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Remnants of an Ancient Pathway to L-Phenylalanine and L-Tyrosine in Enteric Bacteria: Evolutionary Implications and Biotechnological Impact†

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The pathway construction for biosynthesis of aromatic amino acids in *Escherichia coli* is atypical of the phylogenetic subdivision of gram-negative bacteria to which it belongs (R. A. Jensen, Mol. Biol. Evol. 2:92–108, 1985). Related organisms possess second pathways to phenylalanine and tyrosine which depend upon the expression of a monofunctional chorismate mutase (CM-F) and cyclohexadienyl dehydratase (CDT). Some enteric bacteria, unlike *E. coli*, possess either CM-F or CDT. These essentially cryptic remnants of an ancestral pathway can be a latent source of biochemical potential under certain conditions. As one example of advantageous biochemical potential, the presence of CM-F in *Salmonella typhimurium* increases the capacity for prephenate accumulation in a *tyrA* auxotroph. We report the finding that a significant fraction of the latter prephenate is transaminated to L-arogenate. The *tyrA19* mutant is now the organism of choice for isolation of L-arogenate, uncomplicated by the presence of other cyclohexadienyl products coaccumulated by a *Neurospora crassa* mutant that had previously served as the prime biological source of L-arogenate. Prephenate aminotransferase activity was not conferred by a discrete enzyme, but rather was found to be synonymous with the combined activities of aspartate aminotransferase (*aspC*), aromatic aminotransferase (*tyrB*), and branched-chain aminotransferase (*ilvE*). This conclusion was confirmed by results obtained with combinations of *aspC*-, *tyrB*-, and *ilvE*-deficient mutations in *E. coli*. An example of disadvantageous biochemical potential is the presence of a cryptic CDT in *Klebsiella pneumoniae*, where a mutant carrying multiple enzyme blocks is the standard organism used for accumulation and isolation of chorismate. The anomalous presence of CDT accounts for leakiness in its growth requirement for phenylalanine.

Chorismate and prephenate are universal precursors of L-tyrosine (Tyr) and L-phenylalanine (Phe) in both microorganisms and plants (24). In higher plants L-arogenate (25), derived from prephenate by transamination, is the immediate precursor of Tyr and Phe (17). In contrast, *Escherichia coli* utilizes phenylpyruvate and 4-hydroxyphenylpyruvate as the immediate precursors of Phe and Tyr, respectively. However, the *E. coli* pathway construction is not typical of the phylogenetic subdivision of gram-negative bacteria to which it belongs. Members of this subdivision generally possess dual enzymatic routes to Phe and Tyr, a pathway construction typified by that present in *Pseudomonas aeruginosa* (3, 7, 16). In such dual-pathway systems Tyr formation may proceed from either 4-hydroxyphenylpyruvate or L-arogenate, while Phe formation may derive directly from either phenylpyruvate or L-arogenate (Fig. 1).

It has recently become recognized that within the closely related genera of enteric bacteria, considerable diversity exists for pathway construction of aromatic amino acid biosynthesis (2, 4–6). Thus, *Serratia* and *Erwinia* species possess a complete dual-pathway arrangement, whereas other genera possess only chorismate mutase F (CM-F) or cyclohexadienyl dehydratase (CDT), evolutionary remnants of the L-arogenate flow routes that have been discarded by *E. coli* (16). Before this study, it had not been established whether prephenate aminotransferase in enteric bacteria is a discrete enzyme or whether activity is fortuitously conferred by multiple aminotransferase enzymes.

Auxotrophic mutants are used for the microbiological accumulation of chorismate, prephenate, and L-arogenate (Fig. 1). Chorismate is isolated from *Klebsiella pneumoniae* (11). Prephenate is prepared from either *Salmonella typhimurium* (12) or *Neurospora crassa* (26). L-Arogenate is isolated from *N. crassa* (19, 25, 26). In this paper we show that the *S. typhimurium tyrA19* mutant is a more efficient source of L-arogenate than is *N. crassa* ATCC 36373. The biotechnological impact of the presence of prephenate aminotransferase, CM-F, or CDT upon intermediate accumulation in auxotrophic *Salmonella* and *Klebsiella* mutants is elucidated. An evolutionary framework exists within the *E. coli* genealogy for selection of organisms having pathway variations most appropriate for desired biochemical alterations aimed at biotechnological goals.

