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Cloning and Sequencing of a Phenol Hydroxylase Gene of *Pseudomonas pseudoalcaligenes* Strain MH1

A Bacterium Able to Mineralize Various Aromatic Compounds

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Abstract

The phenol-degrading strain *Pseudomonas pseudoalcaligenes* MH1, identified by the rRNA approach, was isolated from wastewater enrichment culture. It utilized phenol up to 1.5 g/L as the sole source of carbon and energy. In addition, cresols (*o*-, *m*-, *p*-), 4-hydroxybenzoic acid, syringic acid, and vanillic acid were metabolized as sole substrates by phenol-grown cells of strain MH1. Using primers, designed on the basis of the sequence of the *dmp* operon of *P. putida* strain CF600, a gene encoding phenol hydroxylase, which catalyzes the hydroxylation of phenol to catechol, was detected in strain MH1. The whole phenol hydroxylase operon of strain MH1 was amplified in a polymerase chain reaction fragment of 5.207 kb that was cloned and sequenced. The total sequence revealed a cluster of six ATG starting open reading frames (ORFs). Analysis of the regulatory signals showed a putative promoter region, 40 bp upstream from the transcriptional start of ORF1, which have a strong homology to a set of positively controlled promoters. Comparison of the MH1 phenol hydroxylase gene sequence with those of other *Pseudomonas* strains revealed higher homology except in the 5' region. Thus, the deduced amino acid sequence of the first subunit of phenol hydroxylase of *P. pseudoalcaligenes* strain MH1 exhibited a difference at the N-terminal region (the first 10 amino acids) compared with that of known phenol hydroxylase of *Pseudomonas* strains.

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Index Entries: Degradation; aromatic compounds; cresol; 4-hydroxybenzoic acid; syringic acid; vanillic acid; phenol hydroxylase; sequence; cloning.

Introduction

Phenol and derivatives, which are toxic to living organisms, are common in the wastes/wastewater from many industrial processes, including those from oil refineries, petrochemical plants, and steel mill coke plants. Many attempts to isolate phenol-degrading microorganisms and to adapt them to biologic wastewater treatment processes have been reported *(1,2)*.

A number of microorganisms have been found to degrade phenol *(3–7)*, in particular *Pseudomonas* spp., which can metabolize most natural and synthetic organic compounds *(8)*. In recent years, interest in the microbial ability to degrade and detoxify aromatic compounds that pollute the environment has markedly increased. Many catabolic pathways of aromatic compound degradation have been elucidated *(9)*, in particular that of phenol. It has been shown that the first step in the catabolism of phenol in an oxygenated environment is its hydroxylation to catechol, a reaction catalyzed by a phenol hydroxylase that incorporates a single hydroxyl group into the substrate *(10)*. This hydroxylation is followed by ring cleavage, which produces aliphatic intermediates that can be readily converted to metabolites of the Krebs cycle, such as pyruvate, succinate, and acetyl coenzyme A.

Two different types of phenol hydroxylases have been identified. A single-chain flavoprotein isolated from the basidiomycetous yeast *Trichosporon cutaneum* functions as phenol monooxygenase *(6)*. A phenol hydroxylase from *Pseudomonas pickettii* also shows the characteristics of a single-chain flavoprotein *(11)*. By contrast, the phenol hydroxylase from most *Pseudomonas* spp. is a complex enzyme consisting of six polypeptides, encoded by six genes forming an operon *(12–15)*.

The aim of the present work was to characterize a strain of *Pseudomonas pseudoalcaligenes* isolated from a contaminated site able to degrade various aromatic compounds. The gene encoding the phenol hydroxylase of this strain was cloned and sequenced. The sequence was analyzed and compared with that of other enzymes with similar properties.

Materials and Methods

Media and Growth Conditions

Escherichia coli JM109 was grown in Luria-Bertani (LB) medium (10 g/L of bactopeptone, 5 g/L of yeast extract, 10 g/L of NaCl) or 2XYT medium (16 g/L of bactopeptone, 10 g/L of yeast extract, 10 g/L of NaCl) at 37°C. Ampicillin at a final concentration of 100 µg/mL was used for selection of plasmids.

The basal medium used for enrichment, isolation, and cultivation contained the following: 2.44 g/L of NaH_2PO_4, 1.25 g/L of KH_2PO_4, 0.5 g/L of

Table 1
Bacterial Strains and Plasmids Used

	Relevant markers and derivation	Reference or source
Strain		
P. pseudoalcaligenes MH1	Phe^+	This study
E. coli JM109	*RecA1 endA1gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lac-proAB*)/*F'* (*traD36 proAB*$^+$ *lacI*^{q} lacZΔM15)	Promega
Plasmid		
pGME^T	Amp^+, cloning vector	Promega
pGME A2	Amp^+, 5.207-kb PCR product cloned in pGME^T	This study

Phe^+, growth in phenol; Amp^+, resistance to ampicillin.

$(NH_4)_2SO_4$, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.05 g/L of $CaCl_2 \cdot 2H_2O$, and 10 mL of trace element solution SL4. The composition of SL4 was as follows: 0.5 g/L of EDTA, 0.2 g/L of $FeSO_4 \cdot 7H_2O$, and 10 mL of trace element solution SL6. SL6 contained the following: 0.1 g/L of $ZnSO_4 \cdot 7H_2O$, 0.03 g/L of $MnCl_2 \cdot 4H_2O$, 0.3 g/L of H_3BO_3, 0.2 g/L of $CoCl_2 \cdot 6H_2O$, 0.01 g/L of $CuCl_2 \cdot 2H_2O$, 0.02 g/L of $NiCl_2 \cdot 6H_2O$, and 0.03 g/L of $NaMoO_4 \cdot 2H_2O$. Phenol (sterilized by filtration) was used as a sole source of carbon and energy. The culture was incubated in a rotary shaker at 180 rpm and 30°C. The pH was adjusted to 7.0 after stream sterilization (121°C, 20 min). Growth was monitored by measuring the turbidity of the culture at 600 nm using a spectrophotometer (Shimadzu UV-160A).

