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Short communication

Chloroplast-type Ferredoxin Involved in Reactivation of Catechol 2,3-Dioxygenase from *Pseudomonas* sp. S-47

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***Pseudomonas* sp. S-47 is capable of degrading catechol and 4-chlorocatechol via the *meta*-cleavage pathway. XylTE products catalyze the dioxygenation of the aromatics. The *xylT* of the strain S-47 is located just upstream of the *xylE* gene. XylT is a typical chloroplast-type ferredoxin, which is characterized by 4 cystein residues that are located at positions 41, 46, 49, and 81. The chloroplast-type ferredoxin of *Pseudomonas* sp. S-47 exhibited a 98% identity with that of *P. putida* mt-2 (TOL plasmid) in the amino acid sequence, but only about a 40 to 60% identity with the corresponding enzymes from other organisms. We constructed two recombinant plasmids (pRES1 containing *xylTE* and pRES101 containing *xylE* without *xylT)* in order to examine the function of XylT for the reactivation of the catechol 2,3-dioxygenase (XylE) that is oxidized with hydrogen peroxide. The pRES1 that was treated with hydrogen peroxide was recovered in the catechol 2,3-dioxygenase (C23O) activity about 4 minutes after incubation, but the pRES101 showed no recovery. That means that the typical chloroplast-type ferredoxin (XylT) of *Pseudomonas* sp. S-47 is involved in the reactivation of the oxidized C23O in the dioxygenolytic cleavage of aromatic compounds.**

Keywords: Catechol 2,3-dioxygenase, Chloroplast-type Ferredoxin, *Pseudomonas* sp. S-47, Reactivation

Introduction

In the aerobic degradation of aromatics and chlorinated aromatics, the metabolic pathway generally leads to the formation of dihydroxy intermediates, such as catechol and other dihydrodiol compounds. For the formation of such aromatic intermediates, two enzymatic steps must be involved (Furukawa *et al*., 1993). The first one is the dihydroxylation step, which is carried out by the insertion of two atoms of oxygen at the adjacent carbon positions by dioxygenase complex. This is followed by a dehydrogenation reaction to produce dihydrodiol compounds that are catalyzed by a dihydrodiol dehydrogenase. The second step is the ring-cleavage of the dihydrodiol aromatics, which is catalyzed by either intradiol dioxygenase or extradiol dioxygenases, depending on the position (*ortho* or *meta*) of the cleavage site that is related to the diol (Kim *et al*., 1997; Noh *et al*., 2000).

Pseudomonas sp. S-47 is a bacterial isolate that degrades 4-chlorobenzoate (4CBA) and benzoate in order to produce 4-chlorocatechol (4CC) and catechol, respectively. The resulting 4CC and catechol can be utilized as the sole carbon and energy source through the complete catabolic pathway (Kim *et al*., 1998; Noh *et al*., 2000). The 4CC was first degraded by an extradiol dioxygenase to produce 5-chloro-2-hydroxy-muconicsemialdehyde (5C-2HMS) before the decholorination reaction, the same as in the *meta*-cleavage of catechol by catechol 2,3-dioxygenase (C23O) (Armengaud *et al*., 2000; Noh *et al*., 2000). The C23O that was encoded by *xylE* is a tetramer that is composed of four identical subunits, each of which contains a ferrous iron atom (Nozaki *et al*., 1968). It is an unstable enzyme that is inactivated upon exposure to oxidizing agents, such as oxygen. The inactivation of C23O also occurs during catalytic turnover when the substrate analogues, such as alkyl- or chloro-catechols (Bartels *et al*., 1984; Cerdan *et al*., 1994).

Polissi and Harayama (1993) reported that a mechanism exists *in vivo* that hinders the irreversible inactivation of C23O. Mutant cells of *Pseudomonas putida* that lack a *xylT* gene lost their ability to grow on *p*-xylene and *p*-toluate as

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carbon sources. In addition, it was found that 4-methylcatechol, which is an intermediate that is produced in the degradation of *p*-xylene and *p*-toluate, irreversibly inactivated the C23O in the *xylT* mutants, while the enzyme remained active in the wild-type. It was, therefore, envisioned that the *xylT* gene product might participate in a mechanism of reactivation of the C23O enzyme. The *xylT* gene product in *Pseudomonas putida* mt-2 was characterized as a chloroplast-type ferredoxin with [2Fe-2S] that specifically reactivates C23O (Hugo *et al*., 1998). Reactivation proceeds through a XylT-dependent reduction of the iron atom that is essentially present in the ferric state in the inactive form of the enzyme (Hugo *et al*., 2000). In this study, we cloned the *xylT* gene from the chromosome of *Pseudomonas* sp. S-47 and analyzed its nucleotide sequences. Then we established that XylT was involved in the reactivation of the oxidized C23O for dioxygenation of 4CC.