MATERIALS AND METHODS

Microbial strains. The *S. typhimurium tyrA19* mutant (22) was obtained from D. B. Sprinson, Columbia University, New York, N.Y. The prototrophic *S. typhimurium* ATCC 15277 was purchased from the American Type Culture Collection, Rockville, Md., as were *E. coli* W ATCC 9637, *K. pneumoniae* ATCC 25304, and *K. pneumoniae* ATCC 25306. *E. coli* aminotransferase mutants are all derivatives of *E. coli* K-12 (14). Strains DG27 (*ilvE tyrB*), DG30 (*ilvE tyrB, aspC*), DG34 (*ilvE aspC*), and DG44 (*tyrB aspC*) were obtained from Barbara Bachmann and carry *E. coli* Genetic Stock Center (Yale University) numbers CGSC 5798, CGSC 5799, CGSC 5801, and CGSC 5802, respectively.

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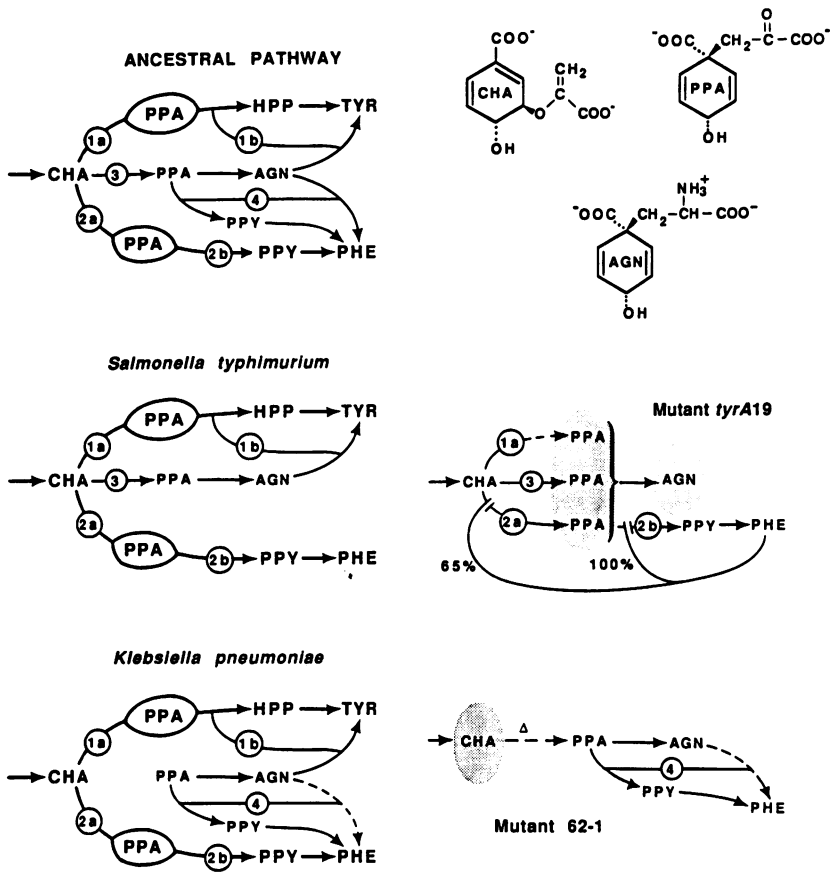

FIG. 1. The putative ancestral pathway of enteric bacteria, still present in contemporary *Serratia* and *Erwinia* species (16), is shown at the upper left. Chorismate (CHA) may be utilized by CM-T (enzyme 1a), CM-P (enzyme 2a), or CM-F (enzyme 3). Enzyme 1b is cyclohexadienyl dehydrogenase, which is capable of transforming prephenate (PPA) to 4-hydroxyphenylpyruvate (HPP) or of transforming L-arogenate (AGN) to L-tyrosine (TYR). Activities 1a and 1b make up the bifunctional T protein. Enzyme 2b is prephenate dehydratase, which converts PPA to phenylpyruvate (PPY). Activities 2a and 2b make up the bifunctional P protein. Enzyme 4 is CDT, which is capable of converting PPA to PPY or of converting AGN to L-phenylalanine (PHE). Other unnumbered arrows indicate aminotransferase enzymes that transaminate PPA, PPY, or HPP. Structures of CHA, PPA, and AGN are shown at the upper right. The middle left panel shows the pathway construction of wild-type *S. typhimurium* (lacks enzyme 4 of the ancestral pathway). The *S. typhimurium tyrA19* mutant possesses a defective T protein which lacks activity 1b, but retains partial activity for 1a. Excess Phe present during PPA and AGN accumulation inhibits enzyme 2a activity 65% and enzyme 2b activity 100%. Wild-type *K. pneumoniae* (lower left) lacks enzyme 3 of the ancestral pathway, which presumably is necessary for AGN formation. *K. pneumoniae* 62-1 (lower right) lacks both the P protein and the T protein. The dotted arrow indicates that CDT, which ordinarily is able to convert AGN to PHE, cannot do so in *K. pneumoniae*. The presence of enzyme 4 provides capability for Phe formation to the extent that CHA is nonenzymatically converted to PPA (dotted arrow) as a rate-limiting step. Shading highlights the compounds that are accumulated behind metabolic blocks.

