Journal of Microbiology (2014) Vol. 52, No. 1, pp. 13–19
DOI 10.1007/s12275-014-3250-x

Molecular Characterization of the Alpha Subunit of Multicomponent Phenol Hydroxylase from 4-Chlorophenol-Degrading *Pseudomonas* sp. Strain PT3

Wael S. El-Sayed[1,2*], Mohamed K. Ibrahim[2], and Salama A. Ouf[1,3]

[1]*Biology Department, Faculty of Science, Taibah University, 344, Almadinah Almunwarrah, KSA*
[2]*Microbiology Department, Faculty of Science, Ain Shams University, 11566, Cairo, Egypt*
[3]*Botany Department, Faculty of Science, Cairo University, Giza 12613, Egypt*

(Received May 6, 2013 / Revised Aug 6, 2013 / Accepted Aug 13, 2013)

Multicomponent phenol hydroxylases (mPHs) are diiron enzymes that use molecular oxygen to hydroxylate a variety of phenolic compounds. The DNA sequence of the alpha subunit (large subunit) of mPH from 4-chlorophenol (4-CP)-degrading bacterial strain PT3 was determined. Strain PT3 was isolated from oil-contaminated soil samples adjacent to automobile workshops and oil stations after enrichment and establishment of a chlorophenol-degrading consortium. Strain PT3 was identified as a member of *Pseudomonas* sp. based on sequence analysis of the 16S rRNA gene fragment. The 4-CP catabolic pathway by strain PT3 was tentatively proposed to proceed *via* a *meta*-cleavage pathway after hydroxylation to the corresponding chlorocatechol. This hypothesis was supported by polymerase chain reaction (PCR) detection of the LmPH encoding sequence and UV/VIS spectrophotometric analysis of the culture filtrate showing accumulation of 5-chloro-2-hydroxymuconic semialdehyde (5-CHMS) with λ_{max} 380. The detection of catabolic genes involved in 4-CP degradation by PCR showed the presence of both mPH and catechol 2,3-dioxygenase (C23DO). Nucleotide sequence analysis of the alpha subunit of mPH from strain PT3 revealed specific phylogenetic grouping to known mPH. The metal coordination encoding regions from strain PT3 were found to be conserved with those from the homologous dinuclear oxo-iron bacterial monooxygenases. Two DE(D)XRH motifs was detected in LmPH of strain PT3 within an approximate 100 amino acid interval, a typical arrangement characteristic of most known PHs.

Keywords: 4-chlorophenol, biodegradation, phenol hydroxylase, catalytic domain, Pseudomonas

*For correspondence. E-mail: waelsme@yahoo.com; Tel.: +8460008-1433

Introduction

Since their discovery, chloroaromatics represented a serious source of potentially hazard materials for both humans and the environment. Tens of thousands of such chemicals have been developed and have invariably found their way into the environment. Some are not readily biodegradable and become persistent. Because of their various health effects and the subsequent increase in public concern, careful monitoring and regulation of these pollutants is now generally required. Chlorinated phenols are common environmental contaminants; they have been extensively used as biocides, detergents, wood preservatives, bleaching agents, and disinfectants. They are subsequently released into the environment as by-products from many industrial activities (Tartakovsky *et al*., 2001).

Chemical, physical, and biological methods have been established for remediation purposes; however, an integrated chemical-biological and/or physical system for treatment of phenolic wastes could be very useful. In this regard, advanced oxidation processes like wet air oxidation, catalytic wet air oxidation, H_2O_2-promoted, catalytic wet air oxidation, activated carbon were found to be a useful option to prepare the phenolic effluents before the biological remediation (Esplugas *et al*., 2002; Rubalcaba *et al*., 2007).

Biological degradation of chlorinated phenols has been reported and pathways for their degradation have also been elucidated (Harayama and Rekik, 1989; Arenghi *et al*., 2001). The key step in the degradation of a phenolic compound was found to be the initial hydroxylation *via* phenol hydroxylase (PH). PHs are known to initiate the catabolism of a vast array of phenolic compounds by hydroxylation to the corresponding catechols (Evans *et al*., 1971; Schwien and Schmidt, 1982). Such partially oxidized aromatic intermediates may undergo *ortho* or *meta* cleavage *via* catechol 1,2-dioxygenase (C12DO) or C23DO, respectively. The produced metabolites would then be mineralized to carbon dioxide and water in the subsequent reactions of the tricarboxylic acid cycle (Powlowski and Shingler, 1994; Cafaro *et al*., 2004).

Enzymatic removal of phenolic compounds has been investigated. It has been shown that peroxidases are able to react with aqueous phenolic compounds and form non-soluble materials that could be easily removed from the aqueous phase, however; these processes suffer from enzyme inactivation (Bodalo *et al*., 2006; Ulson de Souza *et al*., 2007; Zhang *et al*., 2007).

Bacterial multicomponent monooxygenases are a diverse

family of enzymes that hydroxylate many aromatic compounds including chlorinated phenols (Leahy *et al.*, 2003; Notomista *et al.*, 2003). PH belongs to the family of bacterial multicomponent monooxygenases with carboxylate-bridged diiron active sites (Sazinsky *et al.*, 2006). mPHs have been isolated and characterized from different bacteria (Herrmann *et al.*, 1995; Bertoni *et al.*, 1996, 1998; Arenghi *et al.*, 2001; Cafaro *et al.*, 2004). mPH consists of a dimeric hydroxylase of the form $(\alpha\beta\gamma)_2$, a cofactorless regulatory protein and a FAD flavoprotein-[2Fe-2S] reductase that supplies the hydroxylase with electrons *via* NADH (Leahy *et al.*, 2003; Notomista *et al.*, 2003).

