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Cyclohexadienyl Dehydratase from *Pseudomonas aeruginosa*

MOLECULAR CLONING OF THE GENE AND CHARACTERIZATION OF THE GENE PRODUCT*

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The gene encoding cyclohexadienyl dehydratase (denoted *pheC*) was cloned from *Pseudomonas aeruginosa* by functional complementation of a *pheA* auxotroph of *Escherichia coli*. The gene was highly expressed in *E. coli* due to the use of the high-copy number vector pUC18. The *P. aeruginosa* cyclohexadienyl dehydratase expressed in *E. coli* was purified to electrophoretic homogeneity. The latter enzyme exhibited identical physical and biochemical properties as those obtained for cyclohexadienyl dehydratase purified from *P. aeruginosa*. The activity ratios of prephenate dehydratase to arogenate dehydratase remained constant (about 3.3-fold) throughout purification, thus demonstrating a single protein having broad substrate specificity. The cyclohexadienyl dehydratase exhibited K_m values of 0.42 mM for prephenate and 0.22 mM for L-arogenate, respectively. The *pheC* gene was 807 base pairs in length, encoding a protein with a calculated molecular mass of 30,480 daltons. This compares with a molecular mass value of 29.5 kDa determined for the purified enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Since the native molecular mass determined by gel filtration was 72 kDa, the enzyme probably is a homodimer. Comparison of the deduced amino acid sequence of *pheC* from *P. aeruginosa* with those of the prephenate dehydratases of *Corynebacterium glutamicum*, *Bacillus subtilis*, *E. coli*, and *Pseudomonas stutzeri* by standard pairwise alignments did not establish obvious homology. However, a more detailed analysis revealed a conserved motif (containing a threonine residue known to be essential for catalysis) that was shared by all of the dehydratase proteins.

Prephenate, a cyclohexadienyl-ring molecule formed from chorismate by chorismate mutase, is a precursor which is uniquely used for the biosynthesis of L-phenylalanine and L-tyrosine in microorganisms (1). The conversion of prephenate to phenylpyruvate (via prephenate dehydratase) or to 4-hydroxyphenylpyruvate (via prephenate dehydrogenase) was first established in enteric bacteria. Although the latter were assumed for many years to be universal enzyme steps functioning in nature, an alternative route to L-tyrosine biosynthesis was discovered in cyanobacteria in 1974, whereby prephenate was transaminated to L-arogenate via prephenate aminotransferase (2). L-Arogenate, a substrate for arogenate dehydrogenase in cyanobacteria, was initially identified as a precursor of L-tyrosine, but was later recognized as a precursor of L-phenylalanine in many microorganisms and in higher plants (3). Phenylalanine biosynthesis in microorganisms exhibits multiple aspects of diversity. Exclusive use of the phenylpyruvate route (*e.g. Bacillus*) or exclusive use of the L-arogenate route (*e.g. Pseudomonas diminuta*) may be in place. On the other hand, both pathway routes may coexist due to (i) the substrate ambiguity of cyclohexadienyl dehydratase or (ii) the presence of one of the possible pairs made up of prephenate dehydratase, arogenate dehydratase, or cyclohexadienyl dehydratase. Another aspect of diversity is the presence or absence of a physical organization of prephenate dehydratase as one catalytic domain of a bifunctional protein (denoted the P-protein), which also possesses a catalytic domain for chorismate mutase. Two of the three major rRNA Gram-negative superfamilies (Superfamily A and Superfamily B) possess the bifunctional P-protein. Most enteric bacteria (but not *Escherichia coli*) and most members of the entire Superfamily B assemblage possess cyclohexadienyl dehydratase in addition to the P-protein (3, 4).

Pseudomonas aeruginosa, a Superfamily-B organism, was the first example of a microorganism possessing dual pathways to L-phenylalanine (5). This is illustrated in Fig. 1. In addition to the bifunctional P-protein, *P. aeruginosa* possesses chorismate mutase-F and cyclohexadienyl dehydratase. Because the latter two enzymes are unrestrained by allosteric control, they have been referred to as components of an overflow pathway (6). The cyclohexadienyl dehydratase of *P. aeruginosa* has a broad substrate specificity that accommodates both prephenate and L-arogenate as substrates for L-phenylalanine biosynthesis (5). The overflow pathway to L-phenylalanine biosynthesis is widely distributed among Superfamily-B microorganisms (7).

The physiological role of the overflow pathway in nature is essentially unknown. The evolutionary relationship of cyclohexadienyl dehydratase to monofunctional prephenate dehydratase, to monofunctional arogenate dehydratase, or to the dehydratase domain of the bifunctional P-protein remains to be elucidated. In this paper, we report the molecular cloning, expression and nucleotide sequence of the *P. aeruginosa* cyclohexadienyl dehydratase gene, as well as the purification and characterization of its gene product.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M74132.

