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PhhC is an essential aminotransferase for aromatic amino acid catabolism in *Pseudomonas aeruginosa*

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The *phhC* gene of *Pseudomonas aeruginosa* encodes a protein which is a member of the Family I aminotransferases. At high expression levels in the heterologous *Escherichia coli* system, PhhC can compensate for the absence of AspC (which functions in L-aspartate biosynthesis) and TyrB (which functions in aromatic biosynthesis). In the native organism, PhhC is essential for catabolism of either L-tyrosine or L-phenylalanine, as demonstrated by gene inactivation. This catabolic function of PhhC is consistent with its inclusion as the distal gene in the inducible phenylalanine hydroxylase operon. The presence of PhhC for catabolism of aromatic amino acids is required in spite of an existing multiplicity of other *P. aeruginosa* aminotransferases having a similar pattern of broad substrate specificity *in vitro*. This implies a spatial orientation of PhhC that effectively specializes it for aromatic amino acid catabolism.

Keywords: aromatic aminotransferase, phenylalanine catabolism, *phhC*, phenylalanine hydroxylase operon, tyrosine catabolism

INTRODUCTION

PhhC from *Pseudomonas aeruginosa* is an aminotransferase belonging to the α subfamily of Family I aminotransferases (Jensen & Gu, 1996). These aminotransferases participate in aspartate and/or aromatic amino acid biosynthesis. Individual subfamily members may be relatively specific for aromatic amino acids or for aspartate in some cases, but exhibit broad specificity and accommodate both substrate classes in other cases. *P. aeruginosa phhC* is the distal gene in a three-gene operon (Zhao *et al.*, 1994) which includes *phhA* and *phhB*. PhhA is a pterin-dependent phenylalanine hydroxylase and PhhB is a carbinolamine dehydratase that functions in pterin recycling. The stop and start codons of *phhB* and *phhC* overlap and are thus probably translationally coupled. The operon is subject to positive regulation by a divergently transcribed regulatory protein which belongs to the large family of σ^{54}-dependent enhancer-binding proteins (Song & Jensen, 1996).

The organizational inclusion of *phhC* within the *phh* operon implies an *in vivo* function as an aromatic aminotransferase. Operation of the *phh* operon in a catabolic mode would utilize the following initial steps: L-phenylalanine → L-tyrosine → 4-hydroxyphenylpyruvate. The latter supposition suggests that PhhC might be essential for L-phenylalanine and/or L-tyrosine catabolism. In view of the demonstrated expression of up to four additional aromatic aminotransferases in *P. aeruginosa* (Whitaker *et al.*, 1982), the extent to which PhhC might be obligatory for a specialized role in catabolism was examined in this study.

METHODS

Bacterial strains, plasmids and media. All bacterial strains and plasmids used in this study are listed in Table 1. Luria–Bertani (LB) medium was used as enriched medium (Silhavy *et al.*, 1984). The minimal medium used was the M9 formulation, except for *Escherichia coli* DL39, which was grown in the medium described by LeMaster & Richards (1987). Where indicated, media were supplemented with ampicillin at 100 μg ml^{-1}, kanamycin at 35 μg ml^{-1}, thiamin at 17 μg ml^{-1} and $HgCl_2$ at 15 μg ml^{-1}. For solid media, 2% (w/v) agar was added.

Crude-extract preparation and enzyme assays. Cultures of *E. coli* or *P. aeruginosa* were grown at 37 °C with vigorous shaking in LB or minimal medium supplemented with appropriate antibiotics. The cells were harvested in the late-exponential phase of growth by centrifugation and resus-

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Abbreviation: EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
S17-1	[RP4-2 (Tc::Mu) (Km::Tn7) Tra IncP)] *pro hsdR recA thi-1* Tp^r Sm^r	Simon *et al.* (1983)
DL39	λ^- *aspC13 fnr-25 ilvE12 tyrB507*	LeMaster & Richards (1987)
P. aeruginosa		
PAO-1	Prototroph	Holloway (1955)
RW8-24	Phenylalanine auxotroph	Whitaker *et al.* (1982)
JS104	Hg^r *phhC* knockout	This study
Plasmids		
pUC18	Ap^r lac'IPOZ'	Yanisch-Perron *et al.* (1985)
pUC19	Ap^r lac'IPOZ'	Yanisch-Perron *et al.* (1985)
pUFR004	ColE1 Cm^r Mob^+ *mob*(*P*)	DeFeyter *et al.* (1990)
pJS3A	3·7 kb *Bam*HI–*Hin*dIII fragment of *phhB–phhC* subcloned into the *Bam*HI/*Hin*dIII sites of pUC18	This study
pJS3B	3·7 kb *Bam*HI–*Hin*dIII fragment of *phhB–phhC* subcloned into the *Bam*HI/*Hin*dIII sites of pUC19	This study
pDG106	Hg^r Km^r P15A replicon	Gambill & Summers (1985)
pJS101	*Pst*I–*Sma*I fragment of pDG106 inserted into pUC18	Song & Jensen (1996)

