Phenol hydroxylase cloned from *Ralstonia eutropha* strain E2 exhibits novel kinetic properties

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Ralstonia eutropha strain E2 (previously *Alcaligenes* sp.) is a phenol-degrading bacterium expressing phenol-oxygenating activity with a low K_s (the apparent half-saturation constant in Haldane's equation) and an extremely high K_{SI} (the apparent inhibition constant). To identify the molecular basis for these novel cellular kinetic properties, a 9.5 kb DNA fragment that allowed *Pseudomonas aeruginosa* PAO1c (Phl⁻ Cat⁺) to grow on phenol as the sole carbon source was cloned from strain E2 into plasmid pRO1614. PAO1c harbouring this plasmid (designated pROE217) transformed phenol to catechol, indicating that this fragment contains gene(s) for phenol hydroxylase. The cloned genes consist of eight complete ORFs, designated *poxRABCDEFG*. The products are homologous to those of *dmpRKLMNOPQ* of *Pseudomonas* sp. CF600, sharing 30–65% identity: this suggests that the phenol hydroxylase is a multicomponent enzyme. The kinetic constants for phenol-oxygenating activity of PAO1c(pROE217) were determined, and these were compared with those of strain E2. The kinetic constants of PAO1c derivatives expressing different phenol hydroxylases were also determined. A comparison of these kinetic data suggests that phenol hydroxylase, the first enzyme in the phenol-degradative pathway, determines K_s and K_{SI} values for the cellular phenol-oxygenating activity. It is thus suggested that the phenol hydroxylase cloned from strain E2 exhibits the novel kinetic properties that were observed with intact cells of strain E2.

Keywords: phenol-oxygenating activity, phenol hydroxylase, kinetics, *Ralstonia eutropha*

INTRODUCTION

Phenolic compounds are known to be potential environmental pollutants discharged from industrial plants. Biodegradation of phenolics by bacteria has thus been a central subject in environmental microbiology, and a large number of phenol-degrading bacteria have been isolated. In our previous study, phenol-degrading bacteria were isolated from oil-refinery activated sludge, and the kinetics for phenol-oxygenating activities of these isolates and those of several known phenol-degrading bacteria were investigated in intact cells (Watanabe *et al.*, 1996). It was found that the phenol-degrading bacteria tested can be classified into several kinetically different groups according to K_s (the ap-

parent half-saturation constant in Haldane's equation) and K_{SI} (the apparent inhibition constant) values. However, *Ralstonia eutropha* strain E2 (previously *Alcaligenes* sp.), one of our isolates, did not belong to any group, exhibiting novel kinetic properties, i.e. a low K_s value and an extremely high K_{SI} value (Watanabe *et al.*, 1996). These kinetic constants of strain E2 were also different from those previously reported by other researchers (Folsom *et al.*, 1990; Hashimoto *et al.*, 1992). We were thus interested in identifying the molecular basis for the novel kinetics for the cellular phenol-oxygenating activity of strain E2 with an expectation that a novel phenol-degradative enzyme might exist.

Although there have been many reports documenting the kinetic evaluation of phenol-degrading bacteria (Folsom *et al.*, 1990; Hashimoto *et al.*, 1992; Watanabe *et al.*, 1996) and also describing identification of bacterial phenol-degradative enzymes (Nordlund *et al.*, 1990;

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Kukor & Olsen, 1990; Nurk *et al.*, 1991; Ng *et al.*, 1994; Herrmann *et al.*, 1995; Ehrt *et al.*, 1995; Takeo *et al.*, 1995), it has not yet been identified which molecules in the cell determine the cellular kinetic properties for phenol degradation. Among several possibilities, such as phenol-uptake systems and enzymes in the degradative pathways, phenol hydroxylase, the first enzyme in the bacterial phenol-degradative pathway, was assumed to be most likely to determine the K_s and K_{SI} values for phenol degradation by bacteria. In this study, to verify this assumption, genes for phenol hydroxylase of strain E2 were cloned, and the kinetics for phenol-oxygenating activity of *Pseudomonas aeruginosa* PAO1c (Phl⁻ Cat⁺) expressing the phenol hydroxylase of strain E2 were examined. In addition, the genes were sequenced to compare the putative amino acid sequences with those of the products of previously sequenced phenol hydroxylase genes.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Ralstonia eutropha* strain E2 (previously *Alcaligenes* sp.) had been

