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Cloning and characterization of a *p*-cymene monooxygenase from *Pseudomonas chlororaphis* subsp. *aureofaciens*

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Abstract

p-Cymene monooxygenase is the enzyme system that catalyzes the hydroxylation of *p*-cymene to 4-isopropylbenzyl alcohol (*p*-cumic alcohol), the initial step in the assimilation of *p*-cymene by *Pseudomonas chlororaphis* subsp. *aureofaciens*. Cloning and sequencing of single NADH-dependent cytochrome c reductase gene (*cymA*) present in *P. chlororaphis* subsp. *aureofaciens* was described earlier. In this study, analysis of the upstream sequence of *cymA* revealed two open reading frames, designated as *cymB* (495 bp) and *cymM* (1128 bp). Database searches with the *cymM* gene product showed similarity to integral-membrane di-iron enzymes, while that with *cymB* showed no significant similarity to other known proteins with the exception of epoxystyrene isomerases. Expression of all three components (*cymMBA*) in *Escherichia coli* confirmed its ability for *p*-cymene methyl group hydroxylation, while expression of *cymM* and *cymA* along with the partially truncated *cymB* gene showed an 85% decrease in the hydroxylation capacity. Our results suggest for the first time that the small protein, CymB, having no conserved domains in protein databases, is involved as enhancer/activator in *p*-cymene hydroxylation.

Keywords: *p*-Cymene; Methyl hydroxylase; Monooxygenase; Non-heme di-iron enzyme; *Pseudomonas*

1. Introduction

Biogeochemical reserves and industrial activities are the major sources of alkylated aromatic compounds which form an important group of environmental pollutants or their precursors in the ecosystem (Dutta, 2005; Radke, 1987). Microorganisms that mineralize a variety of methyl aromatic compounds have been isolated and their metabolism via side-chain hydroxylation has been well documented (Assinder and Williams, 1990; Defrank and Ribbons, 1977; Dutta et al., 1998; Hopper, 1988). However, the oxygenase system that hydroxylates methyl substituents of the aromatic ring, the catalytic iron center and the reaction cycle largely remains to be defined. Catalytic centers, which contain iron prosthetic groups, have been well documented for several oxygenases. The most abundant center is a unique heme-thiolate monooxygenase which contains more than 500 related protein sequences (Nelson, 2006). A few other well-studied oxygen reaction centers are the $Fe_2(\mu O)_2$ cluster, found in methane monooxygenase, alkane hydroxylase and other non-heme di-iron enzymes (Fox et al., 1988; Schwartz et al., 2008; Shanklin et al., 1994, 2009; Shanklin and Whittle, 2003) and dissociable ferrous, mononuclear non-heme iron coordination sites (Kovaleva and Lipscomb, 2008).

To understand the structural aspect and the mechanistic details, we selected *p*-cymene, an aromatic-terpenoid of biosynthetic origin, as the representative model for alkylated aromatics. *p*-Cymene is a natural aromatic hydrocarbon which occurs in the oils of many gymnospermic and angiospermic

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plants (Benchaar et al., 2008; Singh et al., 1999). Moreover, it has been observed as the dead-end product in anaerobic enrichment cultures of certain non-aromatic monoterpenes (Harder and Probian, 1995).

Oxidative catabolism of *p*-cymene had been studied widely and the assimilating pathway was already established (Defrank and Ribbons, 1977; Eaton, 1997; Lee et al., 2006; Madhyastha et al., 1968). The upper pathway of *p*-cymene metabolism occurs by progressive oxidation of the methyl side-chain to carboxylic acid. Cloning and characterization of the *cym* operon, which was reported to be involved in the conversion of *p*-cymene to *p*-cumate in *Pseudomonas putida* F1 revealed the presence of a two-component methyl monooxygenase (Eaton, 1997). Similar oxidation of methyl groups was reported for toluene and *m*- and *p*-xylenes (Assinder and Williams, 1990; Harayama et al., 1989, 1992). For example, xylene monooxygenase, a two-component system in *P. putida* mt-2, is biochemically most closely related to *p*-cymene monooxygenase, and its terminal component, XylM, carries similarity with *n*-alkane hydroxylase and steroyl-CoA desaturase terminal components (Kok et al., 1989; Shanklin et al., 1994; Suzuki et al., 1991). On the other hand, the flavoprotein reductase, XylA differs from the corresponding alkane and, in part, from desaturase proteins.