pended in 50 mM potassium phosphate buffer (pH 7·0), containing 1 mM DTT. The cells were broken by sonication using an Ultratip Labsonic System (Lab-Line Instruments). The suspension was centrifuged at 150000 ***g*** for 65 min at 4 °C. The supernatant fraction was collected and passed through a DG-10 Sephadex column (1·5 × 5·5 cm), eluting with the same buffer. The resulting desalted preparation was the crude extract.

The ability of PhhC to function as an aspartate aminotransferase was assayed by two methods. (i) A continuous spectrophotometric assay was used to monitor oxaloacetate formation at 260 nm (Bonner & Jensen, 1987). Reaction mixtures contained 2 mM L-aspartate, 10 mM 2-oxoglutarate in 50 mM EPPS [*N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid)] buffer at pH 8·6. (ii) A stopped HPLC assay was also used. Reaction samples were derivatized with *o*-phthalaldehyde in 18% methanol/82% 20 mM potassium phosphate at pH 7·0 and analysed by HPLC, using a C_{18} ODS reverse-phase column (Alltech). Under these conditions, L-aspartate and L-glutamate exhibited retention times of 12·04 and 17·26 min, respectively. Aromatic aminotransferase was assayed as described by Gu *et al.* (1995). L-Phenylalanine and 2-oxoglutarate were used as the substrate combination, unless indicated otherwise. The Bradford assay (Bradford, 1976) was used for determination of protein concentrations.

Purification of cloned PhhC from *E. coli* DL39. *E. coli* DL39 carrying pJS3A was grown at 37 °C in 4 l LB medium supplemented with ampicillin in a gyratory shaker until late-exponential phase. The cells were harvested by centrifugation, and the pellet was resuspended in 50 mM potassium phosphate buffer (pH 7·0) containing 1 mM DTT. The cells were disrupted by sonication and centrifuged at 150000 ***g*** for 65 min at 4 °C. The crude extract was dialysed overnight against 2 l of the above buffer with one change of buffer. A 1184 mg amount of protein was applied to a 3 × 50 cm DE-52 column equilibrated with the buffer. The column was washed with 800 ml buffer, and bound protein was eluted with 800 ml of a linear salt gradient (0–0·4 M KCl) prepared in the same buffer. Fractions (6 ml) were collected and assayed for aromatic aminotransferase activity. Fractions showing high enzyme activity were pooled and concentrated by means of an Amicon YM-10 membrane. The concentrated preparation was dialysed overnight against 5 mM potassium phosphate buffer (pH 7·0) containing 1 mM DTT. A 288 mg aliquot of protein was then loaded onto the bed of a 3 × 16 cm Bio-Gel HTP column previously equilibrated with the above buffer. The column was washed with 500 ml starting buffer and then eluted by application of a potassium phosphate gradient (5–350 mM) in the same starting buffer. Fractions (3·5 ml) were collected and aromatic aminotransferase activity was assayed. Fractions showing high enzyme activity in the wash eluate were pooled and concentrated by means of an Amicon YM-10 membrane. The concentrated preparation was dialysed overnight against 50 mM potassium phosphate buffer (pH 7·0) containing 1 mM DTT and 0·15 M NaCl. Gel filtration was carried out by use of an FPLC-connected Superdex 75 column (Pharmacia) which had been previously equilibrated with the above buffer. Eluate fractions (0·5 ml) were collected; those showing high aromatic aminotransferase activity were pooled and concentrated by an Ultrafree-15 centrifugal filter device (Millipore).