isolated from phenol-acclimatized oil-refinery activated sludge by using agar plates containing dCGY medium after enrichment in a continuous culture fed on phenol as the sole carbon source (Watanabe *et al.*, 1996). Strain E2 was affiliated with *R. eutropha* according to the nucleotide sequence of the 16S rRNA gene (accession number AB007998). Strain E2 was capable of growing on phenol as the sole carbon source, phenol being mineralized by strain E2 through a *meta*-cleavage pathway (Watanabe *et al.*, 1996). *P. aeruginosa* PAO1c is capable of growing on catechol, but not on phenol (Holloway *et al.*, 1979).

Media and growth conditions. The culture media used in this study were LB medium (Sambrook *et al.*, 1989), dLB medium (l⁻¹: 0.5 g Bacto yeast extract, 1 g Bacto tryptone and 1 g NaCl) and an inorganic medium called MP (Watanabe *et al.*, 1996). The strains of *P. aeruginosa* and *Escherichia coli* were grown at 37 °C, while strain E2 was grown at 30 °C. Tetracycline, ampicillin, kanamycin and carbenicillin were used in selective media at concentrations of 50, 50, 50 and 500 µg ml⁻¹, respectively.

Genetic techniques. Plasmid isolation from the *E. coli* strains, restriction endonuclease digestion and transformation of the *E. coli* strains were conducted as described by Sambrook *et al.* (1989). The *P. aeruginosa* strains were transformed as described by Chakrabarty *et al.* (1975).

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant markers and derivation*	Reference/source
Strain		
<i>R. eutropha</i> E2	Phl ⁺	Watanabe <i>et al.</i> (1996)
<i>P. aeruginosa</i> PAO1c	Phl ⁻ Cat ⁺	Holloway <i>et al.</i> (1979)
<i>E. coli</i> VCS257	Host strain of cosmid vector, <i>supF</i> derivative of DP50	Stratagene
<i>E. coli</i> JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lac-proAB)/F' (traD36 proAB⁺ lacI^q lacZΔM15)</i>	Sambrook <i>et al.</i> (1989)
Plasmid		
pLAFR3	Cosmid vector derived from pLAFR1, IncP1, <i>λcos⁺ rlx</i> , Tc ^r	Staskawicz <i>et al.</i> (1987)
pUC18	Ap ^r /Cb ^r , cloning vector	Sambrook <i>et al.</i> (1989)
pUC118	Ap ^r /Cb ^r , cloning vector	Sambrook <i>et al.</i> (1989)
pRO1614	Ap ^r /Cb ^r Tc ^r , cloning vector	Olsen <i>et al.</i> (1982)
pRO1959	Ap ^r /Cb ^r Phl ⁺ , containing cloned <i>phlA</i> and <i>phlR</i> of <i>B. pickettii</i> PKO1	Kukor <i>et al.</i> (1990)
pS04-92	Km ^r Phl ⁺ , containing cloned <i>pheA</i> and <i>pheR</i> of <i>P. putida</i> BH	Fujita <i>et al.</i> (1995)
pROBH01	Ap ^r /Cb ^r , 6.8 kb <i>NruI</i> fragment of pS04-92 cloned into the <i>EcoRV</i> site of pRO1614	This study
pRO1614M	Ap ^r /Cb ^r , multicloning site of pUC18 inserted into <i>EcoRI</i> - <i>HindIII</i> -cleaved pRO1614	This study
pLAFRE201	Ap ^r /Cb ^r , 23 kb <i>Sau3AI</i> partially digested DNA fragment from <i>Ralstonia</i> sp. E2 cloned into pLAFR3	This study
pLAFRE202	Tc ^r , 16 kb <i>EcoRI</i> fragment deleted from pLAFRE201	This study
pUCE203	Ap ^r /Cb ^r , 11.5 kb <i>BamHI</i> fragment of pLAFRE201 cloned into pUC18	This study
pROE204	Ap ^r /Cb ^r , 12.5 kb <i>NheI</i> - <i>KpnI</i> fragment of pLAFRE201 cloned into pRO1614M	This study
pROE206	Ap ^r /Cb ^r , 6.6 kb <i>EcoRI</i> fragment of pLAFRE201 cloned into pRO1614	This study
pUCE207	Ap ^r /Cb ^r , 3.5 kb <i>KpnI</i> - <i>EcoRI</i> fragment of pLAFRE201 cloned into pUC118	This study
pROE216	Ap ^r /Cb ^r , 10.5 kb <i>EcoRI</i> partially digested fragment of pLAFRE201 cloned into pRO1614	This study
pROE217	Ap ^r /Cb ^r , 10 kb <i>Clai</i> - <i>NheI</i> fragment of pLAFRE201 cloned into pRO1614	This study
pROE218	Ap ^r /Cb ^r , 10 kb <i>EcoRI</i> partially digested fragment of pLAFRE201 cloned into pRO1614	This study