The genetic organization of mPH encoding genes from *Pseudomonas* sp. CF600 revealed the presence of polypeptides encoded by the six open reading frames designated *dmpKLMNOP* (Powlowski and Shingler, 1994). The DmpL-DmpN-DmpO proteins are believed to form the oxygenating component of the hydroxylase. DmpN is considered to be the largest subunit of mPH (LmPH) and possesses the enzyme active site, harboring the dinuclear iron center, Fe-O-Fe (Qian *et al.*, 1997; Enroth *et al.*, 1998).

The aim of the present study was to elucidate the catabolic route for 4-chlorophenol degradation in *Pseudomonas* sp. PT3 and characterize the alpha subunit of the corresponding mPH as the catalytic domain responsible for overall enzyme activity.

Materials and Methods

Sampling and culture conditions

Chlorophenol contaminated soil samples were collected from three different oil stations in Almadina almonawarah, KSA, namely Alkurdi, Sabaa masajid, and alburg. Samples were collected in clean and sterile bottles, and then kept at 4°C until further microbiological analysis. Soil samples were enriched in basal mineral media (BMM) (10%) as previously described (Farrell and Quilty, 1999). Media were supplemented with either 100 mg/L phenol or chlorophenol as a sole carbon source and incubated at 30°C. Successive subculturing was performed using a10-fold dilution until soil-free cultures were obtained showing a higher rate of phenol and/or chlorophenol degradation. Chlorophenol concentrations were measured using the 4-aminoantipyrine colorimetric method based on the procedure detailed in Standard Methods for the Examination of Water and Wastewater (Greenberg *et al.*, 1992).

Isolation of 4-chlorophenol-degrading bacteria

Aliquots from each enrichment culture were transferred onto basal mineral media supplemented with 4-chlorophenol and kept at 30°C. The obtained colonies were screened for isolates capable of removal of 4-chlorophenol after subculturing in a liquid medium. Sub-culturing of cultures showing positive chlorophenol degradation in a fresh medium was continued until a stable culture was obtained showing consistent biodegradation of selected chlorinated phenols.

UV/VIS spectrophotometric analysis

For detection of intermediates, cell-free extracts were prepared and assayed for enzymatic activities involved in 4-CP catabolism. Preparation of the cell extract was attained by harvesting cells by centrifugation at 5,000 rpm for 5 min and re-suspension in Tris-HCl buffer (pH 7.5). Crude extract was obtained after cell disruption by discontinuous sonication in an ice bath with a Branson Sonifier 250. The supernatant was recovered by filtration through Whatman No. 1 filters and used for assaying enzymatic activities. 4-CP degradation and production of ring cleavage products were monitored separately as described by Farrell and Quilty (1999) by mixing a crude cell extract with a substrate and measuring the absorbance from 200 to 600 nm with a scanning Unicam UV-300 spectrophotometer, UK. Phenol and 4-CP removal were detected by measuring the decrease in absorbance at 260 nm while formation of hydroxymuconate semialdehyde (HMS) and 5-CHMS were detected by measuring the increase in absorbance at 375 and 380 nm, respectively.

Molecular identification of the 4-CP-degrading isolate

Molecular identification of strain PT3 was performed by amplification of 16S rDNA with eubacterial universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Lane, 1991) and 517R (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer *et al.*, 1993). Amplification was performed in 25-µl reactions using Dream-*Taq* PCR master mix (Fermentas, USA) according to the thermal cycler instruction manual (Applied Biosystems 2720, USA). The PCR conditions were adjusted to 5 min for initial denaturation at 95°C and then 35 cycles of 40 sec at 94°C, 40 sec at 54°C, and 40 sec at 72°C, and finally 10 min at 72°C. Amplified genes were subjected to electrophoresis on a 1% agarose gel loaded alongside size markers (DNA ladder, Promega, USA).

PCR amplification of LmPH encoding gene

The coding sequence of the alpha subunit of mPH was amplified by using primers flanking the LmPHs conserved sequence, pheUf (5′-CCAGG(C/G)(C/G/T)GA(G/A)AA(A/G)GAGA(A/G)GAA(G/A)CT-3′) and pheUr (5′-CGG(A/T)A(G/A)CCGCGCCAGAACCA-3′) (Futamata *et al.*, 2001). Extraction of genomic DNA was performed according to Sambrook *et al.* (1989). PCR was performed with a thermal cycler (Applied Biosystems 2720, USA) using Dream-*Taq* PCR master mix (Fermentas, USA) according to the manufacturer's instructions. The PCR reaction (in 25 µl) used the following program: step 1, 10 min of denaturation 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. Detection of the C23DO encoding sequence by PCR was performed according to El-Sayed (1999). Amplified genes were subjected to electrophoresis on a 1% agarose gel loaded alongside size markers as described above (DNA ladder, Promega).