N-terminal amino acid sequencing of cloned PhhC. The purified enzyme preparation was denatured by SDS and resolved by SDS-PAGE. The protein was transferred to a PVDF membrane (Bio-Rad) by use of a protein miniblotting apparatus. After staining with Coomassie brilliant blue R-250, the band corresponding to PhhC was excised and sequenced by using an Applied Biosystems model 407A protein sequencer with an on-line 120A phenylthiohydantoin analyser at the Protein Core Facility at the University of Florida.

Molecular mass determination. SDS-PAGE was carried out to determine the subunit molecular mass of the enzyme. Lysozyme (14·4 kDa), soybean trypsin inhibitor (21·5 kDa), carbonic anhydrase (31 kDa), ovalbumin (66 kDa) and phos-

phorylase *b* (97·4 kDa) were used as molecular mass standards. Subunit size was also determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. A Vestec LaserTech Research linear instrument (Perceptive Biosystems) employing a nitrogen laser (337 nm), a 1·2 m flight tube, and an accelerating voltage of 10 kV was used. The mass axis was set by calibration with insulin. Samples were diluted into 0·1% trifluoroacetic acid and mixed on the target with an equal volume of matrix consisting of a saturated solution of sinapinic acid dissolved in 40% acetonitrile and 0·1% trifluoroacetic acid. The mean was taken from 153 spectra and assigned using the GRAMS-LaserTech program.

The molecular mass of the native enzyme was estimated by FPLC using a Superdex 75 HR 10/30 column equilibrated with 50 mM potassium phosphate buffer at pH 7·4 containing 0·15 M NaCl, 20% (v/v) glycerol and 0·1 mM pyridoxal phosphate. The enzyme (250 µl enzyme recovered from the DEAE-cellulose step) was eluted with the same buffer at a flow rate of 0·5 ml min^{-1}. Standards used to calibrate the column were alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (30 kDa) and cytochrome C (12·3 kDa).

Gene inactivation. The chromosomal *phhC* gene was insertionally inactivated by single-crossover recombination (Kamoun *et al*., 1992) as described by Song & Jensen (1996). A truncated *phhC* fragment ('*phhC*') was generated by PCR using the upper primer 5′ GTCGAGCAGGAAACCACCAAGA 3′ and the lower primer 5′ GTTGGCTACGCAGGTCGGTGAG 3′. Thus, 174 bp was eliminated from the 5′ end and 372 bp from the 3′ end of the 1·2 kb *phhC* gene. The PCR-generated '*phhC*' fragment was first cloned into pUFR004 (a mobilizable suicide vector) to create pUFR/'*phhC*'. A Hg^r *Hin*dIII-cassette from pJS101 was then cloned into pUFR/'*phhC*' to create pUFR/'*phhC*'/Hg^r, which was used to transform *E. coli* S17-1 (a mobilizing strain). *E. coli* S17-1 harbouring pUFR/'*phhC*'/Hg^r was used as a donor in biparental mating with *P. aeruginosa*, performed as described by Simon *et al*. (1983). The mating mixture was plated onto Pseudomonas isolation agar (Difco) plates containing 15 µg $HgCl_2$ ml^{-1}. Hg^r colonies were isolated and the interruption of the chromosomal *phhC* gene by integration of the suicide vector was confirmed by Southern blot analysis (Sambrook *et al*., 1989) of Hg^r isolates. The knockout mutant selected for further work was denoted strain JS104.

SDS-PAGE and Western-blot analysis. SDS-PAGE was performed with a Mini-PROTEAN II cell (Bio-Rad) by the method of Laemmli (1970). Samples of exponential-phase cells were collected by centrifugation, and the pellets were suspended in gel-loading buffer and heated at 100 °C for 10 min. Samples of 5–10 µl were loaded onto two SDS-12% polyacrylamide gels. After electrophoretic separation, one gel was stained with Coomassie brilliant blue R-250, and the other gel was used for Western blotting. For comparison of different crude extracts, equivalent amounts of protein were applied. Western blots were performed according to Towbin *et al*. (1979).