Growth regimens. Previously described growth protocols were followed for *E. coli* W ATCC 9637 (20) and *K. pneumoniae* ATCC 25304 (21). Aminotransferase-deficient mutants of *E. coli* (DG27, DG30, DG34, and DG44) were grown in a minimal medium with supplementation as described previously (14). *S. typhimurium* strains were grown in minimal salts medium (23) as described by Dayan and Sprinson (12). Plates of minimal medium, with the addition of 50 μg of Tyr ml^{-1} for the *tyrA19* auxotroph, were used for growth of initial cultures at 30°C. Starter cultures for liquid medium (60 ml contained in a 250-ml Erlenmeyer flask) were inoculated from the plate cultures and shaken at 150 rpm for 5 h at 30°C. The *tyrA19* cultures were supplemented with 200 μg of Tyr ml^{-1} for this stage of growth. A 10-ml portion of starter culture was used to inoculate each of six Fernbach (2,800-ml) flasks, each containing 1.25 liters of the same growth medium. The cultures at 30°C were shaken overnight at 150 rpm before centrifugation at 13,600 × *g* for 15 min at 4°C.

Accumulation of prephenate and L-arogenate by the *tyrA19* mutant. The centrifuged cells were washed with accumulation medium and resuspended in 1.5 liters of accumulation medium in each of six Fernbach flasks. The accumulation medium recipe (12) was modified to contain 15.0 g of glucose $liter^{-1}$ and to give a lower content of salts (in grams per liter) as follows: Na_2HPO_4, 12.8; KH_2PO_4, 1.36; NH_4Cl, 2.7; $MgSO_4 \cdot 7H_2O$, 0.25; $CaCl_2$, 0.02; and Trp and Phe, 0.5 each. The pH was maintained between 7.1 and 8.3 by periodic additions of 3.5 M NH_4OH over the course of 20 h at 30°C with shaking at 150 rpm. Cells were harvested by centrifugation as above and stored in a −80°C freezer, and the supernatant was used to determine the concentrations of prephenate and arogenate in the accumulation medium.

Preparation of crude extracts. Cell pellets (stored at −80°C) of *S. typhimurium*, *E. coli*, and *K. pneumoniae* were resuspended in 25 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol. Cells were disrupted sonically in a Lab-Line Ultratip sonicator at 0°C with three 15-s

bursts of energy at an intensity of 100 to 110 W; each burst was followed by 2 min of cooling. After ultracentrifugation at 150,000 × *g* at 4°C for 60 min, the extracts were dialyzed against the extraction buffer (three changes of 1 liter each).

Measurement of intracellular pool sizes of prephenate and L-arogenate. Approximately 500 mg (wet weight) of sedimented cells of *S. typhimurium* were harvested by centrifugation after 6 h of growth, resuspended in 20 mM potassium phosphate buffer (pH 7.5), sonicated, and centrifuged (as above), and concentrations of prephenate and L-arogenate in a crude extract preparation containing 11.6 mg of protein per ml were determined.

Determinations of prephenate and L-arogenate concentrations. The prephenate concentration was determined after the quantitative conversion of prephenate to phenylpyruvate by addition of 0.1 ml of 1 M HCl to 0.1-ml samples of prephenate, followed by incubation at 37°C for 10 min. A 0.8-ml amount of 2.5 M NaOH was added, and the A_{320} was measured. The phenylpyruvate concentration was calculated by using a molar extinction coefficient of 17,500 (11).

L-Arogenate was identified by high-pressure liquid chromatography (HPLC) separation of the *o*-phthalaldehyde derivative on a C-18 ODS column (4.6 by 250 mm; Alltech). Experimental samples of L-arogenate were shown to elute with the same retention time as authentic L-arogenate. Following treatment of 0.1-ml samples with 0.1 ml of 1 M HCl at 37°C for 10 min, no peak was visualized in the position expected for L-arogenate and a new peak appeared which corresponded to Phe, the nonenzymatic acidification product of L-arogenate. The concentration of L-arogenate was determined by quantitating the acidified product with respect to a standard curve constructed from authentic Phe.