Sequencing and phylogenetic analysis

PCR amplicons for both 16S rRNA and LmPH encoding genes were used for subsequent sequence base determination. The nucleotide sequencing was determined by the automated florescent dye terminator sequencing method (Sanger *et al*., 1977) using the ABI310 genetic sequence analyzer (Applied Biosystems, USA) according to the manufacturer's instructions. The obtained sequences were aligned with the published sequences from the BLAST search program supplied by the National Center for Biotechnology Information (NCBI) (Altschul *et al*., 1997) by using CLUSTAL-X (Thompson *et al*., 1997). Sequences were edited by using Genetyx-Win MFC application software version 4.0. Phylogenetic trees were constructed on the basis of the neighbor-joining methods (Saitou and Nei, 1987) by using MEGA version 3 (Kumar *et al*., 2004).

Nucleotide sequence accession numbers

The obtained sequences in this study have been deposited in NCBI GenBank under accession numbers AB824287 and AB824288 for partial 16S rRNA gene sequence and the gene for alpha subunit of mPH of *Pseudomonas* sp. PT3, respectively.

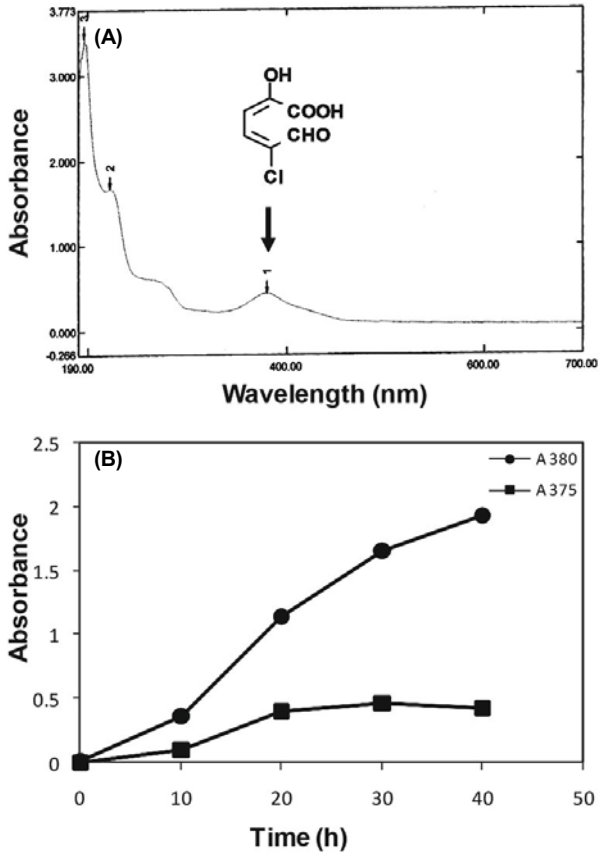

Fig. 1. Catabolic intermediates of 4-CP degradation by strain PT3. (A) UV/VIS spectral analysis of intermediate metabolites of 4-CP degradation showing accumulation of 5-CHMS at a λ_{max} of 380 nm. (B) Accumulation of extradiol *meta* ring cleavage products of phenol and 4-CP degradation by strain PT3.

Results and Discussion

Isolation and characterization of 4-CP-degrading strain PT3

From the screening of 4-CP-degrading bacteria from petroleum oil contaminated soils, strain PT3 was recovered. Soil samples enriched with 100 mg/L 4-CP showed the potential to catalyze biodegradation at a significant rate. Subsequent transfer from liquid to solid mineral agar plates supplemented with 4-CP resulted in the isolation of several different strains. A representative strain PT3 was selected because of its ability to biodegrade 4-CP as a sole carbon source. The ability of strain PT3 to degrade different concentrations of 4-CP was determined in BMM containing 4-CP from 50 to 500 mg/L as a sole carbon source. Growth was measured by cell counting and the decreases in 4-CP concentrations were monitored. Strain PT3 was able to grow and catabolize 4-CP at concentrations up to 150 mg/L, above which, a dramatic decrease in the biodegradation was observed (data not shown). The decrease in biodegradation rate may be attributed to the cytotoxic effects of chlorinated phenols at high concentrations (Herrera *et al*., 2008).

Metabolic pathway for 4-CP degradation by strain PT3

Determination of the metabolic pathway for 4-CP degradation by strain PT3 was based on monitoring the removal of 4-CP and production of potential ring cleavage metabolites spectrophotometrically. UV/VIS spectrophotometric analysis showed that strain PT3 degrades phenol or 4-CP *via* an extradiol *meta*-pathway. Firstly, phenol or 4-CP was hydroxylated to the corresponding catechol, which was then cleaved *via* an extradiol *meta*-pathway to give a typical yellow HMS or 5-CHMS (Fig. 1A).