RESULTS

Catalytic capabilities of PhhC *in vivo*

The ability of *P. aeruginosa phhC* to function in a biosynthetic direction under conditions of high expression in a heterologous system was tested. *E. coli* DL39 is a triple mutant, deficient in *aspC*, *tyrB* and *ilvE*, and thus requiring L-aspartate, L-tyrosine, L-phenylalanine, L-leucine, L-isoleucine and L-valine for growth (LeMaster & Richards, 1987). *E. coli* DL39 was transformed with pJS3A (a construct which carries *phhB* and *phhC* on a *P. aeruginosa* DNA insert), and ampicillin-resistant colonies were selected on LB plates. Purified transformants were spread on minimal plates to provide confluent lawns. Sterile discs containing various combinations of the above-cited amino acids were placed on the lawns to evaluate the nutritional requirements of *E. coli* DL39 expressing *phhC* (Table 2). Growth occurred in the absence of L-tyrosine and L-phenylalanine or in the absence of L-aspartate, thus indicating the ability of *phhC* to compensate for the deficient *E. coli tyrB* and *aspC* genes. On the other hand, L-isoleucine, L-leucine and L-valine were required for growth, indicating that *phhC* could not complement the *ilvE* deficiency.

Table 2. Ability of *phhC* to complement deficiencies of *aspC*, *tyrB* or *ilvE* in *E. coli* DL39/pJS3A

Agar plates contained minimal medium plus ampicillin. + indicates the presence of 50 µg ml^{-1} of the amino acid shown at the top of the column.

Test disc	Supplementation			Growth
	Tyr + Phe	Asp	Ile + Leu + Val	
A	+	+	+	+
B	−	+	+	+
C	+	−	+	+
D	+	+	−	−

Purification of *P. aeruginosa* PhhC from *E. coli*

Crude extracts prepared from *E. coli* DL39/pJS3A possessed high aromatic aminotransferase activity, whereas comparable extracts from strain DL39 exhibited no detectable activity. When the *P. aeruginosa* DNA insert was placed in the opposite orientation (*E. coli* DL39/pJS3B), no aromatic aminotransferase activity was found. Thus, the *phhC* fragment either lacks a native promoter or has a promoter not recognized by *E. coli*, therefore requiring the orientation which allows use of the *lac* promoter in the vector.

PhhC was purified from *E. coli* DL39/pJS3A by use of three chromatography steps (Table 3). The hydroxylapatite chromatography step resolved two peaks of activity (Fig. 1a). An unbound species eluted in the wash fraction, and a second species eluted in the gradient. The leading peak of aminotransferase was of greater purity (Table 3, Fig. 2), and this fraction was applied to Superdex 75 in the final step of purification. The aminotransferase recovered was visualized as a single protein band following SDS-PAGE (Fig. 2). Mass spectrometry of the sample revealed a minor 16·3 kDa protein contaminant. The molecular mass of the major band was estimated to be 43 kDa, in agreement with

Table 3. Purification of the cloned *P. aeruginosa* PhhC from *E. coli* DL39/pJS3A

Purification step	Total protein (mg)	Specific activity *	Purification factor	Yield (%)
Crude extract	1184	1·7	1·0	100
DEAE-cellulose	320	3·5	2·0	55·6
Hydroxylapatite				
Gradient	40	11·9	7·0	23·6
Wash	5	24·5	14·4	6·1
Superdex 75†	1	32·1	18·9	1·6

* Specific activity expressed as μmol min^{-1} mg^{-1}.

† The protein recovered from the hydroxylapatite wash fraction was applied.