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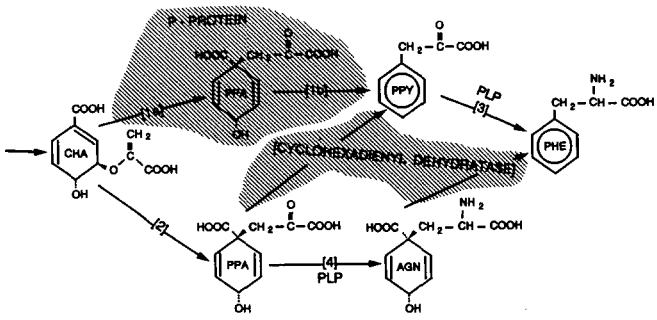

FIG. 1. **Dual biosynthetic routes to L-phenylalanine in *P. aeruginosa*.** The bifunctional P-protein (denoted by *shading*) consists of chorismate mutase (*[1a]*) and prephenate dehydratase (*[1b]*) domains. The broad specificity cyclohexadienyl dehydratase catalyzes the two reactions indicated by shading: prephenate dehydratase or arogenate dehydratase. Enzyme *[2]* is the monofunctional chorismate mutase-F. Reactions *[3]* and *[4]* refer to a multiplicity of aminotransferase enzymes with overlapping substrate specificities (26) which transaminate phenylpyruvate or L-arogenate, respectively. Abbreviations: *CHA*, chorismate; *PPA*, prephenate; *PPY*, phenylpyruvate; *PHE*, L-phenylalanine; *AGN*, L-arogenate; *PLP*, pyridoxal 5′-phosphate.

MATERIALS AND METHODS[1]

RESULTS

Cloning of the Gene Encoding Cyclohexadienyl Dehydratase—Approximately 4000 recombinants were obtained after transformation of *E. coli* JM83 (see "Materials and Methods"). Purified plasmids from these recombinants were used to transform *E. coli* JP2255. The transformants obtained were allowed to grow in LB medium overnight at 37 °C, washed twice with M9 medium, and then plated on M9 plates which were supplemented with L-tyrosine, thiamine, and ampicillin. After incubation for 4 days at 37 °C, two colonies were observed. Plasmids were purified from cultures derived from each of these transformants. Each of the two plasmids isolated (designated as pJZ1 and pJZ2) was found to be capable of transforming *E. coli* JP2255 to phenylalanine independence. The transformants of *E. coli* JP2255 carrying the clones of pJZ1 and pJZ2 were slow growers on M9 medium. However, the transformation of pJZ1 or pJZ2 into *E. coli* KA197 produced faster growing transformants, apparently due to the presence of chorismate mutase encoded by *tyrA*.

The presence of prephenate dehydratase and arogenate dehydratase activities was examined in crude extracts of *E. coli* JP2255 carrying plasmids pJZ1 or pJZ2. A high level of both activities was evident in crude extracts of *E. coli* JP2255 carrying the pJZ1 and pJZ2 plasmids, whereas no enzyme activity was detected in a crude extract of *E. coli* JP2255 carrying pUC18 (Table II).

Digestion of pJZ1 and pJZ2 with *Hin*dIII, *Pst*I, and *Kpn*I showed that they carried two identical DNA fragments estimated to be 5.7 kilobase pairs in length. The plasmid designated as pJZ1 was used for further study.

Southern blot hybridization showed that when the *Sph*I-*Eco*RI fragment of pJZ1 was biotinylated, it hybridized with a 3.5-kilobase pair fragment of *P. aeruginosa* chromosomal DNA obtained by complete digestion with *Sph*I and *Eco*RI, but it did not hybridize with *E. coli* chromosomal DNA which had been digested with *Sph*I and *Sma*I (data not shown).

TABLE II

Expression of the P. aeruginosa pheC gene in E. coli

Specific activity is defined as nanomoles of phenylpyruvate or phenylalanine formed per min/mg of protein. All the clones or subclones listed were first transformed into *E. coli* JP2255, and the crude extracts of the transformed JP2255 were used for enzyme assay.

Plasmid in strain JP2255	Specific activity	
	Prephenate dehydratase	Arogenate dehydratase
pJZ1	621	191
pJZ2	700	211
pJZ1a	0	0
pJZ1b	0	0
pJZ1c	0	0
pJZ1d-S	618	186
pJZ1d-O	5.6	1.7
pJZ1e	0	0
pJZ1f	0	0
pJZ1g	1314	387
pUC18/pUC19	0	0