Materials and Methods

Strains and cultivation *Pseudomonas sp.* strain S-47 was grown at 30°C in a MM2 minimal medium [1 μM $FeSO_4 \cdot 7H_2O$, 100 μM $CaCl_2 \cdot 7H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 8.5 mM NaCl, 18 mM $(NH_4)SO_4$, 10 mM potassium phosphate buffer (pH 7.0)] that contained 0.5 mM 4CBA. *E. coli* XL1-Blue was used as a host strain for recombinant plasmids and grown in a Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37°C. Transformation was accomplished by the calcium chloride method (Sambrook *et al*., 2001). For selection of the transformants, a LB medium was supplemented with a 50 μg/ml final concentration of ampicillin. pBluescript II SK(+) was used as cloning and sequencing vectors.

Cloning and sequencing of the *xylT* gene Plasmid DNA was isolated by the alkali lysis method, as described by Sambrook *et al*. (2001). DNA cleavage by restriction endonuclease and ligation of DNA fragments by T4 DNA ligase were performed by standard procedures, as recommended by the supplier (Kosco Co., Seoul, Korea). The *xylTE* gene that was involved in the catabolism of catechol and 4CC was cloned in pCS1, pCS101, pCS201, and pCS202 from the chromosomal DNA of *Pseudomonas* sp. S-47 (Kim *et al*., 1998). A 7.0 kb fragment of pCS1 that was digested with *Pst*I was introduced into the polyclonal region of pBluescript II SK(+) vector to make pCSP21. The subclones of pRES1, pRES101, and pRES102 were obtained from the pCSP21 by digestion with various enzymes.

For nucleotide sequencing of *xylT*, a subclone of pRES102 was isolated by using Wizard Plus Minipreps (Promega Co., Madison, USA) under standard conditions, as recommended by the supplier. Editing and initial analysis of the *xylT* nucleotide sequences were performed using the DNASIS software (Hitachi version 7.0). A search for nucleotide and amino acid sequence similarities were done using the FASTA and BLAST programs, as well as the EMBL and GenBank Databases.

Preparation of cell extracts and enzyme assays Cultures were grown to the late-exponential growth phase in a LB medium. Bacterial cells were harvested by centrifugation, washed, and resuspended in a 10 mM potassium phosphate buffer (pH 7.5), and disrupted by sonication at 0°C. Cell debris was removed by centrifugation. The catechol 2,3-dioxygenase activity was measured according to the method of Sala-Trepat and Evans (1971). The following wavelength and molar absorption coefficients were used: catechol, 375 nm and 33,000 M^{-1} cm^{-1}, respectively. One unit (U) for each enzyme was defined as the amount that transforms 1 mmol of substrate per min.

Reactivation of catechol 2,3-dioxygenase by XylT *E. coli* XL1-Blue that contained pRES1 or pRES101 cells were grown at 37°C in a LB medium. The cells were harvested, washed, and resuspended in an equal volume of a 10 mM potassium phosphate buffer (pH 7.5). Four ml cell suspensions that contained pRES1 or pRES101 were 50 μM of H_2O_2 and incubated at room temperature. At appropriate times, 100 μl aliquots were taken. Their C23O activities were determined in a 10 mM potassium phosphate buffer (pH 7.5) that contained 600 μM catechol by measuring the change in absorbance at 375 nm. The catechol 2,3-dioxygenase activity was determined by using intact cells that gave almost identical values to those that were determined using sonicated cell extracts.