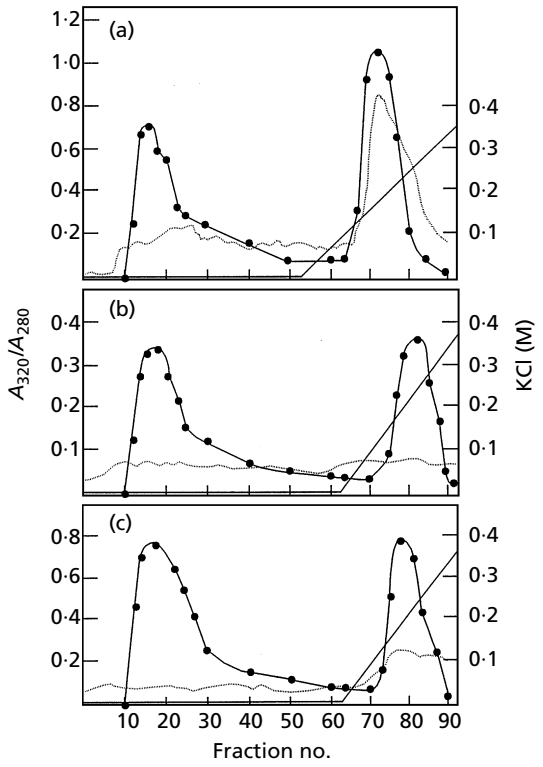

Fig. 1. PhhC hydroxylapatite elution profile. The protein applied was from: (a) the DEAE-cellulose step of purification; (b) the wash fraction shown in (a); (c) the gradient fraction shown in (a). All the gradient peaks eluted at about 0·1 M potassium phosphate. Enzyme activity (●) was measured as formation of phenylpyruvate (A_{320}) following the transamination of L-phenylalanine in the presence of 2-oxoglutarate, as described in Methods. Dotted lines represent the protein profile (A_{280}) of the eluate; the solid line represents the KCl concentration.

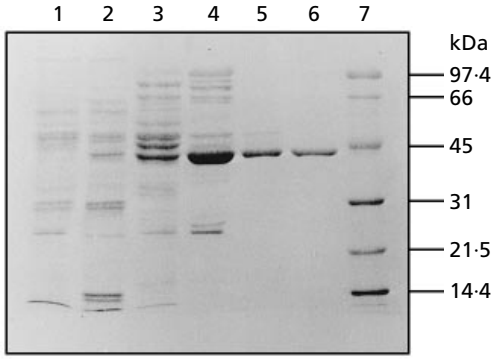

Fig. 2. SDS-PAGE of PhhC from *E. coli* DL39/pJS3A. The protein samples were run in a 12% gel and stained with Coomassie brilliant blue. Lanes: 1, crude extract of DL39/pUC18; 2, crude extract of DL39/pJS3A; 3, fraction collected after DE-52 chromatography; 4, gradient fraction collected after hydroxylapatite chromatography; 5, wash fraction collected after hydroxylapatite chromatography; 6, fraction collected after Superdex 75 chromatography; 7, molecular mass standards.

predictions based upon the DNA sequence. This was further confirmed by mass spectrometry (Fig. 3) which demonstrated a molecular mass of 43248 Da. Since a purification factor of only 19-fold was required to obtain pure enzyme, it can be estimated that about 5% of the total protein in the original clone was PhhC.

The two species of PhhC recovered after hydroxylapatite chromatography appear to be conformational isoforms at equilibrium. Appropriate experimental work was done to rule out trivial explanations such as an artifact of column packing, or that adsorption to the column might be a slow process which was not complete prior to elution. Both species eluted identically when individually subjected to gel filtration, indicating no detectable difference in molecular mass. N-terminal amino acid sequencing of samples of either species showed identical N termini: SHFAKVARVP. This corresponds exactly with the deduced N terminus (Zhao *et al.*, 1994), except that the starting L-methionine had been processed. When either of the two species recovered from hydroxylapatite chromatography was reloaded onto the same column and eluted under the same conditions as before, the original bi-peaked profile was again obtained (Fig. 1b, c). The leading peak was characteristically broad, possibly consistent with a tendency for a new equilibrium of conformational isoforms to form during chromatography. These results were reproducible when

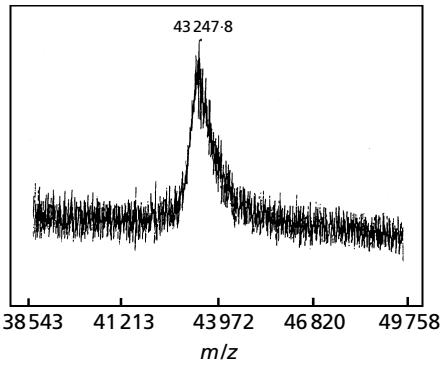

Fig. 3. Determination of the molecular mass of PhhC by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Mass spectrometry was carried out with a sample of purified PhhC from *E. coli* DL39/pJS3A as detailed in Methods.