Localization of the P. aeruginosa pheC Gene and Expression of the Gene in E. coli—Cleavage of pJZ1 with *Eco*RI, *Kpn*I, and *Sma*I yielded subclones denoted pJZ1a, pJZ1b, and pJZ1c, respectively (Fig. 2*A*). These plasmids were unable to complement *E. coli* JP2255 and *E. coli* KA197, and cyclohexadienyl dehydratase activity was not detected in crude extracts of transformants carrying the subclones (Table II), indicating the probable location of the gene within the *Kpn*I-*Kpn*I fragment. The *Kpn*I-*Kpn*I fragment (Fig. 2*A*) was cloned into pUC18 in both possible orientations. When the *Kpn*I-*Kpn*I fragment was cloned in the same orientation as the original clone (designated as pJZ1d-S), the same high level of activities conferred by pJZ1 was found (Table II). However, when this fragment was cloned in the opposite orientation (designated as pJZ1d-O), the activities observed were two orders of magnitude lower (Table II). These results showed that the overexpression of the enzyme activity of these clones was largely dependent on the *lacZ* promoter of the plasmid. The data also indicate that the promoter of the cloned gene was able to function in *E. coli*, although not efficiently. Further localization of the gene was carried out by cloning the two *Sma*I fragments (released upon digestion of pJZ1d-S with *Sma*I) into pUC18 at the *Sma*I site. The two resulting subclones, designated as pJZ1e and pJZ1f (Fig. 2*A*), were unable to complement the *pheA* defects of *E. coli* strains JP2255 and KA197, suggesting that the *pheC* gene was localized within the two *Sma*I fragments. In order to obtain a *Sph*I-*Sma*I-*Sma*I fragment, the original clone was completely digested with *Sph*I and then partially digested with *Sma*I. The *Sph*I-*Sma*I-*Sma*I fragment was isolated and cloned into pUC19, yielding a subclone denoted pJZ1g. Subclone pJZ1g complemented *E. coli* strains JP2255 and KA197 and exhibited a 2-fold increase in enzyme activities when compared with pJZ1d-S (Table II). The increased enzyme activity was probably due to the decrease of the distance between *lacZ* promoter and the transcriptional start site of the gene.

DNA Sequence of the P. aeruginosa pheC Gene and Its Flanking Regions—The complete nucleotide sequence of the 1259-base pair *Sma*I fragment is presented in Fig. 3. The structural gene encoding cyclohexadienyl dehydratase was located within a single open reading frame (807 base pairs in length). The deduced amino acid sequence presented in Fig. 3 indicates that the *P. aeruginosa* cyclohexadienyl dehydratase contained 268 residues with a molecular weight of 30,480. This compares with a value of 29,500 (Fig. 4) determined for

[1] Portions of this paper (including "Materials and Methods," Tables I and III, and Figs. 2, 4, and 5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

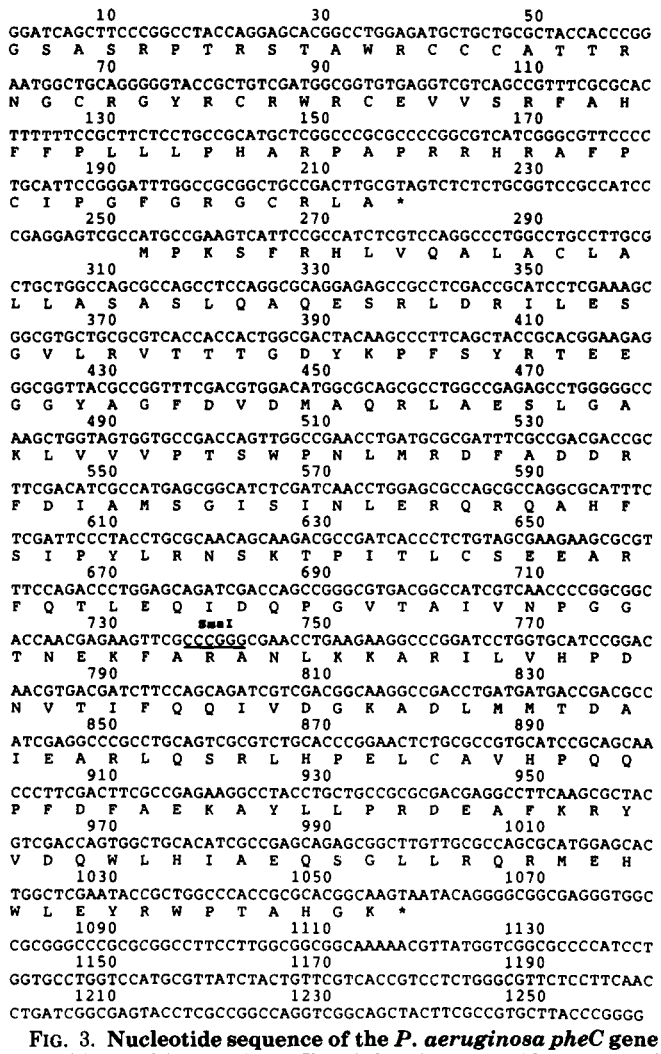

FIG. 3. **Nucleotide sequence of the *P. aeruginosa pheC* gene and of its lanking regions.** The deduced amino acid sequence of the gene along with its upstream flanking region is shown beneath the corresponding codons. The *Sma*I site of cistronic inactivation midway through the *pheC* gene is also shown.

the purified enzyme by SDS-PAGE[2] (19). The sequence GAG-GAG, located 6 base pairs upstream of the start codon is presumbly the ribosome binding site (22). The open reading frame was terminated by a TAA codon.