* Phl, growth on phenol; Cat, growth on catechol.

Cloning and screening strategies. Total DNA of strain E2 was extracted by the procedure of Marmur (1961). A cosmid library was prepared by ligating partially *Sau*3AI-digested total DNA into broad-host-range cosmid vector pLAFR3 (Staskawicz *et al.*, 1987). After *in vitro* packaging into bacteriophage λ , using a Gigapack II *in vitro* packaging kit (Stratagene), *E. coli* VCS257 was transfected with the phage. Approximately 2500 recombinant *E. coli* clones were tested for the formation of the yellow *meta*-cleavage product of catechol by spraying with 5% (w/v) catechol (these clones grew on agar plates containing the dLB medium supplemented with 10 mM MgSO₄, 0.2% maltose, 250 mg phenol l⁻¹ and tetracycline). The catechol *meta*-cleavage activity of *E. coli* JM109 harbouring subfragments was assayed on dLB plates supplemented with phenol (250 mg l⁻¹) and ampicillin.

Phenol utilization by recombinant *P. aeruginosa* strains. DNA fragments of a recombinant pLAFR3 plasmid were subcloned into pRO1614 or pRO1614M, and *P. aeruginosa* PAO1c was transformed with the resultant plasmids. Colonies on LB plates supplemented with carbenicillin were directly inoculated into 5 ml MP medium supplemented with carbenicillin and phenol (250 mg l⁻¹). Growth was monitored by measuring the OD₆₆₀ of the culture (Beckman model DU 640 spectrophotometer). The phenol concentration was measured by using the Phenol Test Wako kit (Wako Pure Chemical) as described previously (Watanabe & Hino, 1996). Catechol in the culture media was detected by HPLC with an 8010 HPLC system (Tosoh) equipped with a UV-8010 detector (Tosoh) and a reverse-phase column (Inertsil ODS-80A, 5 μ m, 4.6 $\times$ 150 mm; GL Science). Cells were removed from each culture by filtering through a 0.22 μ m pore-size membrane, and 100 μ l of the filtrate was injected for HPLC. The mobile phase was acetic acid/water (1:99, v/v) at a flow rate of 1 ml min⁻¹, and the eluate was monitored at 260 nm. Catechol in the medium was identified by comparing its retention time with that of the pure reagent of catechol (Wako Pure Chemical).

Nucleotide sequencing and computer analysis. To determine the nucleotide sequence of the 9.5 kb DNA fragment, subfragments were cloned into the multicloning site of pUC18. Nucleotide sequencing of the subfragments in both orientations was carried out by using the M13 primers, a DNA sequencing kit (Dye Terminator Cycle Sequencing; Perkin Elmer) and a 370A DNA sequencer (Applied Biosystems) according to the manufacturers' instructions. The DNA sequence data and deduced amino acid sequences were analysed using version 2.0x of the FASTA program package (Pearson & Lipman, 1988; Pearson, 1990) and version 1.7 of CLUSTAL W (Thompson *et al.*, 1994).