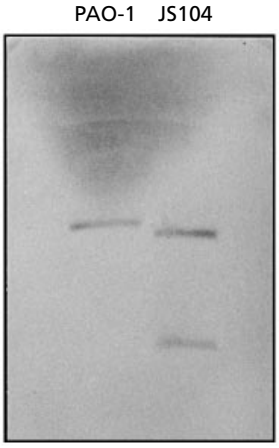

Fig. 4. Southern blot of chromosomal DNA from *P. aeruginosa* PAO-1 wild-type and *phhC* mutant JS104. Chromosomal DNA was completely digested with *Eco*RI and probed at high stringency with the truncated '*phhC*' PCR fragment described in Methods.

the initial column was loaded with lesser amounts of protein, down to a level approaching the sensitivity of peak detection by enzyme assay.

Gel filtration experiments showed a native molecular mass of about 73 kDa. Since a native dimer of 86 kDa is expected (see Discussion), PhhC may exhibit a physical shape that alters its migration in the gel. Gel filtration was carried out by FPLC in the presence of 2-oxoglutarate, L-phenylalanine and pyridoxal phosphate to see whether binding of active-site molecules would influence the elution position. A repetition of several experiments gave the same result: the apparent molecular mass was not influenced by the small molecules tested. In gel-filtration experiments, use of the same buffer which yielded two chromatographic forms from a hydroxylapatite matrix, yielded only a single peak at 73 kDa.

Catalytic capabilities of PhhC *in vitro*

The pH optimum for catalysis was determined over a pH range of 6·0–10·5. Potassium phosphate buffer (50 mM) was used for the range pH 6·0–7·5; 50 mM EPPS buffer was used for the range of pH 8·0–8·8; 50 mM sodium borate buffer was used for the range pH 8·8–10·5. Similar profiles were obtained when assays contained 10 mM 2-oxoglutarate in combination with 2 mM L-phenylalanine, L-tyrosine or L-aspartate. Optimal transamination rates occurred at pH 9·0, with 50% of the optimal rate occurring at pH 10·0 and pH 7·6.

Substrate saturation curves were constructed in assays carried out in 50 mM sodium borate buffer at pH 9·0 and 22 °C. L-Aspartate, L-phenylalanine and L-tyrosine were used as variable substrates in combination with 2-oxoglutarate as the fixed substrate. 2-Oxoglutarate was also used as a variable substrate in combination with L-aspartate as the fixed substrate. K_m values were derived from double-reciprocal plots for L-tyrosine (1·2 mM), 2-oxoglutarate (4·2 mM), L-aspartate (5·0 mM) and L-phenylalanine (14·3 mM). At saturating substrate concentrations in combination with 20 mM 2-oxoglutarate, the ratio of specific activities obtained with L-tyrosine : L-phenylalanine : L-aspartate was 1·0 : 5·7 : 8·3.

Insertional inactivation of phhC

The *phhC* gene was interrupted as described in Methods to give mutant JS104 (Table 1). Gene interruption was verified by Southern-blot analysis of chromosomal DNA from wild-type PAO-1 and knockout-mutant JS104 (Fig. 4). Chromosomal DNA was completely digested with *Eco*RI and probed at high stringency with the '*phhC*' fragment (which lacks an *Eco*RI site). Two bands hybridized with '*phhC*' in mutant JS104, in contrast to the single band visualized with wild-type PAO-1. This is the expected result because of the presence of one *Eco*RI cleavage site in the vector.

Mutant JS104 was tested for its ability to use L-tyrosine or L-phenylalanine as the sole source of carbon. In contrast to the doubling times at 30 °C observed for wild-type PAO-1 on L-tyrosine (doubling time 2·4 h) or L-phenylalanine (doubling time 25·5 h), mutant JS104 was unable to utilize either amino acid as a carbon source. This cannot be attributed to downstream polarity effects of the insertion because the downstream gene *pbpG* is unrelated to aromatic amino acid metabolism and has been characterized recently (Song *et al.*, 1998). The gene downstream of *pbpG* (*yaeJ*) is transcribed divergently. Inactivation of *pbpG* had no effect upon aromatic amino acid catabolism (J. Song, unpublished). Mutant JS104 was able to utilize 4-hydroxyphenylpyruvate (doubling time 7 h) or 4-hydroxyphenylacetate (doubling time 1·8 h) extremely