The G + C content of the cyclohexadienyl dehydratase gene was 65.6% which falls within the 60.6–66.3% range for *P. aeruginosa* genomic genes (23). The codon usage of the gene was typical of *P. aeruginosa* (23), exhibiting a striking preference for G or C in the third base position in 91.1% of the codons. As is the case for most *P. aeruginosa* genes, C (52.4%) was utilized more frequently than G (38.7%) in the third position.

A portion of an unidentified open reading frame encoding a truncated peptide of 74 residues (Fig. 3) was found upstream of *pheC*. A search of GenBank sequences did not reveal obvious homology with any known gene sequences.

Characterization of the P. aeruginosa pheC Gene Product Purified from E. coli and Comparison with the Cyclohexadienyl Dehydratase Isolated Directly from P. aeruginosa—Purification of the cyclohexadienyl dehydratase isolated from *E. coli* JP2255(pJZ1g) is summarized in Table III. The ratio of 3:1 obtained for the activity of prephenate dehydratase compared with that of arogenate dehydratase remained constant throughout the process of purification. Only one major band was resolved by SDS-PAGE after the Sephadex G-200 column (Fig. 4). The subunit molecular weight of the cloned cyclohexadienyl dehydratase was 29,500 as determined by SDS-PAGE, and the molecular weight of the native enzyme was 72,000 as determined by gel filtration on Sephadex G-200.

Purification of the cyclohexadienyl dehydratase from *P. aeruginosa* was essentially carried out under identical conditions as those used for its isolation from *E. coli*. Both of the cylohexadienyl dehydratase preparations failed to bind to DEAE-cellulose at pH values lower than 7.4, and they were found to migrate into equivalent fractions throughout the purification process. A specific activity ratio of prephenate dehydratase to that of arogenate dehydratase of 3:1 was maintained throughout the purification process, as found for the *P. aeruginosa* enzyme isolated from *E. coli* (data not shown). The native molecular weight of this enzyme was found to be 72,000, a value identical to that obtained for the cloned gene product. After the final step of purification, the enzyme preparation obtained was not electrophoretically homogeneous (data not shown).

K_m values of 0.42 mM for prephenate and of 0.22 mM for L-arogenate were obtained for the enzyme produced from the cloned gene (Fig. 5). The corresponding values obtained for cyclohexadienyl dehydratase isolated directly from *P. aeruginosa* were 0.40 and 0.19 mM, respectively. V_{max} values of 307.7 μmol/min/mg for prephenate and 102.8 μmol/min/mg for L-arogenate were obtained for the enzyme produced from the cloned gene. Since the preparation of the cyclohexadienyl dehydratase isolated from *P. aeruginosa* was not homogeneous, the V_{max} values were not determined. Prephenate dehydratase activity of both preparations was competitively inhibited by L-arogenate with a K_i value of 0.2 mM, whereas arogenate dehydratase activity was competitively inhibited by prephenate with a K_i value of 0.40 mM. The *P. aeruginosa* cyclohexadienyl dehydratase was not subject to allosteric control by phenylalanine, tyrosine, and tryptophan when present singly or in combination.

DISCUSSION

The Identity of the Cloned Gene and Its Product—Cyclohexadienyl dehydratase was first described in *P. aeruginosa* (5), and the analysis has now been extended to the molecular-genetic level. The successful cloning of *P. aeruginosa pheC* in *E. coli*, in concert with the purification of a gene product having all of the properties of partially purified cyclohexadienyl dehydratase isolated directly from *P. aeruginosa*, proves that a single protein possesses both prephenate dehydratase and arogenate dehydratase activities as a consequence of ambiguity for substrate recognition. This is consistent with kinetic results showing that prephenate competitively inhibited arogenate dehydratase activity, whereas L-arogenate competitively inhibited prephenate dehydratase activity. Recently, similar biochemical results have been obtained for the cyclohexadienyl dehydratase purified from *Erwinia herbicola* (24).

The in Vivo Function of Cyclohexadienyl Dehydratase—The reluctant auxotrophy of *P. aeruginosa* for phenylalanine following otherwise successful mutagenesis protocols was explained as the consequence of independent dual pathways to phenylalanine (25). A mutant lacking the bifunctional P-protein has been identified (26). The mutant exhibited a leaky requirement for phenylalanine, thus indicating that exclusive dependence upon cyclohexadienyl dehydratase for biosynthesis of phenylalanine is rate-limiting to growth. In contrast,

[2] The abbreviation used is: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

a regulatory mutant of *P. aeruginosa* possessing a tyrosine-insensitive DAHP synthase was found to excrete phenylalanine, presumably through the unregulated overflow pathway (6). Thus, the capacity for generation of phenylalanine via the overflow pathway is dramatically influenced by precursor levels *in vivo*.