Kinetic analysis of phenol-oxygenating activity. The cells used for measuring phenol-oxygenating activity were freshly sampled from a continuous culture immediately before the activity measurement: a TBR-2 fermenter (Sakura Fine Technical) with a capacity of 2 l was used. The culture at 37 °C with the working volume of 1 l was started by inoculating cells grown in 30 ml LB medium supplemented with carbenicillin and then washing with the MP medium. The MP medium, supplemented with phenol (1000 mg l⁻¹), Casamino acids (3000 mg l⁻¹) and carbenicillin, was continuously supplied at a flow rate of 0.2 l d⁻¹, and air was supplied at 1.5 l min⁻¹. The cells were sampled after the culture parameters (pH, dissolved oxygen concentration, phenol concentration and OD₆₆₀) had become stable, i.e. at least 3 d after commencing the culture. The dry cell weight in the culture was determined gravimetrically by filtering through

a 0.22 μ m pore-size membrane according to the method of Machado & Grady (1989). The phenol-oxygenating activity was measured with a Clark-type oxygen electrode (S/6 Oxygraph; Gilson) as described previously (Watanabe *et al.*, 1996). One unit of activity is defined as the amount of material required to catalyse the consumption of 1 μ mol oxygen min⁻¹, while the specific activity is defined as the activity (g dry wt cells)⁻¹. The apparent kinetic constants K_s , K_{SI} and V_{max} in Haldane's equation (Watanabe *et al.*, 1996) were determined by a non-linear regression method as described previously (Watanabe *et al.*, 1996).

RESULTS

Cloning and mapping

One clone which showed a yellowish colour on the plate containing phenol was obtained and designated VCS-257(pLAFRE201); the yellowish colour was deepened by the catechol spray. It is thus considered that this *E. coli* clone transformed phenol to 2-hydroxymuconic semialdehyde via catechol. Mapping of the restriction endonuclease sites (Fig. 1) indicated the size of the insertion fragment in pLAFRE201 to be approximately 23 kb. Subclones were constructed, and the abilities of the subclones to allow PAO1c to grow on phenol as the sole carbon source and to allow JM109 to produce the yellow *meta*-cleavage product from catechol were tested (Fig. 1). As shown in the figure, pROE217 allowed PAO1c to grow on phenol, and pUCE206, pUCE207 and pUCE216 allowed JM109 to produce the yellow product from catechol. These results suggest that the genes for phenol hydroxylase and catechol 2,3-dioxygenase were located in DNA fragments E217 (9.5 kb) and E207 (4.5 kb), respectively.

When PAO1c(pROE217) was grown on a dLB plate containing 250 mg phenol l⁻¹ and carbenicillin, brownish colonies appeared, suggesting that catechol had been produced from phenol. In addition, when PAO1c(pROE217) was grown in the MP medium supplemented with phenol as the sole carbon source, catechol was detected by the HPLC analysis of the fluid of an exponentially growing culture (data not shown), indicating that E217 contains the gene(s) for phenol hydroxylase of strain E2.

Sequence analysis

The nucleotide sequence of the 9.5 kb fragment (E217) was determined. Eight complete ORFs preceded by putative Shine-Dalgarno sequences (Shine & Dalgarno, 1975) were identified in the sequence; these ORFs were designated *poxR* and *poxABCDEFG*. All these ORFs are considered to be transcribed in the same orientation. A possible regulatory region for *poxABCDEFG* is presented in Fig. 2. A putative promoter sequence similar to that of the σ^{54} (RpoN)-dependent -24/-12 type of promoter (Dixon, 1986) was located immediately upstream of *poxA*. The seven ORFs (*poxA-G*) are possibly transcribed from this promoter. A long inverted repeat centred at approximately 130 bp upstream of the puta-

DNA fragment	Activity	
	Phe	C230
E201	ND	+
E202	-	-
E203	ND	-
E204	-	-
E206	-	+
E207	ND	+
E216	-	+
E217	+	-
E218	-	-

Fig. 1. Restriction endonuclease map of the 23 kb DNA fragment from strain E2, and subclones derived therefrom. Details of plasmid construction are provided in Table 1. Phe indicates the ability to allow PAO1c to grow on phenol as the sole carbon source, and C230 indicates the ability to allow *E. coli* strains to produce the yellow pigment from catechol. +, Positive; -, negative; ND, not determined; H, *Hind*III; B, *Bam*HI; N, *Nhe*I; E, *Eco*RI; C, *Cla*I; K, *Kpn*I.