By using cell-free extracts of cultures grown on 4-CP as a sole carbon source, a simultaneous decrease in A_{260} coupled with an increase in A_{375} or A_{380} was observed indicating the degradation of phenol or 4-CP and accumulation of HMS

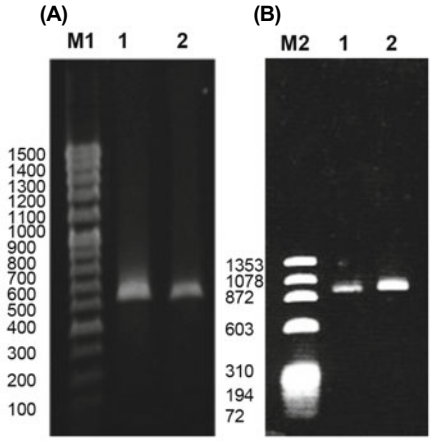

Fig. 2. Detection of the alpha subunit of mPH (*dmpN*) and C23DO encoding sequences from strain PT3 by PCR. (A) Lanes: 1, *dmpN* from strain PT3; 2, *dmpN* from reference *P. aeruginosa* AT2. (B) Lanes: 1, C23DO from strain PT3; 2, C23DO from reference *P. aeruginosa* AT2. Lane M1, M2 represent size markers; DNA ladder and ΦX174 DNA-*Hae*III digest, respectively.

or 5-CHMS, respectively, as a major *meta* ring cleavage products. Figure 1B shows the absorption spectra of the cell suspension supernatant from strain PT3 grown on 4-CP showing the production of 5-CHMS with a λ_{max} at 380 nm.

HMS and 5-CHMS were also detected in cultures of cells growing on phenol or 4-CP, respectively, as a sole carbon source. The highest accumulation of 5-CHMS was achieved by the higher activity of C23DO, the key enzyme for *meta* ring cleavage of chloroaromatic compounds. Therefore, the metabolic pathway for 4-CP degradation by strain PT3 was elucidated to follow the classical *meta* cleavage route by C23DO after initial hydroxylation to the corresponding chlorocatechol by PH. 4-CP biodegradation by strain PT3 *via* the *meta* cleavage pathway was confirmed by detection of the major corresponding catabolic genes. Positive PCR amplification of two major catabolic genes involved in the *meta* cleavage pathway, PH (*dmpN*) and C23DO, were obtained (Fig. 2). Kim *et al.* (2008) have stated that successful mineralization of 4-CP by bacteria is usually accomplished by the oxidation of 4-CP to 4-chlorocatechol, followed by aromatic ring cleavage, removal of chlorine, and transformation into products that are assimilated into the central metabolism of the cell.

Characterization of LmPH encoding sequence

The first and rate-limiting step in the aerobic degradation of 4-CP was found to be a hydroxylation reaction catalyzed by PH (Padilla *et al.*, 2000). There are two types of PHs, single-component and multicomponent enzymes (Harayama *et al.*, 1992). In the present study, we analyzed genes for the largest subunit of mPHs.

Amplification of the catalytic domain encoding sequence of LmPH from strain PT3 was successful using specific primers for LmPH (pheUf and pheUr) targeting positions from 195 to 815 downstream from the initiation site of the LmPH gene. When compared with other sequences, the LmPH active center encoding sequences from strain PT3 was aligned with a high degree of similarity to other LmPH active site encoding sequences reported in GenBank. LmPH-PT3 showed a 97% sequence similarity to the LmPH component of *Pseudomonas* sp. PND-1.

The mPH gene clusters have been cloned and characterized from some bacteria (Heinaru *et al.*, 2000; Cadieux *et al.*, 2002; Jeong *et al.*, 2003; Cafaro *et al.*, 2004; Merimaa *et al.*, 2006; Santos and Sá-Correia, 2007). PCR detection of both mPH and C23DO along with the high sequence similarities of LmPH of strain PT3 with those of the DmpN type suggested that the genetic organization of genes involved in the catabolism of 4-CP by strain PT3 is consistent with that of *Pseudomonas* sp. CF600 type (*dmpKLMNOPQBCDEFGHI*) (Powlowski and Shingler, 1994) (Fig. 3).