L-Arogenate is generated by transamination of prephenate. Five aromatic aminotransferases capable of transamination of prephenate have been isolated from *P. aeruginosa* (27) and shown to have a relatively poor affinity for prephenate compared with the prephenate dehydratase component of the bifunctional P-protein. The most likely source of prephenate molecules for transamination is via the catalytic action of a monofunctional chorismate mutase denoted chorismate mutase-F. However, it has a poor affinity for chorismate compared with the chorismate mutase component of the bifunctional P-protein.[3] Since the cyclohexadienyl dehydratase has a relatively poor affinity for both of its substrates compared with the competing P-protein prephenate dehydratase, it would seem that most phenylalanine is ordinarily synthesized via the bifunctional P-protein and that most phenylalanine molecules are normally derived from phenylpyruvate rather than from L-arogenate. Thus, under ordinary growth conditions, the cyclohexadienyl dehydratase along with the monofunctional chorismate mutase probably does not contribute significantly to phenylalanine biosynthesis.

Perhaps the overflow pathway (the monofunctional chorismate mutase and the cyclohexadienyl dehydratase) exists as a backup system for phenylalanine biosynthesis, possibly in relationship to differential carbon input into aromatic biosynthesis during growth on different carbon sources. It is interesting that *P. stutzeri*, a very close relative of *P. aeruginosa*, lacks the overflow pathway altogether (28). The loss of the prephenate dehydratase activity of the bifunctional P-protein of *P. stutzeri* has yielded a tightly blocked phenylalanine auxotroph (29), in contrast to the bradytrophy of the corresponding mutant of *P. aeruginosa* (26). To have a better understanding of the role of the cyclohexadienyl dehydratase *in vivo*, mutants lacking this enzyme activity would be desirable, and such mutants now can be obtained by using the cloned cyclohexadienyl dehydratase gene to target the corresponding region of chromosome in *P. aeruginosa* through gene-scrambling mutagenesis (30).

Basis for the Ability of P. aeruginosa pheC to Complement pheA Defects in E. coli—E. coli JP2255 was initially employed to select for the clones carrying the bifunctional P-protein gene. Successful complementation of the *pheA* defect by *P. aeruginosa pheC* was not anticipated, because strain JP2255 is deficient in the two chorismate mutase species encoded by *tyrA* and *pheA*. Even though we did not detect chorismate mutase activity under standard assay conditions, a low and perhaps unstable level of enzyme must exist in strain JP2255 as was reported by Baldwin and Davidson (12) in order to explain the slow-growing *pheC* transformants recovered. When the original clones and the resulting subclones were transformed into *E. coli* KA197, fast-growing strains were obtained, an indication that the limitation of phenylalanine *in vivo* was relieved due to the elevation of chorismate mutase activity. Since KA197, a *pheA* mutant lacking a bifunctional P-protein, still possesses an intact bifunctional T-protein (chorismate mutase/cyclohexadienyl dehydrogenase), the prephenate molecules generated *in vivo* were probably derived from the catalytic activity of the chorismate mutase component of the T-protein. Our results indicate that the chorismate mutase component of the T-protein has the potential to participate in phenylalanine biosynthesis, even though the activities of the two domains are tightly coupled (31–33).

Evolutionary Implications—The deduced amino acid sequence of the *P. aeruginosa pheC* gene product was pairwise aligned with those of the *Corynebacterium glutamicum* and *Bacillus subtilis* prephenate dehydratases (34, 35), and with *E. coli* and *P. stutzeri* P-proteins (36, 37). The *P. aeruginosa* enzyme was found to be only marginally similar to these four proteins, ranging in identity from 16.5 to 18.9%. In contrast, the monofunctional prephenate dehydratases of *C. glutamicum* and *B. subtilis* have shown significant identity to the two bifunctional P-proteins of *E. coli* and *P. stutzeri* (34, 35, 37), indicating that the prephenate dehydratases probably share a common evolutionary origin. The marginal similarity of the *P. aeruginosa* cyclohexadienyl dehydratase to the prephenate dehydratases as well as the bifunctional P-proteins might suggest that the cyclohexadienyl dehydratase and the prephenate dehydratases evolved independently. However, a more detailed analysis, focusing on short highly conserved sequence segments (previously established in Ref. 37), rather than the total peptide, revealed a conserved motif which includes the essential threonine residue demonstrated by Hudson and Davidson (36) and a number of flanking residues. This motif, shown in Fig. 6, suggests residues (within *boxes*) that may prove to be common to all of the dehydratases. It is interesting that all of the prephenate dehydratases share the TRF sequence, whereas the cyclohexadienyl dehydratase sequence is TIF. It remains to be seen whether other cyclohexadienyl dehydratases will also possess TIF sequences. Note that in this alignment there was considerable conservation of amino acid sequence between the peptides of the *P. aeruginosa* CDT and the *P. stutzeri* P-protein. These organisms are more closely related to one another than to any of the other organisms examined.