Enrichment and Isolation

The enrichment culture was performed using wastewater from a petrol refinery as the inoculum and a continuous culture in a reactor fed with mineral medium containing phenol (1 g/L) as the sole carbon source. Pure bacterial cultures capable of phenol degradation were obtained by plating out suspension from the reactor in which phenol degradation was active. The ability to utilize phenol was verified in liquid cultures by following the substrate concentration. The isolated strain was identified by sequencing the 16S rDNA gene.

Bacterial Strains and Plasmids

The strains and plasmids used are described in Table 1.

Substrate Utilization

Substrate utilization was tested in 250-mL Erlenmeyer flasks filled with 25-mL of basal medium, supplemented with different aromatic substrates (0.4 g/L). Those substrates (*o*-, *m*-, and *p*-cresol; 4-hydroxybenzoic,

vanillic, and syringic acids) were added as the sole carbon source, inoculated with a suspension of phenol-grown cells, and incubated at 30°C on magnetic stirrers. Substrate concentrations were determined by high-performance liquid chromatography (HPLC) analysis.

Determination of Aromatic Compound Concentration

Aromatic compound concentration was determined by HPLC. Samples were centrifuged at 12,000*g* for 10 min at 4°C. The supernatants were filtered through 0.45-µm pore-size membranes (Whatman). Aromatic compounds were measured using a chromatograph (Shimadzu SPD-6A) equipped with a 5-µm particle size, 4.6 mm id, and C_{18} symmetry column with 250-mm length (Waters). The column temperature was maintained at 35°C. An isocratic mobile phase of 70:30 (v/v) acetonitrile/distilled water was used at a flow rate of 0.6–0.8 mL/min. The volume of the injection loop was 20 µL. Aromatic compounds were quantified at 240 nm with a UV detector (Shimadzu SPD-6A) connected to a Shimadzu CR-6A integrator.

Isolation of Total DNA and Plasmid

Plasmids to be used in cloning procedures were extracted from *E. coli* strains as described by Sambrook et al. *(16)*. Total DNA was isolated from strain MH1 using the alkaline lysis method *(16)*.

Design of Oligonucleotide

The oligonucleotide sequences used for polymerase chain reaction (PCR) amplification (Table 2) were designed from the sequence of the *dmp* operon from *P. putida* strain CF600 that was obtained from GenBank. All primers used were synthesized by Genome Express (Grenoble, France).

PCR Amplification

Thermocycling was performed using a Perkin-Elmer Gene Amp PCR System 2400 (Perkin-Elmer, Norwalk, CT). To each 100-µL reaction mix, the following were added: 100 ng of total DNA; 0.2 m*M* each of dATP, dCTP, dGTP, and dTTP; 25 pmol of each primer; 0.1 vol of *Pfu* 10X buffer (100 m*M* Tris-HCl; 15 m*M* $MgCl_2$; 500 m*M* KCl, pH 8.3), and 1 U of *Pfu* DNA polymerase (Promega, Madison, WI).

Target DNA was amplified with initial denaturation of the DNA at 94°C for 5 min followed by five cycles consisting of denaturation at 94°C for 1 min, primer annealing at 55°C (or 60°C) for 30 s, and primer extension at 72°C for 45 s or 6 min (depending on the length of the amplified fragment); then 35 cycles consisting of denaturation at 94°C for 30 s, primer annealing at 55°C (or 60°C) for 30 s, and primer extension at 72°C for 45 s or 6 min. Samples were incubated at 72°C for 7 min at the end of the amplification cycle to complete the extension reaction.

Table 2
Nucleotide Sequence and Location of Primers Used for Amplification

Primer	Sequence (5'——————————3')	Reference or source	Location[a]	Orientation
8818	ATC ACC GAC TGG GAC AAG TGG GAA GAC C	*17*	2492–2518	Forward
8819	TGG TAT TCC AGC GGT GAA ACG GCG G	*17*	2667–2691	Reverse
71	GTA TCG AGG CCG AGA AGC GCC TGG AGA T	This study	2201–2218	Forward
72	GGC GGC ACC GGA CAT GAA GGG TAC G	This study	2983–3009	Reverse
Phe5	AGATCTTGCCTTCCATGCTCT	This study	249–269	Reverse
Phe12	TCATGGTTCACCTCAGATGC	This study	5393–5410	Forward

[a]Relative to the *dpm* genes for strain *P. putida* CF600.

Agarose Gel Electrophoresis

DNA was resolved on a 0.7 or 1% agarose gel with TAE buffer by electrophoresis, stained with ethidium bromide, and visualized by UV irradiation *(16)*. The DNA concentration was estimated by comparison with smart ladder (Eurogentec).

Identification of Strain

The universal primers Fd1 and Rd1 were used to obtain a PCR product of approx 1.5 kb corresponding to base positions 8–1542 based on *E. coli* numbering of the 16S rDNA *(18)*. Then the PCR product was purified using a QIAquick Kit (Qiagen). The sequencing was performed by the primer walking method using an automated DNA sequencer (Genome Express). Identification was achieved by comparison with other sequences in the GenBank database *(19)* using BLAST *(20)*.