The active site encoding sequence of LmPH was considered

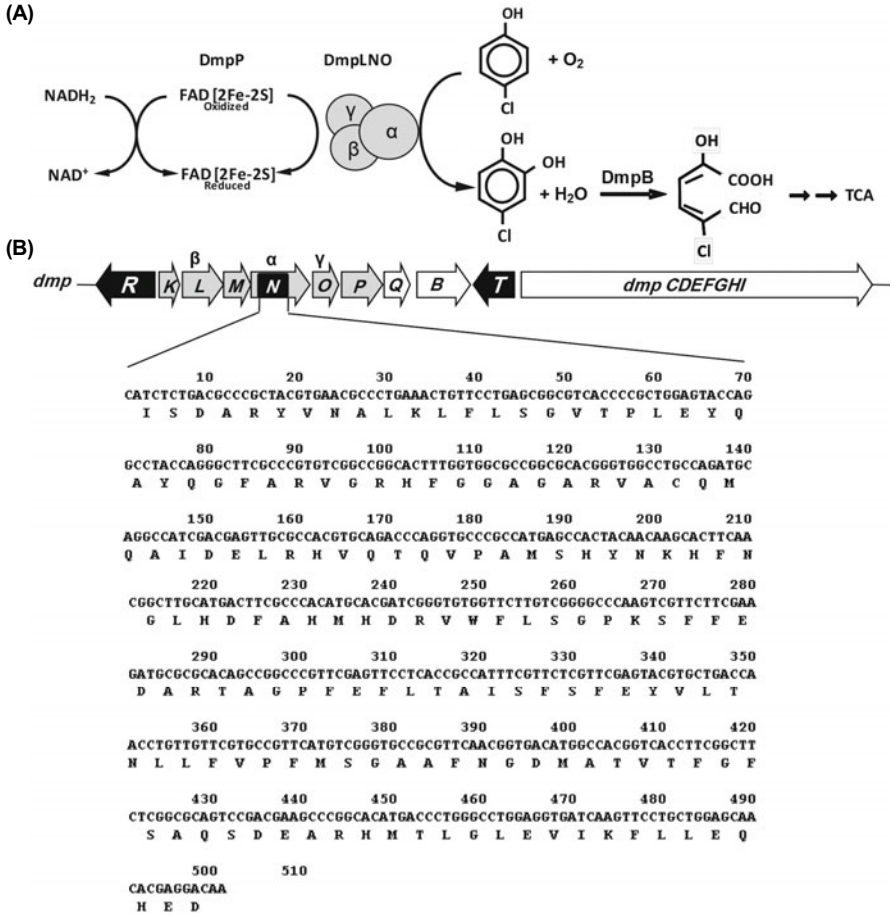

Fig. 3. Genetic organization of mPH components along with their corresponding catalytic reactions. (A) Genes encoding the reductase component (DmpP) that transfers electrons from NADH to a Rieske (2Fe-2S) protein and the α, β, and γ subunits of the oxygenase components (DmpLNO) with their corresponding hydroxylation of the aromatic nucleus. (B) A schematic representation of the gene arrangement of mPH from strain PT3 matching the canonical *dmpKLMNOP* type from *Pseudomonas* sp. CF600. The nucleotide sequence and its deduced amino acids of the alpha subunit of strain PT3 is indicated below the corresponding gene.

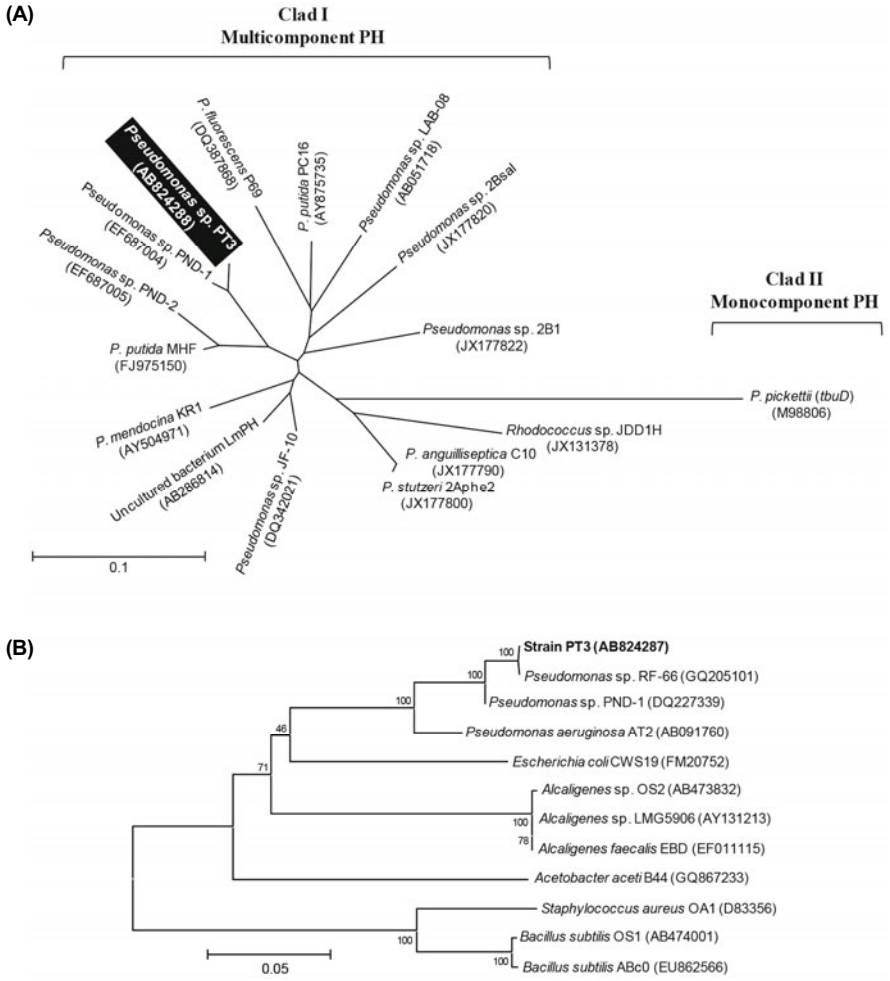

Fig. 4. Neighbor-joining phylogenetic trees. (A) rooted tree of the alpha subunits of mPH from strain PT3 and the alpha subunits of representative members of different groups of PH. (B) consensus tree based on 16S rDNA sequences analysis showing the relationship between isolate PT3 and the representative species of the genus *Pseudomonas* with other related genera. NCBI GenBank accession numbers for all sequences are indicated in parentheses.

to be conserved among most mPHs (Fox *et al*., 1993). Comparing active site encoding sequences from PT3 with other mPH encoding genes from corresponding strains revealed a high degree of conservation especially at the diiron coordination center (Fig. 5). When the metal coordination encoding regions from strain PT3 were compared with the homologous dinuclear oxo-iron bacterial monooxygenases, two typical DE(D)XRH motifs were detected. These motifs are common in several PHs and are suggested to be the diiron-oxo cluster binding site (Sazinsky and Lippard, 2006). These regions were identified by their pair of conserved domains with the amino acid sequence Asp-Glu-X-Arg-His. Two DE(D)XRH motifs were detected in LmPH of strain PT3 with an approximately 100 amino acid interval, a typical arrangement characteristic of most known PHs.