Fig. 2. Nucleotide sequence of a putative regulatory region for the *poxABCDEFG* genes. A bold short arrow indicates the initiation codon for PoxA. A possible ribosome-binding site is indicated with a solid underline. Dotted and dashed underlines indicate a putative promoter region and an IHF-binding region, respectively. Long arrows in both directions indicate a long inverted repeat which is a possible positive-regulator-binding site.

tive promoter was found. Inverted repeats have also been found in regulatory regions at corresponding positions of the *Pseudomonas* TOL upper pathway operon (Abril *et al.*, 1991), the *xylS* gene (Holtel *et al.*, 1992) and the *dmp* operon (Shingler *et al.*, 1993). A sequence homologous to the consensus IHF recognition sequence (Friedman, 1988) was found between the putative promoter sequence and the long inverted repeat. These findings, coupled with the existence of *poxR* (see below), imply that the expression of Pox-ABCDEFG proteins would be controlled by a positive regulatory mechanism that is similar to ones already known. It was found that *poxR* (initiation codon is approximately 2500 bp upstream of that for *poxA*) was also preceded by a typical sequence for the σ^{54} (RpoN)-

dependent -24/-12 type promoter, i.e. TGGC-N8-TGCA, that is located approximately 60 bp upstream of the initiation codon for *poxR*.

Database searches, using the deduced amino acid sequences of PoxR, A, B, C, D, E, F and G, revealed homology with those of previously sequenced multi-component phenol hydroxylases. Table 2 summarizes the identity and similarity of each *pox* product with those of the *dmp* operon in *Pseudomonas* sp. CF600 (Nordlund *et al.*, 1990) and the *mop* operon in *Acinetobacter calcoaceticus* NCIB 8250 (Ehrt *et al.*, 1995). The *dmp* and *mop* phenol hydroxylases are homologous to each other with respect to molecular structure and amino acid sequence, sharing 45-72% identity. In addition to these two strains, the genes for multi-component phenol hydroxylases have also been cloned and sequenced from several phenol-degrading bacteria, including *Pseudomonas putida* P35X (Ng *et al.*, 1994), *P. putida* strain H (Herrmann *et al.*, 1995) and *P. putida* BH (Takeo *et al.*, 1995); these gene products are highly homologous to the *dmp* products, sharing 86-100% identity. PoxR is homologous to DmpR (Shingler *et al.*, 1993), suggesting that PoxR belongs to the NtrC family of transcriptional activators (Kustu *et al.*, 1989). When bound to an effector molecule such as phenol, PoxR may positively regulate the expression of PoxA to PoxG through binding to the upstream inverted repeat sequence. Proteins PoxA to PoxF are considered to be components of the phenol hydroxylase, while PoxG

Table 2. Summary of the *pox* products and comparison with isofunctional proteins

Product	No. of residues	Possible function	Identity/similarity (%)
PoxR	568	Regulator	DmpR (50/77), MopR (46/74)
PoxA	74	Phenol hydroxylase	DmpK (30/49), MopK (33/47)
PoxB	331	Phenol hydroxylase	DmpL (52/75), MopL (47/76)
PoxC	90	Phenol hydroxylase	DmpM (50/81), MopM (49/87)
PoxD	504	Phenol hydroxylase	DmpN (65/86), MopN (66/85)
PoxE	119	Phenol hydroxylase	DmpO (45/69), MopO (38/63)
PoxF	356	Phenol hydroxylase	DmpP (60/77), MopP (53/77)
PoxG	101	Ferredoxin-like protein	DmpQ (40/61)

Fig. 3. Phenol-oxygenating activities of PAO1c(pROE217) (○), PAO1c(pRO1959) (■) and PAO1c(pROBH01) (▲). Each datum point and bar represent the mean and standard error of three determinations, respectively.

(homologous to DmpQ) may be a ferredoxin-like protein having a similar function to XylT, i.e. the reactivation of catechol 2,3-dioxygenase (Polissi & Harayama, 1993). The results of the homology analysis suggest that the phenol hydroxylase of strain E2 belongs to the multicomponent enzyme group.

