcaH* gene (CP001068) *pcaH* gene (CU914168)	*R. pickettii* *R. solanacearum*

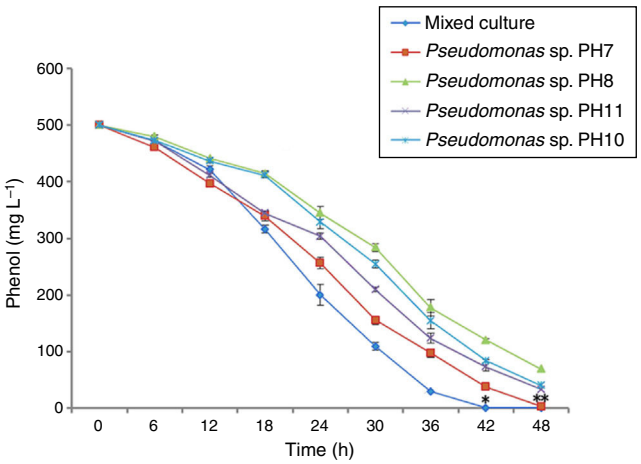

Fig. 2 – Degradation curve of phenol using pure and mixed cultures. Phenol (500 mg L^{-1}) was added to the MM medium, inoculated with 2% (v/v) cultures and incubated at 30 °C and 200 rpm. Statistical analyses were performed with Student's t test in SPSS 17.0 software (SPSS Inc, Chicago, IL, USA), and the error bars represent the means (±SD) of three independent experiments. * The phenol degradation rate of the mixed culture is significantly higher than the rates for strains PH7, PH8, PH11, and PH10 ($p < 0.001$). ** The phenol degradation rate of strain PH7 is significantly higher in comparison with strains PH8, PH11, and PH10 ($p < 0.001$).

Discussion

Different methods have been used for the elimination of phenol and phenolic compounds, but the use of biodegradation methods is universally preferred because of their lower costs and the possibility of complete mineralization.[2] Bacteria that have the ability to use phenol can be used for biodegradation within environments that are contaminated with phenolic compounds. In this study, we describe the isolation and screening of 29 selected phenolic compound-degrading bacterial isolates and the characterization of the dioxygenase genes from three strains by PCR amplification. These strains mostly belong to *Pseudomonas*, *Acinetobacter* and *Alcaligenes*. PCR assays revealed that the three genes were not equally distributed in the isolated strains, and the catechol 2,3-dioxygenase gene was found in only *Pseudomonas* sp. PH7 and PH9. The mixed culture involved three dioxygenase genes, and it exhibited rapid phenol degradation.

The phylogenetic analysis showed that the 29 phenolic compound-degrading bacteria shared high 16S rDNA gene sequence similarities with one another and belonged to two clusters. Members of Cluster 1 possessed much broader specificity, and they could use anywhere from 5 to 11 substrates with 4 substrates that are consistently conserved throughout these strains (Table 2). Approximately 70% of these strains were members of three genera: *Pseudomonas* (8 strains, 27.5%), *Acinetobacter* (6 strains, 20.5%), and *Alcaligenes* (6 strains, 20.5%). Among the phenol-degrading gram-negative bacteria, *Pseudomonas*, *Acinetobacter* and *Alcaligenes* are regarded as the most common species to be isolated from contaminated sites. The abilities of these species to utilize phenolic compounds in particular have been widely documented.[21–23] Cluster 2 has only two members. Interestingly, this small group can utilize at least four substrates (Table 2). The results indicate that bacteria that are capable of degrading aromatic compounds are phylogenetically diverse.

In this study, more than ten carbon sources were tested as sole carbon substrates for the 29 isolates, including various monoaromatic compounds. All the strains could utilize 2-hydroxybenzoic acid, which is considered an intermediate product of aromatic substrate degradation via the salicylate route pathway.[20] In comparison with the other 7 genera, the strains that were members of the *Pseudomonas*, *Acinetobacter* and *Alcaligenes* genera were able to grow on many more carbon sources (Table 2). Because of their abilities to use a wide diversity of carbon energy substrates, they can compete effectively with other bacteria and become dominant culturable members that are capable of utilizing the phenolic compounds found in contaminated sludge.[24]