Phylogenetic analysis

The identity of strain PT3 was determined by using 16S rRNA partial gene sequencing. Partial sequencing was performed to cover up the hyper-variable (V3) region where sequences have diverged over time through evolution. On the basis of 16S rRNA gene sequence comparisons, strain PT3 was found to belong to the genus *Pseudomonas*. Strain PT3 shared 99% sequence similarity to *Pseudomonas* sp. RF-66. Phylogenetic analysis allowed examination of the relationship between strain PT3 and representative species of the genus *Pseudomonas* with other related genera (Fig.4B). In the neighbor-joining phylogenetic tree, strain PT3 was clustered at the same phylogenetic branch with *Pseudomonas* sp. RF-66. Strain PT3 was also closely related to *Pseudomonas* sp. PND-1 and the chlorophenol-degrading isolate *Pseudomonas aeruginosa* AT2, a typical strain known to catabolize chlorophenols *via* a typical *meta*-cleavage pathway (El-Sayed *et al*., 2003). The phylogenetic relationship between genes of LmPH of *Pseudomonas* sp. PT3 with known sequences of LmPH and PHs found at NCBI GenBank was studied using the neighbor-joining phylogenetic approach (Fig. 4A). In Rooted tree, LmPH-PT3 was clustered at the same phylogenetic branch with that of LmPH from *Pseudomonas* sp. PND-1. The sequence was clustered away from analogous mPHs of the *ortho*-pathway (*mopN*) and essentially had no relation to monocomponent PHs. Although monocomponent PHs and mPHs are present, the multicomponent type is considered the predominant one (Peters *et al*., 1997; Watanabe *et al*., 1998). Phylogenetic analysis confirmed the relation of LmPH-PT3 as a member of mPHs of the *meta* pathway type. This also suggests that the catabolic genes for chlorophenol degradation in strain PT3 is consistent with those of phenol degradation with genetic organization characteri-

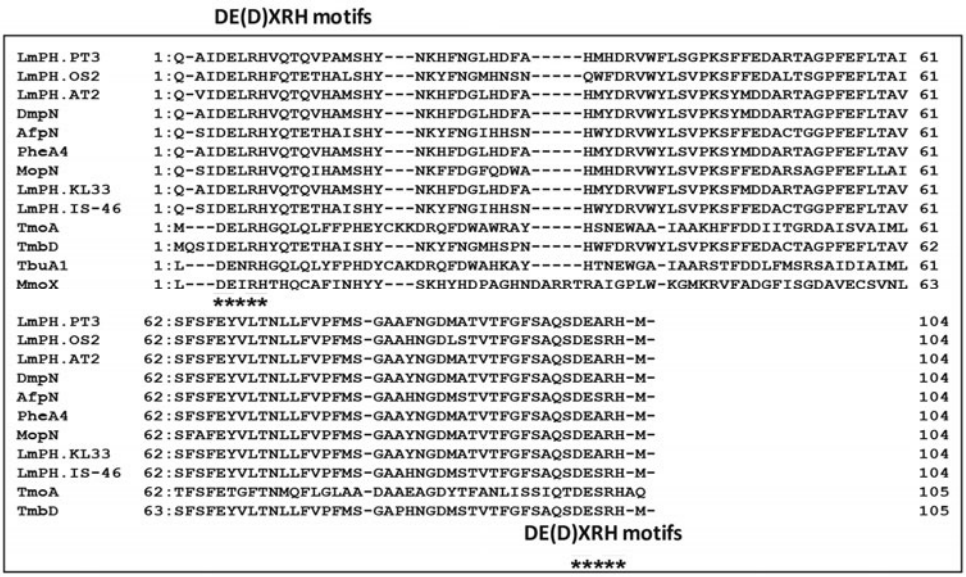

Fig. 5. Multiple sequence alignment of the deduced amino acid sequence of the alphasubunit (LmPH active site) and metal coordination encoding regions from *Pseudomonas* sp. PT3 with homologous dinuclear oxo-iron monooxygenases showing the conservation of the two typical DE(D)XRH motifs.

stics similar to mPHs and the subsequent *meta*-degradation pathway typical of *Pseudomonas* sp. CF66 type (*dmpKLMN OPQBCDEFGHI*) (Powlowski and Shingler, 1994).

Conclusion

A bacterial strain designated strain PT3 was isolated from oil-contaminated soil. Strain PT3 was able to grow using phenol and/or chlorophenol as sole carbon source. Strain PT3 was identified as a member of *Pseudomonas* sp. based on sequence analysis of the 16S rRNA gene fragment. *Pseudomonas* sp. PT3 was able to cataboliz chlorinated phenols *via* a *meta*-cleavage pathway after hydroxylation to the corresponding chlorocatechol. Both mPH and C23DO encoding sequences were detected by PCR as catabolic genes for chlorophenol catabolism by strain PT3. Nucleotide sequence analysis of the alpha (large) subunit of mPH from strain PT3 revealed the presence of the metal coordination system homologous to dinuclear oxo-iron bacterial monooxygenases. Two DE(D)XRH conserved motifs characteristic of most known PHs were detected in LmPH of strain PT3 indicating its phylogenetic grouping with mPHs.