Kinetics for the phenol-oxygenating activity of PAO1c(pROE217)

The phenol-oxygenating activity of PAO1c(pROE217) was measured, and the data were compared with those of PAO1c(pRO1959) and PAO1c(pROBH01). The pRO1959 plasmid contains *phlA* and *phlR* of *Burkholderia pickettii* PKO1 and allowed PAO1c to grow on phenol as the sole carbon source (Kukor & Olsen, 1990), the *phlA* gene encoding a single-component phenol hydroxylase (Kukor & Olsen, 1990). The pROBH01 plasmid contains *pheR* and *pheA123456* of strain BH and allowed PAO1c to grow on phenol as the sole

carbon source. The multicomponent enzyme of BH is highly homologous to that of *P. putida* CF600 (the *dmp* products), there being only one amino acid substitution in PheA6 (Takeo *et al.*, 1995). In addition, strains BH and CF600 expressed almost identical kinetics for the phenol-oxygenating activity (Watanabe *et al.*, 1996). These three recombinant derivatives of PAO1c showed phenol-dependent oxygen consumption, although PAO1c itself did not. The phenol-oxygenating activities of the three recombinant strains were measured at phenol concentrations from 0.5 μ M to 20 mM and are plotted against phenol concentrations (Fig. 3). By using the data in Fig. 3, the apparent kinetic constants for the phenol-oxygenating activities of PAO1c(pROE217), PAO1c(pRO1959) and PAO1c(pROBH01) were determined (Table 3): these values were then compared with each other and with those of strains E2, PKO1 and BH previously reported (Watanabe *et al.*, 1996). This table shows that the three recombinant derivatives of PAO1c exhibited different K_s and K_{SI} values. It is also shown that the K_s and K_{SI} values of PAO1c(pROE217), PAO1c(pRO1959) and PAO1c(pROBH01) are almost identical to those of strains E2, PKO1 and BH, respectively. The V_{max} values of the recombinant strains were low compared with those of the authentic phenol degraders, while the degree of reduction in the V_{max} value of the BH phenol hydroxylase was much less than those of the other phenol hydroxylases. This may have been due to the different phylogenetic distances between PAO1c and the authentic hosts of the phenol hydroxylases.

DISCUSSION

A comparison of the kinetic data suggests that, regardless of the enzyme structures, phenol hydroxylases, the first catabolic enzymes in bacterial phenol-degradative pathways, determine K_s and K_{SI} values for phenol-oxygenating activity expressed by bacterial cells. Thus it is suggested that the phenol hydroxylase cloned from strain E2 exhibits the novel kinetic properties, i.e. the low K_s and the extremely high K_{SI} values, that had been observed with intact cells of E2 (Watanabe *et al.*, 1996).

Table 3. Apparent kinetic constants in Haldane's equation for specific phenol-oxygenating activities of PAO1c(pROE217), PAO1c(pRO1959) and PAO1c(pROBH01)

Data are estimated values $\pm$ SE.

	K_s (μ M)	K_{SI} (μ M)	V_{max} [units (g dry wt cells) $^{-1}$]
PAO1c(pROE217)	0.47 ± 0.11	7700 ± 1400	6.2 ± 0.2
PAO1c(pRO1959)	1.80 ± 0.50	2200 ± 500	2.2 ± 0.1
PAO1c(pROBH01)	3.65 ± 0.42	2600 ± 400	11.0 ± 0.3
E2*	0.36 ± 0.06	7360 ± 500	155.0 ± 7.0
PKO1*	1.49 ± 0.42	1700 ± 370	57.0 ± 3.0
BH*	3.20 ± 0.44	2820 ± 760	40.0 ± 3.0

* Data are cited from a reference (Watanabe *et al.*, 1996).

Fig. 4. Alignment of the deduced amino acid sequences of hydroxylase components in multicomponent phenol hydroxylases. Identical and similar amino acids among all aligned sequences are indicated with asterisks and colons below the sequences, respectively. Solid lines above the sequences indicate two DE(D)XRH motifs. Sequences: Dmp, *Pseudomonas* sp. CF600 (Nordlund et al., 1990); Phe, *P. putida* BH (Takeo et al., 1995); PhL, *P. putida* H (Herrmann et al., 1995); Phh, *P. putida* P35X (Ng et al., 1994); Mop, *A. calcoaceticus* NCIB 8250 (Ehrt et al., 1995); Pox, *R. eutropha* E2 (this study).