Cloning of PCR Product

A 5-µL aliquot of PCR mixture was analyzed by agarose gel electrophoresis. The reaction mixture was incubated at 72°C for 90 min with 0.2 m*M* of each dNTP in a 50-µL reaction containing 2.5 U of *Taq* DNA polymerase. The DNA was again concentrated and washed using a QIAquick column (30-µL final). An aliquot of this solution was then ligated overnight at 4°C to a pGEM^T vector.

The ligation reaction contained 80 ng of the PCR product, 50 ng of pGEM^T vector, 1X ligase buffer (100 m*M* Tris-HCl; 5 m*M* $MgCl_2$; 1 m*M* dithioerythritol; 1 m*M* adenosine triphosphate; pH 7.5), and 3 U of T4 DNA ligase in a 10-µL reaction volume.

Ligation products were used to transform competent *E. coli* JM109. Recombinant (white) colonies were selected from LB plates containing 100 µg/mL of ampicillin, 40 µg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase, and 20 µg/mL of isopropylthio-β-D-galactosidase. Plasmid DNA from such colonies was prepared as mentioned previously, and the presence of the desired PCR product was verified by amplification using internal primer (71–72) and agarose gel electrophoresis.

DNA Sequencing

The 5.207-kb fragment contained in the pGEM A2 plasmid and the PCR product presenting the rDNA 16S gene were sequenced in both strands. The sequencing was performed using the primer walking method with an automated DNA sequencer (Genome Express). The sequence was compiled and analyzed using Gene Works 2.5.1 (Intelli Genetics, Mountain View, CA).

Results and Discussion

Enrichment and Isolation of Phenol-Degrading Strain

The enrichment culture that allowed isolation of phenol-degrading strains was carried out in a continuous reactor fed with increasing concen-

Table 3
Effects of Initial Phenol Concentration on Lag Period and Growth Rate (μ) in Batch Degradation of Phenol by *P. pseudoalcaligenes* MH1

	Initial phenol concentration (g/L)	Lag period (h)	Growth rate μ (h^{-1})
Shaken Erlenmeyer	0.6	2	0.29
	0.8	6.5	0.15
	1	16.5	0.15
	1.5	19	0.14
	2	>72	0.00
High compact reactor	1	7	0.17

trations of phenol. The most efficient strain in degrading phenol (MH1) was selected and further characterized as belonging to the species *P. pseudoalcaligenes* by sequencing of 16S rDNA genes and comparing to bacterial sequences already deposited.

Batch Degradation at Various Phenol Concentrations by P. pseudoalcaligenes *Strain MH1*

P. pseudoalcaligenes strain MH1 grew on phenol as the sole carbon source up to 1.5 g/L. Increasing the phenol concentration in the medium resulted in a significant increase in the lag period, while the growth rate decreased (Table 3), obviously because of the known inhibition of microbial growth by phenol. At concentrations >1.5 g/L, phenol was not degraded and death of the cells was observed.

Batch degradation was studied in a high compact reactor *(21)*, in mineral medium supplemented with 1 g/L of phenol as the sole source of carbon and energy. After inoculation with phenol pregrown cells of strain MH1, a reduction of the lag period from 16.5 h in an Erlenmeyer flask to 7 h in the reactor was observed. This result can be explained by a better oxygen transfer occurring in the reactor. Strain MH1 showed high degradative capacities (1 g/L of phenol was degraded in 20 h) as compared to *P. putida* strain EKII isolated by Hinteregger et al. *(1)*, which required more than 40 h to degrade 1 g/L of phenol. Similarly, to degrade 1 g/L of phenol, Gonzalez et al. *(22)*, working with another strain of *P. putida* (ATCC 17484), showed that it took 12 and 10 d when cells were grown in suspended culture (in a stirred tank) and in an immobilized system (in a fluidized-bed reactor), respectively.

Substrate Specificity of P. pseudoalcaligenes *Strain MH1*

The degradation of other aromatic compounds was tested separately in Erlenmeyer flasks filled with basal medium and inoculated with phenol pregrown cells. All isomers of cresols (*o*-, *m*-, *p*-) and *p*-hydroxybenzoic,