Results and Discussion

Nucleotide sequence of *xylT* and its amino acid homology *E. coli* XL1-Blue that harbored pCSP21, pRES1, and pRES101, respectively, showed a yellow color as a proof of C23O activity. This was the same as *E. coli* XL1-Blue that harbors pCS1, when catechol was sprayed over the colonies, as reported by Kim *et al.* (1998). The physical maps and

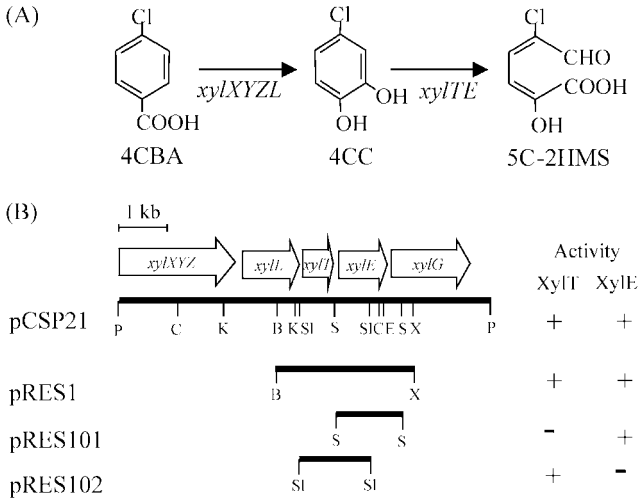

Fig. 1. Degradative pathway of 4-chlorobenzoate (4CBA) by *Pseudomonas* sp. S-47 (A), and Genetic maps of pCSP21 and its clones (B). Abbreviations: 4CBA, 4-chlorobenzoate; 4CC, 4-chlorocatechol; 5C-2HMS, 5-chloro-2-hydroxymuconic semialdehyde; B, *Bam*HI; C, *Cla*I; E, *Eco*RV; K, *Kpn*I; P, *Pst*I; SI, *Sal*I; S, *Sac*II; X, *Xho*I.

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XylT S-47    --MNSAGYEVFEVLSGQSFRCAEGQSVLRAMEAQGKRCIP  38
XylT mt-2    --MNSAGYEVFEVLSGQSFRCAEGQSVLRAMEAQGKRCIP  38
DmpQ CF600   --MNRAGYEIRETVSGQTFRCLPDQSVLSAMEQQGKRCVP  38
PhhQ P35X    --MSSPPFQVHETNSGQSFTCRPDQSVLRAMEEQGKRCVP  38
NahT G-7     ---MSEVFEITVQPGGERFVCQPQQSALIAMETQGKRCLP  37
AtdS YAA     ---MGESYQITEQCSGQRFPCKAGQSVLKAMEQQGLECAP  37
TbuW PKO1    MLIPDPRWTYAWNRPRSTTHARPPKASLTGMLRLGRKGIP  40
                                        *  *   *   *

XylT S-47    VQCRGGGCGLCRVRVLSGAYRSGRMSRGHVPAKAAAEGFA  78
XylT mt-2    VQCRGGGCGLCRVRVLSGAYRSGRMSRGHVPAKAAAEALA  78
DmpQ CF600   VQCRGGGCGLCKVRVLSGTYQCIKMSCNIVPPEAAKQGLA  78
PhhQ P35X    VQCRGGGCGLCKVRVLSGDYQCGRMSCSQVPPEAGEQGLA  78
NahT G-7     VQCRGGGCGLCKVRVLAGDYESGRVSCKHLPVEAREQGYA  77
AtdS YAA     VQCRGGGCGLCKVTVREGDYECGKMSRVIAPPEALAQGEV  77
TbuW PKO1    VQCYNGGCGVCKVRVLDGSTRLGRRQPCPRQRRRRSAGLT  80
             ***  ****** * * *  *

XylT S-47    LACQVFPQTDLTIEYF----RHVGGNKPDNMNYEEVTS-  112
XylT mt-2    LACQVFPQTDLTIEYF----RHVGGNKPDNMNYEEVTS-  112
DmpQ CF600   LACQLFPQTDLNIECL----RRQGPGDIINKNQQEVSS-  112
PhhQ P35X    LACQLYPRADLYIESL----RQVRSNP-------------  101
NahT G-7     LACRLFARSDLCIERY---SKPCSESTVDQQQRE-----  108
AtdS YAA     LACRIYPLSDLIIECR---PRQSAAGLANEYTTTKAMR-  112
TbuW PKO1    LACREAPLTAVRLAVLGKFEKPFSRASAFAGAQSDTKPQ  119
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Fig. 2. Alignment of amino acid sequences of XylT from *Pseudomonas* sp. S-47 and other strains. Asterisks indicate the conserved amino acid residues that were identical in all enzymes. The amino acid sequences in the box indicate the consensus amino acid pattern for chloroplast-type ferredoxin. Multiple alignment was carried out using the Clustal X software. The GenBank accession numbers of the genes are shown in parenthesis. S-47, *Pseudomonas* sp. S-47 (AF320981); mt-2, *Pseudomonas putida* mt-2 (X61467); CF600, *Pseudomonas* sp. CF600 (X60657); P35X, *Pseudomonas putida* P35X (X79063); G-7, *Pseudomonas putida* G7 (X61466); YAA, *Acinetobacter* sp. YAA (AB008831); PKO1, *Ralstonia pickettii* PKO1 (U20258).