Acknowledgements

We acknowledge the Deanship of the Scientific Research, Taibah University, KSA, for supporting this work by a grant (429/284).

References

Altschul, S.F., Thomas, L., Madden, A.A., Schäffer, Z.Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Res*. **25**, 3389–3402.

Arenghi, F.L., Berlanda, D., Galli, E., Sello, G., and Barbieri, P. 2001. Organization and regulation of *meta*-cleavage pathway gene for toluene and oxylene derivative degradation in *Pseudomonas stutzeri* OX1. *Appl. Environ. Microbiol*. **67**, 3304–3308.

Bertoni, G., Bolognesi, F., Galli, E., and Barbieri, P. 1996. Cloning of the genes for and characterization of the early stages of toluene catabolism in *Pseudomonas stutzeri* OX1. *Appl. Environ. Microbiol*. **62**, 3704–3711.

Bertoni, G., Martino, M., Galli, E., and Barbieri, P. 1998. Analysis of the gene cluster encoding toluene/o-xylene monooxygenase from *Pseudomonas stutzeri* OX1. *Appl. Environ. Microbiol*. **64**, 3626–3632.

Bodalo, A., Gomez, J.L., Gomez, E., Hidalgo, A.M., and Yelo, A.M. 2006. Removal of 4-chlorophenol by soybean peroxidase and hydrogen peroxidase in a Discontinuous tank reactor. *Desalination* **195**, 51–59.

Cadieux, E., Vrajmasu, V., Achim, C., Powlowski, J., and Munck, E. 2002. Biochemical andEPR studies of the diiron cluster of phenol hydroxylase from *Pseudomonas* sp. strain CF600. *Biochemistry* **41**, 10680–10691.

Cafaro, V., Izzo, V., Scognamiglio, R., Notomista, E., Capasso, P., Casbarra, A., Pucci, P., and Donato, A.D. 2004. Phenol hydroxylase and toluene/o-xylene monooxygenase from *Pseudomonas stutzeri* OX1: interplay between two enzymes. *Appl. Environ. Microbiol*. **70**, 2211–2219.

Esplugas, S., Gime´nez, J., Contreras, S., Pascual, E., and Rodrı´guez, M. 2002. Comparison of different advanced oxidation processes for phenol degradation. *Water Res*. **36**, 1034–1042.

El-Sayed, W.S. 1999. Biodegradation of certain Egyptian industrial wastes by locally isolated bacteria, Ph. D. thesis. Ain Shams University, Cairo, Egypt.

El-Sayed, W.S., Ibrahim, M.K., Abu-Shady, M., El-beih, F., Ohmura, N., Saiki, H., and Ando, A. 2003. Isolation and characterization of phenol-catabolizing bacteria from a coking plant. *Biosci. Biotechnol. Biochem*. **67**, 2026–2029.

Enroth, C., Neujahr, H., Schneider, G., and Lindqvist, Y. 1998. The crystal structure of phenol hydroxylase in complex with FAD and phenol provides evidence for a concerted conformational change in the enzyme and its cofactor during catalysis. *Structure* **6**, 605–617.

Evans, W.C., Smith, B.S.W., Fernley, H.N., and Davies, J.I. 1971. Bacterial metabolism of 2,4-dichlorophenoxyacetate. *Biochem. J*. **122**, 543–551.

Farrell, A. and Quilty, B. 1999. Degradation of mono-chlorophenols by a mixed microbial community *via* a *meta*-cleavage pathway. *Biodegradation* **10**, 353–362.

Fox, B.G., Shankin, J., Somerville, C., and Munck, E. 1993. Stearyl acyl carrier protein desaturate from *Ricinus communis* is a diiron-oxo protein. *Proc. Natl. Acad. Sci. USA* **90**, 2486–2490.

Futamata, H., Harayama, S., and Watanabe, K. 2001. Group-specific monitoring of phenol hydroxylase genes for a functional assessment of phenol-stimulated trichloroethylene bioremediation. *Appl. Environ. Microbiol.* **67**, 4671–4677.

Greenberg, A.E., Clesceri, L.S., and Eaton, A.D. 1992. "Standard methods for the examination of water and wastewater", 18th ed., APHA, WEF & AWWA, Washington, D.C., USA.

Harayama, S., Kok, M., and Neidle, E.L. 1992. Functional and evolutionary relationships among diverse oxygenases. *Annu. Rev. Microbiol.* **46**, 565–601.

Harayama, S. and Rekik, M. 1989. Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. *J. Biol. Chem.* **264**, 15328–15333.

Heinaru, E., Truu, J., Stottmeister, U., and Heinaru, A. 2000. Three types of phenol and *p*-cresolcatabolism in phenol- and *p*-cresol-degrading bacteria isolated from river water continuously polluted with phenolic compound. *FEMS Microbiol. Ecol.* **31**, 195–205.

Herrera, Y., Okoh, A.I., Alvarez, L., Robledo, N., and Trejo-Herna´ndez, M.R. 2008. Biodegradation of 2,4-dichlorophenol by a *Bacillus* consortium. *World J. Microbiol. Biotechnol.* **24**, 55–60.