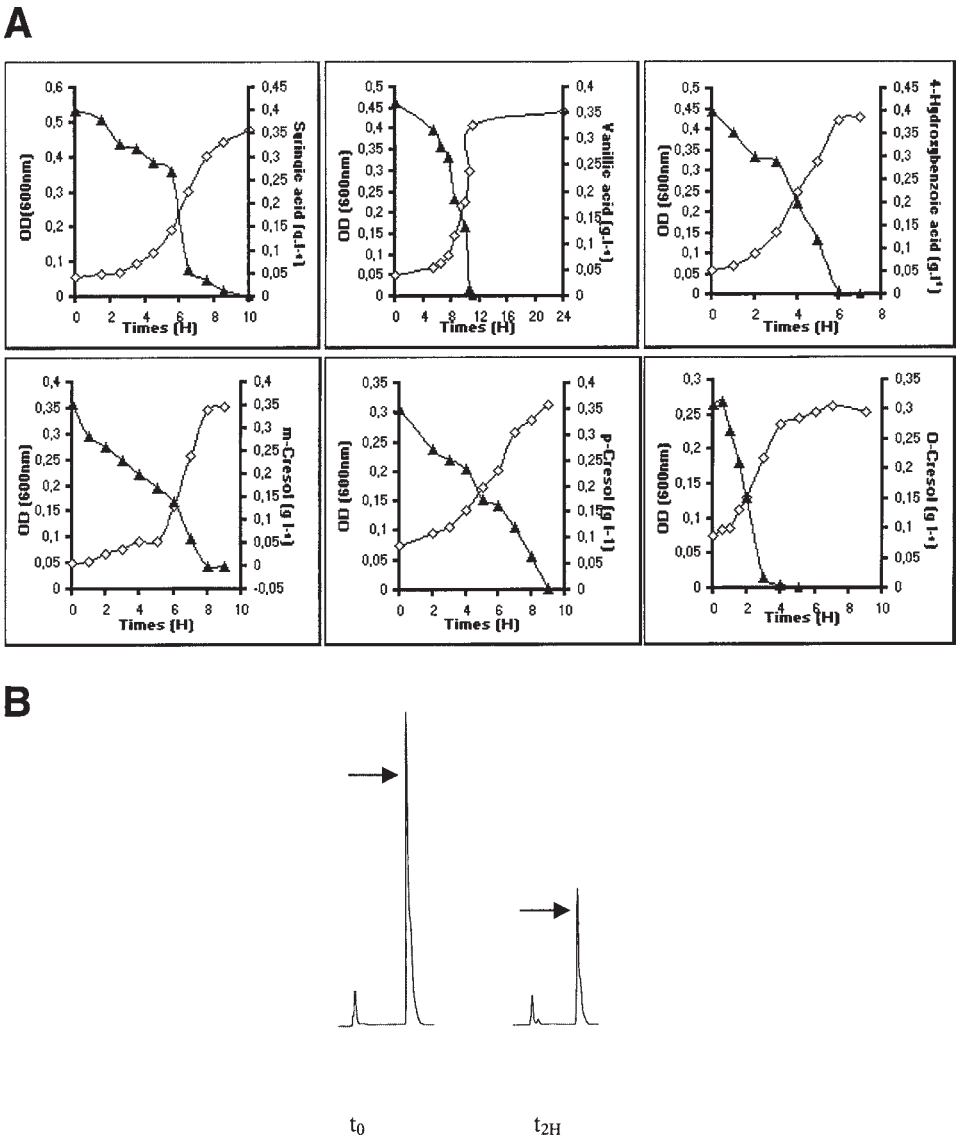

Fig. 1. **(A)** Degradation of cresols and benzoic derivatives in culture of *P. pseudoalcaligenes* MH1. Experiments were carried out in Erlenmeyer flasks containing mineral medium supplemented with aromatic compounds as the sole source of carbon and energy. Optical density (OD) (□); aromatic compound (▲) concentration. **(B)** HPLC chromatograms of *o*-cresol medium obtained before (t_0) and 2 h after inoculation with *P. pseudoalcaligenes* strain MH1. Arrows indicate the peak of *o*-cresol.

syringic, and vanillic acids were completely degraded at an initial concentration of 0.4 g/L (Fig. 1). HPLC analysis showed that aromatic intermediates were not detected for all the compounds tested.

As an example, Fig. 1B shows the analysis of the supernatant of *o*-cresol MH1 culture before and after 2 h of inoculation. This result, which shows

a removal of 50% of *o*-cresol in 2 h, confirmed the fast rate of metabolization of aromatic compounds by MH1.

The range of metabolized substrates of MH1 differed from that reported for the other *Pseudomonas* strains: *P. putida* strain CF600, *P. putida* strain BH, *P. putida* strain H, *P. pickittii* strain PKO1, and *P. putida* strain EKII. Indeed, *P. putida* CF600 grows efficiently on phenol, cresols, and 3,4-dimethylphenol as the sole carbon and energy sources *(9)*. *P. putida* strain BH metabolizes completely phenol, benzoate, cresols, protocatechuate, *p*-hydroxybenzoate, and *m*-toluate *(23)*. *P. putida* strain H can also utilize phenol as well as the cresol isomers *(24)*. Kukor and Olsen *(25)* reported that *P. pickittii* strain PKO1 metabolized only phenol and cresol while *P. putida* strain EKII completely degraded all isomers of cresol and monochlorophenol but only in cometabolism with phenol, as mentioned by Hinteregger et al. *(1)*.

P. pseudoalcaligenes strain MH1 is characterized by a broader range of aromatic compounds degraded. We determined the first enzyme involved in the degradation of these compounds and then cloned and sequenced it.

Amplification and Cloning of Phenol Hydroxylase from P. pseudoalcaligenes *Strain MH1*

The genes for multicomponent phenol hydroxylase have been cloned and sequenced from several phenol-degrading bacteria, including *P. putida* strain P35X *(13)*, *P. putida* BH *(14)*, *P. putida* strain H *(15)*, *P. putida* strain CF600 *(12)*, *P. pickettii (11)*, *Acinetobacter calcoaceticus (26)*, *Bacillus thermoleovorans (27)*, and *Ralstonia eutropha* strain E2 *(28)*. Since phenol hydroxylase genes are highly homologous, we designed a series of oligonucleotide primers according to the sequence of the *dmp* operon of *P. putida* strain CF600 (Table 2).

A 200-bp fragment was amplified from total DNA of strain MH1 using primers 8818 and 8819 (Table 2). The nucleotide sequence of this PCR product displayed significant homology with the corresponding region (2492–2691) of *dmp*N, the gene encoding for the fourth subunit of the multicomponent phenol hydroxylase of *P. putida* strain CF600. Likewise, PCR amplification using several primers, synthesized according to the nucleotide sequence of the *dmp* operon, produced the expected size of amplified DNA. These results suggested that strain MH1 possessed phenol hydroxylase. Then primers Phe5 and Phe12 were designed to amplify the entire enzyme, using the *Pfu Taq* polymerase. The band close to the expected size was chosen for gel purification and cloning.