C23O activities of these subclones are shown in Fig. 1. A nucleotide sequence of *xylT* in the 1.2 kb *Sal*I fragment of pRES102 was analyzed; it consisted of 339 bp. The *xylT* gene is located immediately upstream of *xylE*. These genes show translational coupling, since the stop codon of *xylT* overlaps the start codon of *xylE*. The GenBank accession number of the *xylT* is AF320981.

The XylT sequence contains 112 amino acid residues. These include six cysteines, four of which are located at positions 41, 46, 49, and 81 (Fig. 2). This chloroplast-type [2Fe-2S] motif, $CX_4CX_2CX_nC$, is not generally found in reductases (Harayama *et al*., 1991; Armengaud *et al*., 2000). This type of ferredoxin, therefore, differs significantly from the Rieske-type [2Fe-2S] ferredoxins that contain a [2Fe-2S] cluster that is bound to the polypeptide by two histidine residues. Since the *xylT* gene was first discovered from *Pseudomonas putida* (Harayama *et al*., 1991), a number of related genes that encode biodegradation of aromatic compounds [like naphthalene (*nahT*), phenol (*phhQ*), substituted phenols (*dmpQ*), toluene (*tbuW*), and aniline

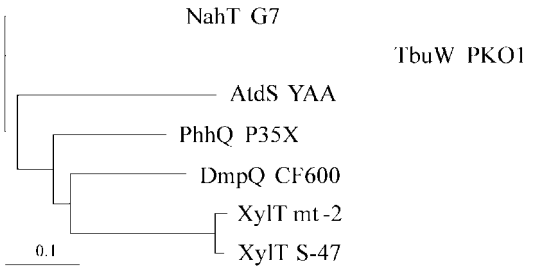

Fig. 3. The levels of homology among the amino acid sequences of chloroplast-type ferredoxins (XylT) from different strains. The GenBank accession numbers of the genes are shown in Fig. 2.

(*atdS*)] have been identified (Harayama *et al*., 1991; Shingler *et al*., 1992; Ng *et al*., 1994; Kukor and Olson, 1996).

An amino acid sequence that was deduced from the *xylT* nucleotide sequence of strain S-47 was aligned with those of 6 corresponding enzymes from other bacterial strains (Fig. 3). The deduced amino acid sequence of the chloroplast-type ferredoxin from *Pseudomonas* sp. S-47 exhibited a 98% identity with that of the TOL plasmid in *Pseudomonas putida* mt-2 (Furukawa *et al*., 1993). However, there was only about a 40 to 60% identity with those of *Pseudomonas* sp. CF600 (Shingler *et al*., 1992), *Pseudomonas putida* P35X (Ng *et al*., 1994), *Acinetobacter* sp. YAA (Fujii *et al*., 1997), *Pseudomonas putida* G7 (Harayama *et al*., 1991), and *Ralstonia pickettii* PKO1 (Kukor and Olson, 1996).

Reactivation of catechol 2,3-dioxygenase by XylT C23O is inactivated by exposure to air or during the catalytic cycle, especially when substituted catechols, such as 3- and 4-methylcatechol, were used as substrates (Bartels *et al*., 1984; Polissi and Harayama, 1993; Cerdan *et al*., 1994). Inactivation of the enzyme is primarily caused by the oxidation of ferrous iron that is present at the active site of the enzyme, but ultimately the oxidized iron atom may be released from the enzyme (Wasserfallen, 1989; Kim *et al*., 2000; Kim *et al*., 2001).