Herrmann, H., Müller, C., Schmidt, I., Mahnke, J., Petruschka, L., and Hahnke, K. 1995. Localization and organization of phenol degradation genes of *Pseudomonas putida* strain H. *Mol. Gen. Genet.* **247**, 240–246.

Jeong, J.J., Kim, J.H., Kim, C.K., Hwang, I., and Lee, K. 2003. 3- and 4-alkylphenol degradationpathway in *Pseudomonas* sp. strain KL28: genetic organization of the lap gene cluster and substrate specificities of phenol hydroxylase and catechol 2,3-dioxygenase. *Microbiology* **149**, 3265–3277.

Kim, K.K., Lee, K.C., Oh, H.M., Kim, M.J., Eom, M.K., and Lee, J.S. 2008. *Arthrobacter defluvii* sp. nov., 4-chlorophenoldegrading bacteria isolated from sewage. *Int. J. Syst. Evol. Microbiol.* **58**, 1916–1921.

Kumar, S., Tamura, K., and Nei, M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform.* **5**, 150–163.

Lane, D.J. 1991. 16S/23S rRNA sequencing. *In* Stackebrandt, E. and Goodfellow, M. (ed.), Nucleic acid techniques in bacterial systematics, pp. 115-148. John Wiley & Sons, Inc., New York, N.Y., USA.

Leahy, J.G., Batchelor, P.J., and Morcomb, S.M. 2003. Evolution of the soluble diiron monooxygenases. *FEMS Microbiol. Rev.* **27**, 449–479.

Merimaa, M., Heinaru, E., Liivak, M., Vedler, E., and Heinaru, A. 2006. Grouping of phenolhydroxylase and catechol 2,3-dioxygenase genes among phenol and *p*-cresol-degrading *Pseudomonas* species and biotypes. *Arch. Microbiol.* **186**, 287–296.

Notomista, E., Lahm, A., Di Donato, A., and Tramontano, A. 2003. Evolution of bacterial and archaeal multicomponent monooxygenases. *J. Mol. Evol.* **56**, 435–445.

Muyzer, G., De Waal, E.C., and Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700.

Padilla, L., Matus, V., Zenteno, P., and González, B. 2000. Degradation of 2,4,6 trichlorophenol via chlorohydroxyquinol in *Ralstonia eutropha* JMP134 and JMP222. *J. Basic Microbiol.* **4**, 243–249.

Peters, M., Heinaru, E., Talpsep, E., Wand, H., Stottmeister, U., Heinaru, A., and Nurk, A. 1997. Acquisition of a deliberately introduced phenol degradation operon, pheBA, by different indigenous *Pseudomonas* species. *Appl. Environ. Microbiol.* **63**, 4899–4906.

Powlowski, J. and Shingler, V. 1994. Genetics and biochemistry of phenol degradation by *Pseudomonas* sp. CF 600. *Biodegradation* **5**, 219–236.

Qian, H., Edlund, U., Powlowski, J., Shingler, V., and Sethson, I. 1997. Solution structure of phenol hydroxylase protein component P2 determined by NMR spectroscopy. *Biochemistry* **36**, 495–504.

Rubalcaba, A., Suárez-Ojeda, M.E., Stüber, F., Fortuny, A., Bengoa, C., Metcalfe, I., Font, J., Carrera, J., and Fabregat, A. 2007. Phenol wastewater remediation: advanced oxidation processes coupled to a biological treatment. *Water Sci. Technol.* **55**, 221–227.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Plainview.

Sanger, F., Nicklen, S., and coulson, A.R. 1977. DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Santos, P.M. and Sá-Correia, I. 2007. Characterization of the unique organization and coregulation of a gene cluster required for phenol and benzene catabolism in *Pseudomonas* sp. M1. *J. Biotechnol.* **131**, 371–378.

Sazinsky, M.H., Dunten, P.W., McCormick, M.S., DiDonato, A., and Lippard, S.J. 2006. X-ray structure of a hydroxylase-regulatory protein complex from a hydrocarbon-oxidizing multicomponent monooxygenase, *Pseudomonas* sp. OX1 phenol hydroxylase. *Biochemistry* **45**, 15392–15404.

Sazinsky, M.H. and Lippard, S.J. 2006. Correlating structure with function in bacterial multicomponent monooxygenases and related diiron proteins. *Acc. Chem. Res.* **39**, 558–566.

Schwien, U. and Schmidt, E. 1982. Improved degradation of monochlorophenols by a constructed strain. *Appl. Environ. Microbiol.* **44**, 33–39.

Tartakovsky, B., Manuel, M.F., Beaumier, D., Greer, C.W., and Guiot, S.R. 2001. Enhanced selection of an anaerobic pentachlorophenol-degrading consortium. *Biotechnol. Bioeng.* **73**, 476–483.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882.

Ulson de Souza, S.M., Forgiarini, E., and Ulson de Souza, A. 2007. Toxicity of textile dyes and their degradation by enzyme horseradish peroxidase (HRP). *J. Hazardous Materials* **147**, 1073–1078.

Watanabe, K., Teramoto, M., Futamata, H., and Harayama, S. 1998. Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. *Appl. Environ. Microbiol.* **64**, 4396–4402.

Zhang, J., Ye, P., Chen, S., and Wang, W. 2007. Removal of pentachlorophenol by immobilized horseradish peroxidase. *Int. Biodeter. Biodegrad.* **59**, 307–314.