PCR analysis was performed to detect dioxygenase genes encoding catechol 2,3-dioxygenase, catechol 1,2-dioxygenase, and protocatechuate 3,4-dioxygenase, which oxidize catechol or protocatechuate via the α-ketoacid and the β-ketoadipate pathways. The identity of the PCR-amplified fragments was further verified through a sequence analysis of selected strains. The three dioxygenase genes were amplified, which means that both the α-ketoacid and β-ketoadipate pathways serve as general mechanisms for the catabolism of catechol or protocatechuate derived from phenolic compounds.[25] However, the three genes are not equally distributed in the isolated strains. The catechol 2,3-dioxygenase gene is found in only two *Pseudomonas* strains, PH7 and PH9, but the catechol 1,2-dioxygenase gene is found in all the gram-negative bacteria except for *Burkholderia* sp. PH15 and *Pseudomonas* sp. PH8 and PH13. The majority of studies on the detection of catabolic genes in contaminated environments have focused on gram-negative bacteria, such as *Pseudomonas*, *Burkholderia*, *Acinetobacter* and *Sphingomonas*.[26] These strains usually carry one or two dioxygenase genes,[27] but catechol 2,3-dioxygenases constitute a group of enzymes that are considered crucial for the degradation of a wide range of aromatic compounds in contaminated habitats, and they are present in most aromatic compound-degrading strains.[28,29] Because these dioxygenase genes are commonly distributed between plasmids of different sizes and are found on the chromosome,[30] a suggestion has been made that these genes may spread to divergent bacteria by horizontal transfer.[31] The detection of these genes within the strains isolated from sludge that was contaminated with phenolic compounds provides evidence that these genes may have differentiated during the evolution of different gram-negative bacterial communities.[32]

An analysis of Table 3 also shows that there are 4 genotypes. Most strains are either PNP or PNN, only three strains are NNP and two strains are PPN. In using a step-wise model of gene acquisition and loss, it is easy to order these genotypes in a parsimonious order (NNP-PNP-PNN-PPN), with the most common genotypes being central. Of the genotypes that were not found (NNN, NPN, NPP, and PPP), only PPP and NNN can be formed from these central genotypes. Clearly, NNN may

not be able to degrade phenol and was thus never enriched. PPP may be 'too costly' to maintain, but there was an exception (*S. maltophilia* KB2) that carries the three dioxygenase genes.[33] NPN and NPP may be feasible, but because of the prevalence of these genes in this environment, they may occur rarely and by chance and may not related to fitness. Alternatively, catechol 1,2-dioxygenase is under strong selection, with protocatechuate 3,4-dioxygenase under medium selection and catechol 2,3-dioxygenase under weak selection. The frequencies of these genotypes reflect the stable phenotypes that could exist. This finding could be linked to the results presented in Table 2. Strains with two dioxygenase genes can grow on many more aromatic compounds than strains with one dioxygenase gene, except strain PH11. However, *Azospirillum* strain PH16, which also carries two dioxygenase genes, utilizes only 4 aromatic compounds because of phylogenetic diversity as mentioned above. It is possible that the presence of a second dioxygenase gene reflects differences in the substrate degradation by the cells, as previously noted.[34] It is not clear whether these genes are plasmid and/or chromosomally mediated. Further experimentation is required.

To decrease the phylogenetic differences in terms of degradation activity, four *Pseudomonas* strains (PH11, PH7, PH10 and PH8) that carry different dioxygenase genes and represent the four genotypes of PNN, PPN, PNP and NNP were chosen for phenol biodegradation analysis. The experimental data indicated that the ternary mixed culture (involving three genes) and *Pseudomonas* sp. PH7 (involving two genes) had fairly high phenol degradation potential. When cells are introduced into a toxic environment, there are a number of different response mechanisms that could have an effect on the degradation and growth properties, and the accumulation of intermediates and/or dead-end products can inhibit enzyme activity. For example, *cis*-muconate and adipic acid can inhibit catechol 1,2-dioxygenase activity,[35,36] and 2-oxopent-4-dienoate can inhibit catechol 2,3-dioxygenase activity.[37] The bacteria in the mixed culture and *Pseudomonas* sp. PH7 carry a total of three and two dioxygenase genes, respectively. The bacteria can degrade phenol via multiple pathways. As a result, a low level of feedback repression in the intermediate products is maintained.[38] *Pseudomonas* sp. PH10 also consists of two parallel branches. One branch starts at catechol and the other branch begins at protocatechuic acid. Catechol is cleaved by catechol 1,2-dioxygenase and protocatechuic acid is cleaved by protocatechuate 3,4-dioxygenase. The two branches converge at the same intermediate, or β-ketoadipate enol-lactone, which acts as feedback inhibitor of dioxygenase activity.[13,39,11]

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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