The *E. coli* XL1-Blue cells that harbored pRES1 or pRES101, which were inactivated with H_2O_2, showed an immediate decrease in C23O activity (Fig. 4). The cells that harbored pRES1 with both *xylT* and *xylE* were reactivated about 4 minutes after inactivation with H_2O_2 (Fig. 4A). However, the activity of the cells that harbored pRES101 with only *xylE* was not reactivated. This means that *xylT* was necessary for the reactivation of the inactivated C23O (XylE) in pRES1. The C23O of pRES101, which was inactivated with H_2O_2, was also reactivated when pRES102 was added in the cell extract (Fig. 4B). These results, therefore, demonstrate that XylT is involved in the reactivation of catechol 2,3-dioxygenase, which was inactivated by H_2O_2.

XylT specifically mediates the reduction of oxidized iron in the active site of catechol 2,3-dioxygenase (C23O), resulting in the reactivation of the enzyme. This XylT function is based on the characterization of the *xylT* deletion mutants that were

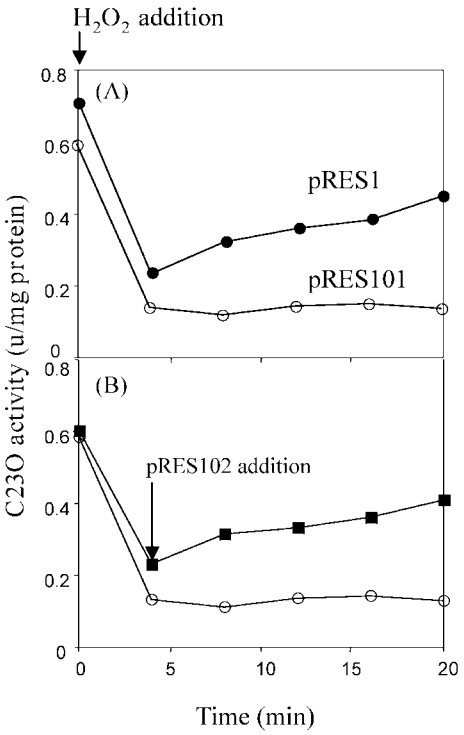

Fig. 4. Reactivation of C23O (XylE) in the pRES1 and pRES101, which were treated with H_2O_2. The C23O in the pRES1 was reactivated (●) about 4 minutes after treatment (A), but not in pRES101 (○). However, the C23O in the inactivated pRES101 was reactivated (■) when pRES102 was added (B).

made by Polissi and Harayama (1993). Therefore, the results in this study proved that the *xylT* product in *Pseudomonas* sp. S-47 is chloroplast-type ferredoxin, which functions for the reactivation of the oxidized C23O. XylT contains 4 cysteine residues at the typical positions of 41, 46, 49, and 81, as observed in the chloroplast-type ferredoxins from other bacterial strains (Harayama *et al*., 1991; Armengaud *et al*., 2000; Hugo *et al*., 2000).

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References

Armengaud, J., Gaillard, J. and Timmis, K. N. (2000) A second [2Fe-2S] ferredoxin from *Sphingomonas* sp. Strain RW1 can function as an electron donor for the dioxin dioxygenase. *J. Bacteriol*. **182**, 2238-2244.

Bartels, I., Knackmuss, H. J. and Reineke, W. (1984) Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. *Appl. Environ. Microbiol*. **47**, 500-505.

Cerdan, P., Wasserfallen, A., Rekik, M., Timmis, K. N. and Harayama, S. (1994) Substrate specificity of catechol 2,3-dioxygenase encoded by TOL plasmid pWW0 of *Pseudomonas putida* and its relationship to cell growth. *J. Bacteriol*. **176**, 6074-6081.

Fujii, T., Takeo, M. and Maeda, Y. (1997) Plasmid-encoded genes specifying aniline oxidation from *Acinetobacter* sp. strain YAA. *Microbiology* **143**, 93-99.

Furukawa, K., Hirose, J., Suyama, A., Zaiki, T. and Hayashida, S. (1993) Gene components responsible for discrete substrate specificity in the metabolism of biphenyl (*bph* operon) and toluene (*tod* operon). *J. Bacteriol*. **175**, 5224-5232.

Harayama, S., Polissi, A. and Rekik, M. (1991) Divergent evolution of chloroplast-type ferredoxins. *FEBS Lett*. **285**, 85-88.