Nucleotide Sequence of Cloned PCR Product, Phenol Hydroxylase–Encoding Region

To characterize the organization and the structure of the genes encoding the phenol hydroxylase in *P. pseudoalcaligenes* strain MH1, we deter-

mined the nucleotide sequence of the 5.207-kb PCR product, which contained the entire operon. The complete nucleotide sequence was determined using the primer walking methods. A total of 5.207 kb was sequenced including the 4675-nucleotide (nt) coding sequence, 524 nt upstream and 5181 nt downstream surrounding the structural genes. Translation of the sequence in all of the possible reading frames revealed a cluster of six ATG starting ORFs. All of the ORFs were preceded by a potential ribosome-binding site sequence having complementarity to the 3' end of the 16S rRNA of both *P. aeruginosa* and *E. coli* in front of the ATG start codon, as shown in Table 4. These ORFs were designated *phm* (ABCDEF). The designation refer to "phenol hydroxylase of MH1." The transcriptional stop codon was TAA for *phm*B and TGA for the other genes. Comparison of the nucleotide sequence of *phm* with that of *dmp* of *P. putida* strain CF600 revealed differences in the 5' region, as shown in Fig. 2.

The nucleotide sequence was also analyzed by potential regulatory signals. A putative promoter region, 40 bp upstream from the transcriptional start of ORF1, was identified (Fig. 2). This promoter shows a strong homology to a set of positively controlled promoters proposed to be transcribed by a *Pseudomonas* σ^{54}-like RNA polymerase holoenzyme *(29)*. This putative promoter sequence for *phm* has considerable homology with (1) the XylCAB and XylS promoters of toluene plasmid pWWO and *dmp* promoter of pVI150 catabolic plasmid for strain CF600, and (2) a putative promoter sequence upstream of the *tbu*D gene encoding phenol/cresol hydroxylase from *P. pickittii* strain PKO1 (Fig. 3). This class of promoter is recognized on the basis of a minimum sequence of GG-10bp-GC at positions -24 to -12 from the mRNA start site *(30)*. The six ORFs (*phm*A–F) are possibly transcribed from this promoter. A long inverted repeat (15 nucleotides) at approx 99 bp upstream of the putative promoter was found (Fig. 2). This structure may play a role in the transcription termination of the region upstream of the *phm* operon.

Comparison of six predicted polypeptide sequences with a nonredundant sequence database using BLAST at National Centre for Biotechnology Information (NCBI) revealed a high degree of similarity with the six subunits of multicomponent phenol hydroxylase from *P. putida* CF600. However, we found that *phm*A showed no similarity, at the beginning of the ORF, with the *dmp* K (Fig. 4). The difference detected between the two sequences was 10 amino acids at the N-terminal region of the first subunit of phenol hydroxylase. The molecular masses of the gene products calculated from predicted sequences are presented in Table 4.

From comparison of studies between the substrate specificities of the enzymes from the strain *P. pseudoalcaligenes* MH1 described herein and the other strains, *P. putida* BH, *P. putida* strain H, and *P. putida* strain CF600, with respect to the difference in the 5' region sequences, we can conclude at present that MH1 strain is able to grow on the main aromatic compounds tested for *P. putida* BH, *P. putida* strain H, and *P. putida* strain CF600, which are phenol and methyl phenol. Moreover, MH1 strain is able to metabolize other aromatics such as *p*-hydroxybenzoic, syringic, and vanillic acids,

Table 4
Summary of ORFs and Their Products of Phenol Hydroxylase Operon of Strain MH1

Gene	Coordinates (bp)	Intergenic spacing[a] (bp)	Putative ribosome binding site[b] 5'————————————3'	No. of amino acid residues	Predicted molecular mass (kDa)	Function[c]	Source
*phm*A	524–793		ACCAGCCAGGAACCGGCCGAGA**ATG**	89	10.3	PH component	This study
*phm*B	846–1841	52	CAACAAGAGGGTACGGTTGAT**ATG**	331	36.6	PH component	This study
*phm*C	1845–2117	3	AAAAGCCGCGAGGAATAAAGC**ATG**	90	10.15	PH component	This study
*phm*D	2129–3739	11	GAAGAACTAGGAGACAAGCTC**ATG**	367	60.6	PH component	This study
*phm*E	3750–4109	10	CAAGAACATGAGGGTTCGATC**ATG**	117	13.17	PH component	This study
*phm*F	4120–5181	10	TCGTGCAGCTGAGAGGTGTGTC**ATG**	353	38.47	PH component	This study

[a]Numbers of base pairs between the indicated gene and the gene listed below it in column 1.
[b]Nucleotide residues with overscores and underscores indicate sequences that are complementary to the 3' end of the 16S rRNA of *E. coli* and *P. aeruginosa*, respectively.
[c]PH, phenol hydroxylase.

Fig. 2. Nucleotide and deduced amino acid sequences of *phm* operon from *P. pseudoalcaligenes* MH1. Underlining indicates putative ribosome-binding sites. Boxes indicate the stop codon of each ORF. Dashed arrows indicate inverted repeated. Dark highlighting sequences indicate the putative promoter region. Bold letters indicate the nucleotide variations between *phm* operon (our strain MH1) and *dmp* operon (*P. putida* strain CF600).