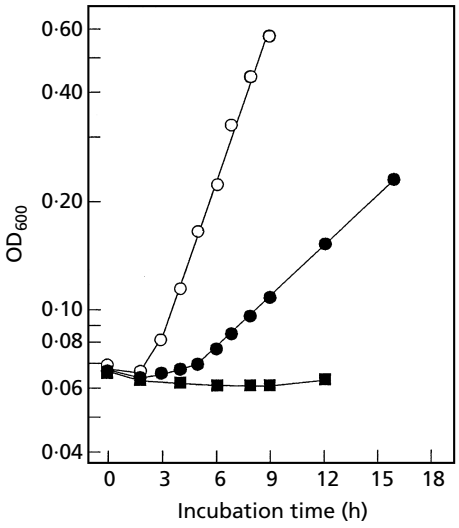

Fig. 5. Inability of *phhC*-inactivated mutant JS104 to utilize L-tyrosine as the sole source of carbon. A glucose-grown culture in the exponential phase was centrifuged and the pellet resuspended in minimal medium containing L-tyrosine (■), 4-hydroxyphenylpyruvate (●), or 4-hydroxyphenylacetate (○). Turbidities of cultures at 30 °C in sidearm flasks were monitored by measuring OD_{600} at the indicated sampling times.

well as carbon sources, as shown in Fig. 5. Since growth is supported by 4-hydroxyphenylpyruvate but not by L-tyrosine, it would appear that little, if any, of the exogenously supplied L-tyrosine is converted to 4-hydroxyphenylpyruvate by other aminotransferases that coexist with PhhC in *P. aeruginosa*.

DISCUSSION

Apparent physical isoforms of PhhC

Two different species of PhhC were eluted following hydroxylapatite column chromatography. The two separable species of PhhC obtained must have originated from a single gene since it was purified from an *E. coli* clone lacking an aromatic aminotransferase background. Each species exhibited an identical molecular mass (43 kDa) and an identical N-terminal amino acid sequence. When rechromatographed on the same column, each species was again resolved into a nearly equal mixture of the two isoforms. Taken together, these results ruled out the possibilities that interconvertible molecular-mass forms of the enzyme might be in equilibrium, that limited proteolysis might generate a second still-active form, or that some form of covalent modification, such as phosphorylation, might be in operation.

Since the clone used also expresses *phhB*, to which *phhC* is translationally coupled (Zhao *et al*., 1994), we initially suspected the existence of a dissociable PhhB/PhhC complex. Although the complex should then possess a greater molecular mass, the small size (13 kDa) of PhhB might have masked the difference. However, this was ruled out because the dissociated form of the enzyme (PhhC) should have no possibility to generate the PhhB/PhhC complex upon rechromatography. Furthermore, no PhhB was detected by Western analysis of either chromatographic peak using anti-PhhB polyclonal antibody.

We conclude that an equilibrium distribution of PhhC isoforms exists whose charge (or other) properties are discriminated by the hydroxylapatite matrix. Such protein conformers have been rigorously documented in the literature (Cao *et al*., 1998). *P. aeruginosa* PhhC may exemplify the 2-state class of dimer (Xu *et al*., 1998).

Dimeric state of PhhC

PhhC belongs to the Family I aminotransferase homology group (Jensen & Gu, 1996). Well-studied members of this family share a mechanism of catalysis which requires homodimer formation. As is common for many dissociable enzyme oligomers (Traut, 1994), crucial catalytic sites are organized at subunit interfaces and are disrupted by subunit dissociation. Since *P. aeruginosa* PhhC is nested well within a homology group of four proteins for which crystal structures demonstrate an identical three-dimensional fold (Jeffery *et al*., 1998), the existence of a homodimer structure seems certain. Although a variety of size-exclusion chromatography experiments consistently yielded estimates of molecular mass (73 kDa) somewhat smaller than expected (86 kDa), the existence of the expected homodimer is indicated. Proteins having an asymmetrical shape often exhibit aberrant migration behaviour on a gel filtration matrix.

Potential for biosynthetic function of PhhC *in vivo*

In the native *P. aeruginosa*, PhhC appears to be uniquely oriented to aromatic amino acid catabolism. However, when highly expressed in *E. coli*, PhhC was also shown to suppress the deficiencies caused by mutant *aspC* and *tyrB* genes. Thus, PhhC has the potential to function in both L-aspartate biosynthesis and aromatic amino acid biosynthesis, at least when expressed at high levels. Inactivation of *phhC* does not result in an inability to synthesize aromatic amino acids (or aspartate), consistent with the conclusion that it is not a biosynthetic enzyme.