Hugo, N., Armengaud, J., Gaillard, J., Timmis, K. N. and Jouanneau, Y. (1998) A novel [2Fe-2S] ferredoxin from *Pseudomonas putida* mt-2 promotes the reductive reactivation of catechol 2,3-dioxygenase. *J. Biol. Chem*. **273**, 9622-9629.

Hugo, N., Meyer, C., Armengaud, J., Gaillard, J., Timmis, K. N. and Jouanneau, Y. (2000) Characterization of three XylT-like [2Fe-2S] ferredoxins associated with catabolism of cresols or naphthalene: evidence for their involvement in catechol dioxygenase reactivation. *J. Bacteriol*. **182**, 5580-5585.

Kim, S. and Shin, H.-Y. (2000) Reduction of Azobenzene by purified bovine liver quinone reductase. *J. Biochem. Mol. Biol*. **33**, 321-325

Kim, K. P., Seo, D. I., Lee, D. H., Kim, Y. and Kim, C. K. (1998) Cloning and expression in *E. coli* of the genes responsible for degradation of 4-chlorobenzoate and 4-chlorocatechol from *Pseudomonas* sp. strain S-47. *J. Microbiol*. **36**, 99-105.

Kim, S. I., Kim, S.-J., Leem, S.-H., Oh, K.-H., Kim, S. and Park, Y.-M. (2001) Site-directed mutagenesis of two cysteines (155, 202) in catechol 1,2-dioxygenase I_1 of *Acinetobacter lwoffi* K24. *J. Biochem. Mol. Biol*. **34**, 172-175.

Kim, S. I., Kweon, S. M., Kim, S. and Ha, K.-S. (1997) Expression and characterization of *catA*$_1$ (catechol 1,2-dioxygenase I_1) of *Acinetobacter lwoffi* K24 in *Escherichia coli*. *J. Biochem. Mol. Biol*. **30**, 342-345.

Kukor, J. J. and Olsen, R. H. (1996) Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments. *Appl. Environ. Microbiol*. **62**, 1728-1740.

Manson, J. R. and Cammack, R. (1992) The electron-transport proteins of hydroxylating bacterial dioxygenases. *Annu. Rev. Microbiol*. **46**, 277-305.

Mars, A. E., Kingma, J., Kaschabek, S. R., Reineke, W. and Janssen, D. B. (1999) Conversion of 3-chlorocatechol by various catechol 2,3-dioxygenases and sequence analysis of the chlorocatechol dioxygenase resion of *Pseudomonas putida* GJ31. *J. Bacteriol*. **181**, 1309-1318.

Ng, L. C., Shingler, V., Sze, C. C. and Poh, C. L. (1994) Cloning and sequences of the first eight genes of the chromosomally encoded (methyl) phenol degradation pathway from *Pseudomonas putida* P35X. *Gene* **151**, 29-36.

Noh, S. J., Kim, Y., Min, K. H., Karegoudar, T. B. and Kim, C. K. (2000) Cloning and nucleotide sequence analysis of *xylE* gene responsible for *meta*-cleavage of 4-chlorocatechol from *Pseudomonas* sp. S-47. *Mol. Cells* **10**, 475-479.

Nozaki, M., Ono, K., Nakazawa, T., Kotani, S. and Hayashi, O. (1968) Metapyrocatechase. II. The role of iron and sulfhydryl groups. *J. Biol. Chem*. **243**, 2682-2690.

Polissi, A. and Harayama, S. (1993) *In vivo* reactivation of catechol 2,3-dioxygenase mediated by a chloroplast-type ferredoxin: a bacterial strategy to expand the substrate specificity of aromatic degradative pathways. *EMBO J.* **12**, 3339-3347.

Sala-Trepat, J. M. and Evans, C. W. (1971) The *meta* cleavage of catechol by *Azotobacter* species. 4-Oxalocrotonate pathway. *Eur. J. Biochem*. **20**, 400-413.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (2001) *Molecular Cloning: A Laboratory Manual*. 3rd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shingler, V., Powlowski, J. and Marklund, U. (1992) Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. *J. Bacteriol*. **174**, 711-724.

Wasserfallen, A. (1989) Biochemical and genetical study of the specificity of catechol 2,3-dioxygenase from *Pseudomonas putida*. Ph.D. thesis, University of Geneva.