The reductase sequence of *p*-cymene monooxygenase from *Pseudomonas aureofaciens* reclassified as *Pseudomonas chlororaphis* subsp. *aureofaciens* (Peix et al., 2007) was reported in our previous communication (Dutta and Gunsalus, 1997). Moreover, its high similarity with various oxygenase-coupled flavoproteins of both prokaryotic and eukaryotic origin including systems of heme and non-heme monooxygenases, aromatic dioxygenaes and reductases involved in other biological functions had also been demonstrated. The present study reports the genetic characterization and expression of the three-component *p*-cymene monooxygenase, including a small open reading frame, *cymB*, involved in *p*-cymene methyl hydroxylation.

2. Materials and methods

2.1. Organism, culture conditions and plasmid

P. chlororaphis subsp. *aureofaciens* strain PJC was cultured in PAS medium (Gunsalus and Wagner, 1978) with *p*-cymene (1 g l^{-1}) as the sole source of carbon and energy as described earlier (Dutta and Gunsalus, 1997). Recombinant strain *Escherichia coli* JM109 (Yanisch-Perron et al., 1985) was grown in Luria-Bertani (LB) medium (Davis et al., 1980) overnight at 37 °C. Bacto agar (1.5%), Difco Laboratories, Detroit, MI was added for solid media.

2.2. Cloning and DNA manipulation of the p-cymene monooxygenase genes

The N-terminal amino acid sequence and the near C-terminal conserved sequences of related iron-sulfur flavoproteins associated with aromatic methyl hydroxylase and ring-hydroxylating monooxygenases were used to design primers for PCR processing (Dutta and Gunsalus, 1997). The PCR product used as a probe in southern and colony hybridization for the 8 kb (*Bgl*II fragment) clone of the total cell DNA in pLV59 (O'Connor and Humphries, 1982) has been described previously (Dutta and Gunsalus, 1997). The 8 kb insert in pLV59 (pTD101) was digested with *Hin*dIII and *Kpn*I, gel-purified and inserted into the *Hin*dIII and *Kpn*I unique sites within the *lacZ*α gene of the plasmid pBluescriptII SK (Fig. 1). The recombinant plasmid (pTD102) was transformed in *E. coli* on selective medium containing ampicillin (100 μg ml^{-1}), IPTG (0.25 mM) and X-gal (0.02%). White colonies were picked for further analysis. To delete the *Hin*dIII-*Bgl*II region which is from plasmid pLV59 and to delete the *Pst*I site from the pBluescriptII SK, the recombinant plasmid was digested with *Bgl*II and *Spe*I (*Spe*I is one of the multiple cloning sites of pBluescriptII SK). The overhangs were filled by the action of Klenow fragment of DNA polymerase I, ligated with T4 DNA ligase and transformed into *E. coli* under similar selection pressure. The white colonies carrying the *Bgl*II-*Kpn*I region of the original 8 kb insert in pBluescriptII SK (pTD103, Fig. 1) which essentially lost the *Bgl*II and *Spe*I sites due to Klenow filling, was further digested with *Hin*cII and *Pst*I to delete a fragment (123 bp, nucleotide 1599 to 1721) from the *cymB* gene. *Hin*cII produced the blunt end but the 3′ overhang from *Pst*I was repaired by the 3′→5′ exonuclease activity of T4 DNA polymerase incubated in the presence of all four dNTPs, ligated (pTD104, Fig. 1) and transformed into *E. coli*. The variants containing different inserts were confirmed by restriction analysis of the respective plasmids and DNA sequencing analysis.

2.3. DNA sequencing and analysis

DNA sequences were determined by the dideoxy chain termination method using double-stranded DNA as the template (Sanger et al., 1977). Sequencing was accomplished according to the manufacturer's specifications for *Taq* DNA polymerase-initiated cycle sequencing reactions using fluorescently labeled di-deoxynucleotide terminators and primers on an Applied Biosystem Model 373A DNA sequencer. (Perkin–Elmer Applied Biosystems, Inc.). Sequence data were aligned and edited using DNASTAR (DNASTAR Inc., Madison, WI, USA). Searches for specific nucleotide or amino acid sequences in databases were carried out with the BLAST program (Altschul et al., 1990), while ClustalX v1.81 (Thompson et al., 1997) was used to obtain multiple sequence alignments for protein sequences.

2.4. Expression of recombinant strains and metabolite analysis

Recombinant strains were grown in LB medium at 37 °C, supplemented with ampicillin (100 μg ml^{-1}). After 2 h incubation, inducer, IPTG was added to a final concentration of 1 mM. Induction proceeded for 3 h at 37 °C and then cells were collected by centrifugation, washed twice using