As has been reported for other phenol-degrading bacteria (Powlowski & Shingler, 1990), the purification of multicomponent phenol hydroxylases is recognized to be very difficult. In the case of strain E2, the phenol-oxygenating activity became undetectable immediately after disrupting the cells, even in the presence of cofactors, i.e. Fe^{2+} and NAD(P)H (unpublished data). This may be the major reason why the kinetic properties of bacterial phenol hydroxylases have not been well understood. This study demonstrated that an *in vivo* system with PAO1c is very useful for determining the K_s and K_{SI} values of labile phenol hydroxylases.

Our previous study showed that the oil-refinery activated sludge from which strain E2 had been isolated exhibited E2-type kinetics for phenol-oxygenating activity when it was fed on phenol as the sole carbon source (Watanabe & Hino, 1996). This fact, coupled with the results of the present study, suggests that the phenol hydroxylase cloned from strain E2 (Pox) was functionally dominant in the activated-sludge microbial community. Although it has not yet been elucidated why the Pox-type phenol hydroxylase played the dominant role in the activated sludge, it would be interesting to investigate the distribution of the Pox-type phenol hydroxylase in other microbial communities. The nucleotide sequence determined in this study will be useful to design specific probes for detecting the *pox* genotype in the environment.

The fact that phenol hydroxylase determines the kinetic constants for cellular phenol-oxygenating activity implies that the engineering of phenol hydroxylase would allow bacterial activity for phenol degradation to be improved. We have reported that bacterial phenol-oxygenating activity could be classified into several groups according to the K_s and K_{SI} values (Watanabe *et al.*, 1996), implying that several kinetically different groups of phenol hydroxylases exist in nature. Cloning and sequence analysis of these phenol hydroxylases will provide useful information for engineering phenol hydroxylases and thus for constructing the desired phenol-degrading bacteria.

The results of the nucleotide sequence analysis suggest that the *pox* product is a multicomponent enzyme that is structurally analogous to the well-characterized *dmp* products (Shingler, 1996), although the deduced amino acid sequences of the *pox* products were unexpectedly different from those of the *dmp* products. The known multicomponent phenol hydroxylases are classified into two types according to the genetic organization of the operons, i.e. (i) the *dmp* type followed by genes for a ferredoxin-like protein and a catechol 2,3-dioxygenase (Shingler, 1996), and (ii) the *mop* type followed by a gene for catechol 1,2-dioxygenase without a gene for a ferredoxin-like protein (Ehrt *et al.*, 1995). Based on this criterion, *pox* belongs to the *dmp* type, although it is the only known example of the gene which is not highly homologous to equivalent genes of the other members of the *dmp* group.

Genetic and biochemical analyses of the phenol hy-

droxylase of CF600 indicate that six polypeptides are required for growth on a phenolic substrate, while five polypeptides (not DmpK) were indispensable for phenol hydroxylase activity *in vitro* (Powlowski & Shingler, 1990). Among these six polypeptides, DmpLNO have been suggested to be components of hydroxylase (Shingler, 1996). Hence, the deduced amino acid sequences of the P1, P3 and P4 components (corresponding to DmpLNO, respectively) of the known phenol hydroxylases were compared to identify the conserved regions (Fig. 4). Among the three components, P3, the largest, was found to be most conserved; 55% of the amino acids were identical among all the phenol hydroxylases. In particular, the middle part of this component (from amino acids 126 to 249) that contains two DE(D)XRH motifs exhibited a high degree of conservation (74%). Two DE(D)XRH motifs with an approximately 100 amino acid interval have been suggested to be the di-iron-oxo cluster binding site (Fox *et al.*, 1993); this cluster is considered to be the catalytic centre of the oxygenases. Therefore, it would be reasonable to assume that amino acids around this conserved region may interact with substrates and may thus be involved in determining the kinetic properties of phenol hydroxylases. A comparison of the deduced amino acid sequence of the phenol hydroxylase of strain E2 with those of the other phenol hydroxylases shown in this paper would facilitate the identification of amino acids that determine the kinetic properties of phenol hydroxylases.

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