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  A  M  L  T  E  F  M  R  D  W  Y  G  E  S  T  R  W  V  D  A  M
TCAAGACCGTGCTTGCCGAAAATGACGCTAACCGTGAGCAGGTGCAGGCCTGGCTGGAGGTCTGG     1712
F  K  T  V  L  A  E  N  D  A  N  R  E  Q  V  Q  A  W  L  E  V  W
GAGCCGCGTGCCTACGAGGCATTGTTGCCCCTGGCCGAGGAAGCCACCGGTATCGCCGCGCTGGA     1777
 E  P  R  A  Y  E  A  L  L  P  L  A  E  E  A  T  G  I  A  A  L  D
TGAAGTCCGCAGCGCCTTCGCTACTCGCCTGCAGAAAATCGGCCTGAAAAGCCGCGAGGAATAAA     1842
  E  V  R  S  A  F  A  T  R  L  Q  K  I  G  L  K  S  R  E  E
GCATGTCATCACTCGTCTACATCGCCTTCCAGGATAACGACAACGCGCGTTACGTGGTGGAAGCG     1907
   M  S  S  L  V  Y  I  A  F  Q  D  N  D  N  A  R  Y  V  V  E  A
ATCATCCAGGACAACCCCCACGCCGTCGTCCAGCACCACCCGGCGATGATCCGTATCGAGGCCGA     1972
 I  I  Q  D  N  P  H  A  V  V  Q  H  H  P  A  M  I  R  I  E  A  E
GAAGCGCCTGGAGATCCGCAGGGAAACCGTGGAAGAGAACCTCGGCCGCGCCTGGGACGTCCAGG     2037
  K  R  L  E  I  R  R  E  T  V  E  E  N  L  G  R  A  W  D  V  Q
AAATGCTGGTGGACGTAATCACCATCGGCGGCAACGTCGACGAGGACGATGACCGCTTCGTCCTC     2102
E  M  L  V  D  V  I  T  I  G  G  N  V  D  E  D  D  D  R  F  V  L
GAGTGGAAGAACTAGGAGACAAGCTCATGGCTACCCACAACAAGAAACGCCTCAACCTGAAAGAC     2167
 E  W  K  N                 M  A  T  H  N  K  K  R  L  N  L  K  D
AAATACCGCTACCTGACCCGCGATCTGGCCTGGGAAACGACCTACCAGAAGAAAGAAGACGTGTT     2232
 K  Y  R  Y  L  T  R  D  L  A  W  E  T  T  Y  Q  K  K  E  D  V  F
CCCGCTGGAGCACTTCGAGGGCATCAAGATCACCGACTGGGACAAGTGGGAAGACCCCTTCCGCC     2297
  P  L  E  H  F  E  G  I  K  I  T  D  W  D  K  W  E  D  P  F  R
TGACCATGGACACCTACTGGAAATACCAGGCGGAGAAAGAGAAGAAGCTCTACGCGATCTTCGAC     2362
L  T  M  D  T  Y  W  K  Y  Q  A  E  K  E  K  K  L  Y  A  I  F  D
GCCTTTGCCCAGAACAATGGTCATCAGAACATTTCCGATGCGCGCTACGTCAACGCCCTGAAGCT     2427
 A  F  A  Q  N  N  G  H  Q  N  I  S  D  A  R  Y  V  N  A  L  K  L
GTTCCTCACCGCCGTTTCACCGCTGGAATACCAGGCCTTCCAGGGCTTCTCGCGGGTTGGCCGGC     2492
  F  L  T  A  V  S  P  L  E  Y  Q  A  F  Q  G  F  S  R  V  G  R
AGTTCAGTGGCGCCGGTGCGCGGGTCGCCTGTCAGATGCAGGCGATCGACGAGCTGCGCCATGTG     2557
Q  F  S  G  A  G  A  R  V  A  C  Q  M  Q  A  I  D  E  L  R  H  V
CAGACGCAAGTCCACGCCATGAGCCATTACAACAAGCACTTCGATGGTTTGCATGACTTCGCCCA     2622
 Q  T  Q  V  H  A  M  S  H  Y  N  K  H  F  D  G  L  H  D  F  A  H
CATGTACGACCGGGTCTGGTACCTCTCGGTACCCAAGTCCTATATGGACGATGCGCGGACCGCCG     2687