Two cyclohexadienyl dehydratases, one from *E. herbicola* (24) and the other from *P. aeruginosa*, have now been characterized in detail. The two enzymes are similar with respect to a lack of allosteric control, the broad substrate utilization, and the relative affinity for L-arogenate and prephenate. However, the *E. herbicola* enzyme is a homotetramer, whereas the *P. aeruginosa* enzyme appears to be a homodimer. Furthermore, the subunit molecular weights of the two enzymes also differed considerably, one being 18,000 (*E. herbicola*) and one being 30,480 (*P. aeruginosa*). Since the two organisms studied are relatively close to each other phylogenetically, such results were unexpected. One explanation might be that a small ancestral gene (retained in *E. herbicola*) underwent a tandem duplication and fusion following divergence of the lineage leading to *P. aeruginosa*. This would explain the larger subunit size and the dimer instead of tetramer in *P. aeruginosa*. However, a comparison of amino-terminal sequence with carboxyl-terminal sequence did not reveal any striking

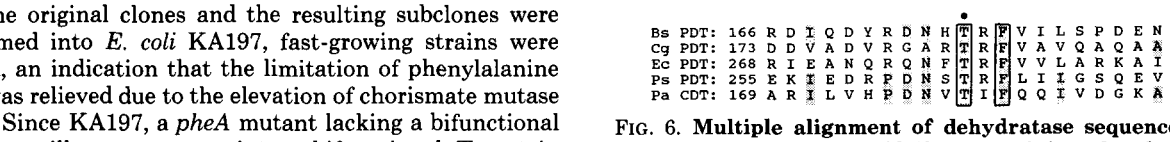

FIG. 6. **Multiple alignment of dehydratase sequences oriented to the sequence motif (37) containing the threonine residue (●) shown to be essential for catalysis (12, 36).** Amino acid residues, beginning as numbered on the left, are compared for *B. subtilis* PDT (*Bs PDT*), *Corynebacterium glutamicum* PDT (*Cg PDT*), *E. coli* P-protein (*Ec PDT*), *P. stutzeri* P-protein (*Ps PDT*), and *P. aeruginosa* CDT (*Pa CDT*). Identities between the residues of one or more of the *P. aeruginosa* CDT with those of the other four dehydratases are shown by *shading*. Residues invariant in all sequences are represented by *boxing*.

[3] G. Zhao, T. Xia, R. S. Fischer, and R. A. Jensen, unpublished results.

identities, and the motif illustrated in Fig. 6 was not present in two places. If the *pheC* gene in *P. aeruginosa* enlarged by gene duplication and gene fusion, the subsequent divergence has been extensive. It should be instructive to obtain the *pheC* gene sequence from *E. herbicola*.

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SUPPLEMENTARY MATERIAL TO

Cyclohexadienyl Dehydratase from *Pseudomonas aeruginosa*: Molecular cloning of the Gene and Characterization of the Gene Product

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MATERIALS AND METHODS

Strains, Plasmids, and Media—All bacterial strains and plasmids used in this study are listed in Table I.

TABLE I

Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source
E. coli K-12		
JM83	*ara* Δ(*lac-proAB*) *rpsL* φ80 *lacZ*ΔM15	BRL
JP2255	*aroF*363 *pheA*361 *pheO*352 *tyrA*382 *thi strR*712 *lacY*1 *xyl*5	(12)
KA197	*thi*1 *pheA*97 *relA*1 *spoT*1	CGSC 5243
P. aeruginosa		
PAO1	Prototroph	(38)
Plasmids		
pUC18	Ap^r *lacZ*	(10)
pUC19	Ap^r *lacZ*	
pJZ1, pJZ2	Original clone carrying *pheC* gene isolated from PAO1 library	This study
pJZ1a	2.0-kb derivative of pJZ1 generated by removal of a 3.8-kb EcoRI fragment	This study
pJZ1b	4.0-kb derivative of pJZ1 generated by removal of a 1.8-kb KpnI-KpnI fragment	This study
pJZ1c	4.5-kb derivative of pJZ1 generated by removal of a 1259-bp SmaI-SmaI fragment	This study
pJZ1d-S	1.8-kb KpnI fragment of pJZ1 subcloned into pUC18 with the same orientation as pJZ1	This study
pJZ1d-0	1.8-kb KpnI fragment of pJZ1subcloned into pUC18 with the opposite orientation of pJZ1	This study
pJZ1e	741-bp SmaI fragment of pJZ1d-S subcloned into pUC18	This study
pJZ1f	515-bp SmaI fragment of pJZ1d-S subcloned into pUC18	This study
pJZ1g	1110-bp SphI-SmaI fragment of pJZ1d-S subcloned into pUC19	This study

LB (8) was used as a rich growth medium, and M9 (8) was used as a minimal medium for *E. coli* and *P. aeruginosa*. Ampicillin (50 μg/ml), tyrosine (50 μg/ml), and thiamine (17 μg/ml) were supplemented when appropriate. Agar was added at 15 g/liter for solid medium.

