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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

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