  M  Y  D  R  V  W  Y  L  S  V  P  K  S  Y  M  D  D  A  R  T  A
GTCCGTTCGAGTTCCTCACCGCCGTCTCGTTCTCCTTCGAGTACGTGCTGACCAACCTGTTGTTC     2752
G  P  F  E  F  L  T  A  V  S  F  S  F  E  Y  V  L  T  N  L  L  F
GTACCCTTCATGTCCGGTGCCGCCTACAACGGCGATATGGCCACGGCCACCTTCGGTTTCTCCGC     2817
 V  P  F  M  S  G  A  A  Y  N  G  D  M  A  T  A  T  F  G  F  S  A
GCAGTCGGACGAGGCGCGGCACATGACCCTGGGTCTGGAAGTGGTCAAGTTCATGCTCGAACAGC     2882
  Q  S  D  E  A  R  H  M  T  L  G  L  E  V  V  K  F  M  L  E  Q
ATGAAGACAACGTGCCCATCATCCAGCGCTGGATCGATAAGTGGTTCTGGCGCGGTTACCGCCTG     2947
H  E  D  N  V  P  I  I  Q  R  W  I  D  K  W  F  W  R  G  Y  R  L
CTGACCCTGATCGGCATGATGATGGATTACATGCTGCCGAACAAAGTGATGTCCTGGTCTGAGGC     3012
 L  T  L  I  G  M  M  M  D  Y  M  L  P  N  K  V  M  S  W  S  E  A
CTGGGGGGTCTACTTCGAGCAGGCCGGTGGCGCGCTGTTCAAGGATCTGGAGCGCTATGGCATCC     3077
  W  G  V  Y  F  E  Q  A  G  G  A  L  F  K  D  L  E  R  Y  G  I
GGCCGCCGAAATACGTCGAGCAGACCACCATCGGCAAGGAGCACATCACCCACCAGGTGTGGGGG     3142
R  P  P  K  Y  V  E  Q  T  T  I  G  K  E  H  I  T  H  Q  V  W  G
GCCTTATATCAATACAGCAAGGCCACCAGCTTCCATACCTGGATACCCGGCGACGAGGAACTGAA     3207
 A  L  Y  Q  Y  S  K  A  T  S  F  H  T  W  I  P  G  D  E  E  L  N
CTGGCTGTCGGAGAAATACCCGGACACCTTCGACAAATACTACCGCCCGCGCTTCGAGTTCTGGC     3272
  W  L  S  E  K  Y  P  D  T  F  D  K  Y  Y  R  P  R  F  E  F  W
GTGAGCAGCAGGCCAAGGGTGAGCGCTTCTACAACGACACCCTGCCGCACCTCTGCCAGGTGTGC     3337
R  E  Q  Q  A  K  G  E  R  F  Y  N  D  T  L  P  H  L  C  Q  V  C
CAGTTACCGGCGATTTTCACCGAGCCGGACGATCCGACCAAGCTCAGCCTGCGCAGCCTGGTGCA     3402
 Q  L  P  A  I  F  T  E  P  D  D  P  T  K  L  S  L  R  S  L  V  H
CGAGGGGGAGCGCTATCACTTCTGCTCGGATGGCTGCTGCGACATCTTCAAGAACGAGCCGGTGA     3467
  E  G  E  R  Y  H  F  C  S  D  G  C  C  D  I  F  K  N  E  P  V
AGTACATCCAGGCCTGGCTGCCGGTGCACCAGATCTACCAGGGCAACTGCGAAGGCGGGGATGTC     3532
K  Y  I  Q  A  W  L  P  V  H  Q  I  Y  Q  G  N  C  E  G  G  D  V
GAAACGGTGGTGCAGAAGTACTACCACATCAAAAGCGGCGTGGACAATTTGGAGTACCTGGGCTC     3597
 E  T  V  V  Q  K  Y  Y  H  I  K  S  G  V  D  N  L  E  Y  L  G  S
GCCCGAGCACCAGCGCTGGCTGGCCCTGAAAGGTCAGACCCCACCAACTGCCGCCCCGGCGGACA     3662
  P  E  H  Q  R  W  L  A  L  K  G  Q  T  P  P  T  A  A  P  A  D
AGAACCTGGGCGCCGCCTGAGGCAGAACCAGCCGCTCAGGGGTGAAGCACCGCCCCTGAGCCATT     3727
K  N  L  G  A  A
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Fig. 2. *(continued)*