Isolation of P. aeruginosa (PAO1) Chromosomal DNA and Construction of a Gene Library—Chromosomal DNA was isolated (9) and was partially digested with Sau3A. Fragments of 5 to 10 kilobases (kb) were isolated from agarose gel after electrophoresis. The library was constructed by ligation of these fragments into the dephosphorylated BamHI site of pUC18 (10). The ligation mixture was transformed into *E. coli* JM83. The transformants obtained on LB plates (supplemented with 50 μg/ ml ampicillin) were collected by means of a glass spreader and stored in 50% glycerol at -75°C. Recombinant plasmids were purified from this library as described by Davis et al. (11). The recombinant plasmids were used to transform *E. coli* JP2255 (12) to independence of its L-phenylalanine requirement in order to select for clones having the *pheC* insert.

DNA Manipulations—All restriction enzymes, T4 DNA ligase, and calf intestine phosphatase were obtained from Gibco BRL and Promega, and were used according to the manufacturer instructions. Analyses of restriction sites and subcloning were conducted by standard methods (8). Southern blot hybridization, using biotinylated probes, was conducted under conditions of high stringency following the instructions of Promega.

DNA Sequencing and Data Analysis—The subclones of pJZ1e, pJZ1f, and pJZ1g were first purified on a CsCl gradient (13), and then sequenced in both directions (14) at the DNA Core Facility of the University of Florida. Nucleotide sequence, along with the corresponding deduced amino acid sequence, was analyzed by using the University of Wisconsin Genetics Computer Group (GCG) package (15).

Crude Extract Preparation and Enzyme Assay—Cultures of *E. coli* JP2255 transformed by various plasmids were grown at 37°C in 450 ml of LB broth supplemented with ampicillin, and harvested by centrifugation during the late exponential phase of growth. The cells were suspended in 3 ml of 50 mM potassium phosphate buffer, pH 7.5, and disrupted by sonication. The resulting suspension was centrifuged at 150,000 *g* for 60 min at 4°C. The supernatant fraction, after passage through a PD-10 Sephadex column to remove small molecules, is referred to as crude extract.

Prephenate dehydratase was assayed as described by Cotton and Gibson (16). Reaction mixtures (200 μl) contained 1.0 mM prephenate, enzyme and 50 mM potassium phosphate (pH 7.5). An extinction coefficient of 17,500 was used for calculation of phenylpyruvate formation (16). Arogenate dehydratase was assayed by measuring the formation of phenylalanine using HPLC (high performance liquid chromatography) (17). A reaction mixture of 200 μl contained 1.0 mM arogenate, enzyme and 50 mM potassium phosphate (pH 7.5). Reactions were incubated at 37°C for 20 min. Protein was measured as described by Bradford (18). One unit of enzyme activity was defined as the formation of 1 nanomole of phenylpyruvate or phenylalanine per min at 37°C.

Purification of the Cyclohexadienyl Dehydratase Encoded by the Cloned pheC Gene—*E. coli* JP2255 carrying the subclone pJZ1g was grown in 2 liters of LB broth supplemented with 50 μg/ml of ampicillin at 37°C, and harvested by centrifugation during late exponential growth phase. The cells were washed once with 20 mM potassium phosphate, 1 mM DTT, pH 8.5 (Buffer A), resuspended in the same buffer, and disrupted by sonication. The resulting suspension was centrifuged at 150,000 *g* for 60 min. The supernatant was applied to a DEAE-cellulose column (2.5 x 30 cm) that was previously equilibrated with Buffer A. The column was first washed with 100 ml of Buffer A, and then eluted with a 1000-ml linear KCl gradient from 0-to-300 mM in Buffer A. Fractions of 2.8 ml were collected, and those showing high cyclohexadienyl dehydratase activity were pooled and concentrated by use of an Amicon PM-10 membrane. The concentrated preparation was washed twice with Buffer B (20 mM potassium phosphate, 1 mM DTT, pH 7.2). It was then applied to a hydroxylapatite column (1.5 x 25 cm) which was equilibrated with Buffer B. The column was eluted with a 600-ml linear gradient of phosphate from 20 mM to 300 mM. Fractions of 2.8 ml were collected, and those showing high cyclohexadienyl dehydratase activity were pooled. The pooled fractions were concentrated as described before, and then applied to a Sephadex G-200 column (2.5 x 98 cm) previously equilibrated with Buffer B. The column was eluted with Buffer B, and the fractions exhibiting cyclohexadienyl dehydratase activity were collected for further study.