Based upon a combination of *P. aeruginosa* mutant characterizations, and regulatory responses of multiple aminotransferase species to different growth conditions (Whitaker *et al*., 1982), two aminotransferase species (AT-2 and AT-4) were proposed to carry out aromatic biosynthesis under normal conditions. Under limiting conditions of growth imposed in the absence of AT-2, an aspartate aminotransferase was shown to support aromatic amino acid biosynthesis (similar to the ability of *E. coli aspC* to suppress a deficiency of *tyrB* (Gelfand & Steinberg, 1977). One might predict that a constitutive mutant of the *phh* operon (i.e. showing elevated expression levels of *phhC*) would be able to suppress

auxotrophic or bradytrophic phenotypes of mutants lacking aminotransferases normally engaged in aromatic biosynthesis in *P. aeruginosa*.

Catabolic function of PhhC *in vivo*

In contrast to the multiplicity of aminotransferase species which apparently can participate in aromatic biosynthesis, PhhC is absolutely required for utilization of either L-phenylalanine or L-tyrosine as the sole source of carbon. Expression of the structural genes in the *phh* operon is induced by well over an order of magnitude in the presence of aromatic amino acids (J. Song & R. A. Jensen, unpublished). The previously described (Whitaker *et al*., 1982) aminotransferase AT-3 was the only species induced in the presence of aromatic amino acids (although only threefold), and could be synonymous with PhhC.

In contrast to L-phenylalanine and L-tyrosine, catabolism of the closely related L-arogenate has been shown not to depend upon expression of *phhC* (Fischer *et al*., 1997). Although L-arogenate can be catabolized through conversion to L-phenylalanine via periplasmic cyclohexadienyl dehydratase (in which case PhhC would be functional), an alternative and more robust pathway to 4-hydroxyphenylpyruvate apparently exists via its conversion to prephenate by oxidative deamination.

The *phh* operon is effectively induced by growth on L-tyrosine as a sole source of carbon (J. Song & R. A. Jensen, unpublished). This meets physiological expectations in view of the essentiality of PhhC for tyrosine catabolism. The concomitant induction of PhhA and PhhB, however, must be gratuitous under these conditions, since no apparent need for phenylalanine hydroxylase exists.

The absolute dependence of L-phenylalanine or L-tyrosine catabolism upon PhhC is striking in view of the multiplicity of aminotransferases present in *P. aeruginosa* that are capable of utilizing aromatic amino acids as substrates. This implies some sort of compartmentalization or physical sequestration, whereby PhhC specifically links with succeeding enzymes of catabolism. In this connection it is suggestive that 4-hydroxyphenylpyruvate dioxygenase, the enzyme using the product of PhhC-mediated catalysis, is an inner-membrane protein (G. Xie & R. A. Jensen, unpublished). It is logical that initial enzymes of aromatic catabolism might be spatially associated with the cell exterior since many of them utilize molecular oxygen, and the cytoplasm is a highly reducing environment.

The genome sequencing project for *P. aeruginosa* (www.pseudomonas.com), which is near completion, will provide a basis for the systematic study at the molecular level of all of the aromatic aminotransferase enzymes. One can anticipate full elucidation of the primary roles of all of these aminotransferases and the extent to which they exhibit functional overlap under normal and forced environmental conditions.

Substrate specificity of PhhC

PhhC resembles its *E. coli* homologues in possessing broad substrate specificity, which potentially allows it to function as either an aromatic aminotransferase or an aspartate aminotransferase. PhhC has a significantly greater affinity for L-tyrosine than for either L-phenylalanine or L-aspartate *in vitro*. L-Tyrosine is presumably the physiological substrate, even when L-phenylalanine is catabolized as the carbon source (i.e. PhhA first converts L-phenylalanine to L-tyrosine). At saturating substrate levels, however, both L-phenylalanine and L-aspartate yield greater specific activities than does L-tyrosine.

Among homologues of PhhC, AspC and TyrB from *E. coli* have been studied to determine the variation of catalytic residues which influence whether L-aspartate or aromatic amino acids are favoured as substrates (Onuffer & Kirsch, 1995). PhhC is clearly dedicated to function with aromatic substrates. In view of this, it is curious that the pattern of critical catalytic residues identified by Onuffer & Kirsch (1995) as important for substrate discrimination resembles the pattern of AspC more than TyrB.

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