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CCAAGAACATGAGGGTTCGATCATGACTGTCAACTCAATCGGCGAATACACCGCCACGCCACGGG      3792
                      M  T  V  N  S  I  G  E  Y  T  A  T  P  R
ATGTGCAGGCCAACTTCAACGGCATGCAACTGCTCTACCTCTACTGGGAAGAGCACCTGATGTAC      3857
D  V  Q  A  N  F  N  G  M  Q  L  L  Y  L  Y  W  E  E  H  L  M  Y
TGCTGCGCGCTCGCGCTCTTGGTAGCCCCCGGCATGCCCTTTGCCGAGTTCCTCGAGCAGGTGCT      3922
 C  C  A  L  A  L  L  V  A  P  G  M  P  F  A  E  F  L  E  Q  V  L
CAAGCCCGCGATCCACGCCCATCCGGACAGCGCGAAGATCGATTTCAGCCAGGCGCTCTGGCAGC      3987
  K  P  A  I  H  A  H  P  D  S  A  K  I  D  F  S  Q  A  L  W  Q
TGAACGACCAGCCGTTCACCCCGGACTACGCCGCCAGCCTGGAAGCCAACGGCATCGACCACAAA      4052
L  N  D  Q  P  F  T  P  D  Y  A  A  S  L  E  A  N  G  I  D  H  K
AGCATGCTGCGTCTGAACACCCCGGGCCTGAACGGCATCCAGGGTTCGTGCAGCTGAGAGGTGTG      4117
 S  M  L  R  L  N  T  P  G  L  N  G  I  Q  G  S  C  S
CATGAGTTACAACGTCACCATTGAACCGACCGGCGAAGTGATCGAAGTGGAGGACGGCCAGACC       4182
  M  S  Y  N  V  T  I  E  P  T  G  E  V  I  E  V  E  D  G  Q  T
ATCCTCCAGGCCGCTCTGCGCCAGGGCGTCTGGCTGCCGTTCGCCTGCGGCCACGGCACCTGCGC      4247
 I  L  Q  A  A  L  R  Q  G  V  W  L  P  F  A  C  G  H  G  T  C  A
CACCTGCAAGGTGCAGGTGGTCGAGGGCGAAGTGGATATCGGCGAAGCCTCGCCGTTCGCCCTGA      4312
  T  C  K  V  Q  V  V  E  G  E  V  D  I  G  E  A  S  P  F  A  L
TGGACATCGAGCGCGACGAGCGCAAGGTGCTGGCCTGCTGCGCCATTCCGCTGTCCGACCTGGTG      4377
M  D  I  E  R  D  E  R  K  V  L  A  C  C  A  I  P  L  S  D  L  V
ATCGAAGCCGACGTCGATGCCGACCCGGACTTCCTCGGCCATCCGGTGGAGGATTACCGGGGGGT      4442
 I  E  A  D  V  D  A  D  P  D  F  L  G  H  P  V  E  D  Y  R  G  V
GGTCAGCGCCCTGGTTGACCTGTCGCCGACCATCAAGGGCCTGCACATCAAGCTGGATCGGCCCA      4507
  V  S  A  L  V  D  L  S  P  T  I  K  G  L  H  I  K  L  D  R  P
TGCCGTTCCAGGCCGGGCAGTACGTCAACCTGGCATTGCCGGGCATCGACGGCACCCGCGCCTTC      4572
M  P  F  Q  A  G  Q  Y  V  N  L  A  L  P  G  I  D  G  T  R  A  F
TCGCTGGCCAACCCGCCGAGCCGGAACGACGAAGTCGAGTTGCACGTGCGCCTGGTCGAGGGCGG      4637
 S  L  A  N  P  P  S  R  N  D  E  V  E  L  H  V  R  L  V  E  G  G
TGCGGCCACCGGCTTTATCCACAAGCAACTGAAAGTCGGCGACGCGGTGGAGCTGTCCGGGCCTT      4702
  A  A  T  G  F  I  H  K  Q  L  K  V  G  D  A  V  E  L  S  G  P
ATGGGCAGTTCTTCGTGCGCGATTCGCAGGCCGGCGACCTGATCTTCATCGCCGGCGGCTCGGGC      4767
Y  G  Q  F  F  V  R  D  S  Q  A  G  D  L  I  F  I  A  G  G  S  G
TTATCGAGCCCGCAGTCGATGATCCTCGATCTGCTTGAACGCGGCGATACGCGGCGGATCACCCT      4832
 L  S  S  P  Q  S  M  I  L  D  L  L  E  R  G  D  T  R  R  I  T  L
GTTCCAGGGCGCGCGCAACCGCGCCGAGCTGTACAACTGCGAACTGTTCGAGGAACTGGCCGCGC      4897
  F  Q  G  A  R  N  R  A  E  L  Y  N  C  E  L  F  E  E  L  A  A
GCCACCCCAACTTCAGTTACGTGCCGGCACTCAACCAGGCCAACGACGATCCCGAATGGCAGGGT      4962
R  H  P  N  F  S  Y  V  P  A  L  N  Q  A  N  D  D  P  E  W  Q  G
TTCAAGGGCTTCGTCCACGACGCCGCCAAGGCGCATTTCGACGGCCGCTTCGGCGGGCAGAAAGC      5027
 F  K  G  F  V  H  D  A  A  K  A  H  F  D  G  R  F  G  G  Q  K  A
CTACCTGTGCGGCCCACCGCCGATGATCGACGCGGCCATCACCACCCTGATGCAAGGTCGCTTGT      5092
  Y  L  C  G  P  P  P  M  I  D  A  A  I  T  T  L  M  Q  G  R  L
TCGAGCGCGACATCTTTATGGAGCGCTTCTACACCGCCGCCGATGGTGCCGGCGAGAGCACCCGT      5157
F  E  R  D  I  F  M  E  R  F  Y  T  A  A  D  G  A  G  E  S  T  R
TCGGCCCTGTTCAAGCGCATCTGAGGTGAACAATCGAATTCCCGCGGCCG                     5207
 S  A  L  F  K  R  I
```

Fig. 2. *(continued)*

Fig. 3. Putative regulation signals of phenol hydroxylase encoding region. R indicates A or G, N indicates any nucleotide, K indicates G or T, and W indicates T or A. The consensus sequence of -24, -12 region is in bold.

Fig. 4. Comparison of amino acid sequence of *phm*A (deduced from the nucleotide sequence) with those of *dmp*L, *phl*A, and *phe*A1 from *P. putida* strain CF600, *P. putida* strain H, and *P. putida* strain BH, respectively. Dark highlighting indicates residues that are identical in at least four sequences.

which are not tested or reported for the other strains. Thus, a direct relationship between the difference in the 5' region sequence and the substrate specificities could not be established. A larger number of groups of aromatics should be tested for growth of MH1 and the other strains in order to confirm the meaning of these differences.

Conclusion

Biologic remediation of industrial wastewater for toxic organics is a promising technology as an alternative to more costly physical and chemical methods. In cases in which contaminants are difficult to degrade biologically, it may be desirable to enrich the indigenous bacteria by inoculation of specially adapted microorganisms such as strain MH1 (bioaugmentation) in order to enhance the rate and extent of decontamination.

The experimental results obtained in our study showed that is was possible to treat wastewaters containing phenol in a high compact reactor with a high removal efficiency using *P. pseudoalcaligenes* strain MH1. In addition, the broad degradation spectrum of strain MH1 prompted us to use this strain for treatment of different industrial effluents containing not only phenol but other aromatic compounds.

Hydroxylated aromatic molecules have commanded considerable interest in industry because of their many uses in the manufacture of plastics and liquid crystals in the synthesis of dyes, and in the pharmaceutical industry. A serious need exists for a selective enzymatic process for the hydroxylation of phenol into hydroquinone whereas chemical reactions result in the production of pyrocatechol *(31)*. One potential route leading to the production of these compounds is biotransformation. Therefore, expression of phenol hydroxylase genes in *E. coli* or other *Pseudomonas* strains was required for further applications in bioconversion reactions of aromatic compounds.

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