For purification of cyclohexadienyl dehydratase from *P. aeruginosa* PAO1, a 3,000-ml culture grown in minimal medium was harvested by centrifugation during the late exponential phase of growth. The crude extract was prepared in Buffer B as described before. The supernatant fraction collected after ultracentrifugation was adjusted to 40% of saturation with solid ammonium sulfate. After stirring for 10 min, the insoluble protein was removed by centrifugation. The supernatant fraction was collected and brought to 60% of saturation with ammonium sulfate. The precipitate was dissolved in 20 ml of Buffer A and dialyzed with several changes against 20 volumes of the same buffer. The dialyzed enzyme preparation was applied to a DEAE-cellulose column and purified as described in the previous paragraph.

Estimates of native molecular mass were made by use of a calibrated Sephadex G-200 column, and subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (19).

Biochemicals and Chemicals—Prephenate was prepared from a tyrosine auxotroph of *Salmonella typhimurium* (20), and L-arogenate was prepared from a triple auxotroph of *Neurospora crassa* (21). Sephadex G-200 was obtained from Sigma Chemical Company, DEAE-cellulose was obtained from Whatman, and hydroxylapatite was obtained from Bio-Rad. Molecular weight standards for SDS-polyacrylamide gel electrophoresis (α-lactalbumin, 14,400; soybean trypsin inhibitor, 20,100; carbonic anhydrase, 30,000; ovalbumin, 43,000; bovine serum albumin, 67,000; and phosphorylase, 94,000), and for gel filtration (carbonic anhydrase, 30,000; bovine serum albumin, 67,000; alcohol dehydrogenase, 150,000; and β-amylase, 200,000) were obtained Pharmacia Fine Chemicals and Sigma Chemical Company, respectively. Agar and LB medium were obtained from Difco. All other biochemicals were purchased from Sigma Chemical Company.

TABLE III

Purification of the cyclohexadienyl dehydratase expressed in *E. coli* JP2255(pJZ1g)

	Total protein (mg)	Specific activity (nmol/min/mg) PDT	ADT	Ratio of PDT/ADT	Purification factor
Crude extract	1,118	1,319	381	3.46	1
DEAE-cellulose	83.5	10,616	3,518	3.02	8.1
Hydroxyl-apatite	4.83	87,750	26,340	3.32	67
Gel filtration	3.25	158,242	48,352	3.27	120

Abbreviations: PDT, prephenate dehydratase; ADT, arogenate dehydratase.

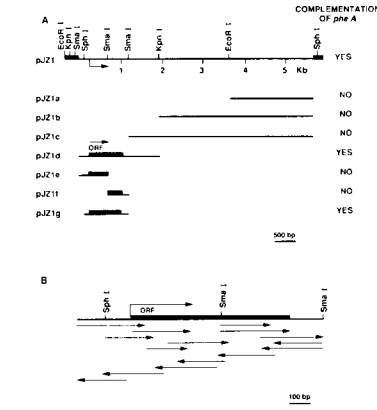

Fig. 2. **A.** Physical localization of the *P. aeruginosa pheC* gene. Linear maps of the various plasmids are shown, and the ability to complement the *pheA* defects of *E. coli* strains JP2255 and KA197 is also indicated. Restriction sites are labeled in the *P. aeruginosa* DNA and in the flanking pUC18 multiple cloning site (depicted by heavy lines). Abbreviation: ORF, open reading frame. **B.** The sequencing strategy used for the *pheC* gene.

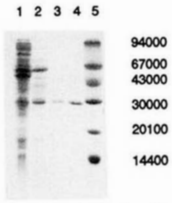

Fig. 4. SDS-polyacrylamide gel electrophoresis of the *pheC* gene product purified from *E. coli* JP2255(pJZ1g). The protein samples were run on a 15% gel and stained with Coomassie blue. The first four lanes show results obtained when samples collected after each of the fractionation steps shown in Table III were applied: lane 1, crude extract; lane 2, DEAE-cellulose; lane 3, hydroxylapatite; lane 4, gel filtration. The molecular-weight standards used are shown in lane 5.

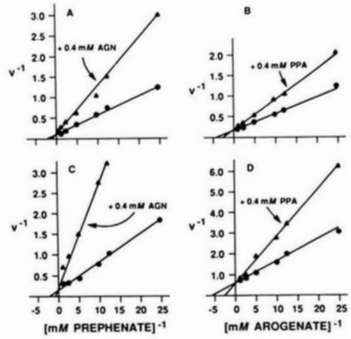

Fig. 5. Double reciprocal plots of *P. aeruginosa* cyclohexadienyl dehydratase purified either from *E. coli* JP2255(pJZ1g) (panels A and B) or directly from *P. aeruginosa* (panels C and D). When assayed as prephenate (PPA) dehydratase (panels A and C), v is expressed as nmoles of phenylpyruvate formed per min in the presence (▲) or absence (●) of 0.4 mM *L*-arogenate (AGN). When assayed as arogenate dehydratase (panels B and D), v is expressed as nmoles of *L*-phenylalanine formed per min in the presence (▲) or absence (●) of 0.4 mM PPA.