DEAE-cellulose chromatography. A 193-mg amount of protein was loaded on a DEAE-cellulose column (2.5 by 23 cm) which was equilibrated with the potassium phosphate extraction-dialysis buffer. The column was washed with 240 ml of equilibration buffer prior to application of a step gradient of 0.12 M KCl in equilibration buffer. Next, three successive linear salt gradients (0.12 to 0.18 M KCl, 400 ml total volume; 0.18 to 0.23 M KCl, 300 ml total volume; and 0.23 to 0.50 M KCl, 600 ml total volume) were applied to the column, followed by a 1 M KCl wash (200 ml) to elute any remaining protein.

Enzyme assays. Prephenate aminotransferase was assayed by HPLC following *o*-phthalaldehyde derivatization of product formed as described by Bonner and Jensen (9). The total volume for all aminotransferase reaction mixtures was 100 μl. Prephenate aminotransferase activity in crude extracts was determined after incubation at 37°C for 20 min with 50 μl of extract, 20 mM prephenate, and 10 mM indicated amino donor substrate. When L-glutamate was the amino donor, L-arogenate formation (measured as Phe following acidification of the reaction sample) was determined on a C-18 ODS HPLC column (5 μm) with a 55% methanol–45% 20 mM potassium phosphate buffer (pH 7.2). Direct quantitation of the L-arogenate product was accomplished by separating it from L-glutamate in the reaction mixture on the above HPLC column by changing the methanol and potassium phosphate buffer concentrations to 15 and 85%, respectively. However, this modification was time-consuming for routine analyses (with each sample requiring about 40 min) because an additional methanol wash was required to elute Phe arising from decomposition of L-arogenate or contaminating L-arogenate fractions. Prephenate aminotransferase activity in crude extracts was also determined on a 10-μm C-18 ODS column with 10 mM Phe as the amino acid donor by measuring the peak of L-arogenate eluted by 60% methanol. When chromatography column fractions were screened for prephenate aminotransferase activity, reaction mixtures containing 65 μl of eluate, 5 mM L-glutamate, and 3 mM prephenate were incubated at 37°C for 20 min, and then the amount of Phe formed from L-arogenate in 50% methanol after acidification was measured.

Aromatic aminotransferase (TRD) was assayed with 5 mM phenylpyruvate, 10 mM L-glutamate, and 50 μl of eluate from the DEAE-cellulose column, and Phe was quantitated by HPLC in 50% methanol. Aspartate aminotransferase (TRA) was assayed by measurement of L-glutamate by HPLC, using a 5-μm ODS column eluted with 15% methanol, following application of a reaction mixture containing 10 mM 2-ketoglutarate, 10 mM L-aspartate, and 50 μl of column eluate. Branched-chain aminotransferase (TRB) activity was assayed in column fractions by HPLC measurement of L-glutamate in 60% methanol, using 50 μl of eluate, 10 mM 2-ketoglutarate, and 10 mM L-leucine. Aminotransferase assays were carried out at 37°C for 10 min.

Prephenate dehydratase was assayed as described by Fischer and Jensen (13) with 2 mM prephenate and 50 μl of column eluate at 37°C for 15 min in a total volume of 200 μl. Chorismate mutase was assayed by the method of Gibson (15) with 50 μl of column eluate and 0.5 mM chorismate at 37°C for 15 min.

Protein concentrations were estimated by the Bradford assay with bovine serum albumin as the protein standard (10).

Biochemicals. L-Arogenate (90% pure) was isolated from a multiple auxotroph of *N. crassa* (ATCC 36373) as described by Zamir et al. (28). Chorismate (85% pure) was prepared by the method of Gibson (15) from the multiple auxotroph *K. pneumoniae* 62-1 (ATCC 25306). Barium prephenate, used in enzyme assays, was prepared from culture supernatants of the *tyrA19* auxotroph of *S. typhimurium* (12) and was converted to the potassium salt with a twofold excess of K_2SO_4 before use. Negligible amounts of organic impurities were present. Bradford reagent was obtained from Bio-Rad, Rockville Center, N.Y. DEAE-cellulose and HPLC-grade methanol were purchased from Fisher Scientific Co., Orlando, Fla. Dithiothreitol was purchased from Research Organics, Inc., Cleveland, Ohio. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Formation of L-arogenate by a prephenate-accumulating *Salmonella* auxotroph. Since a prephenate-accumulating mutant of *Neurospora crassa* was shown to produce the additional cyclohexadienyl derivatives L-arogenate (19), *spiro*-arogenate (26), and prephenyllactate (27), the possible presence of these prephenate derivatives in the *S. typhimurium tyrA19* mutant was examined. Detectable levels of prephenyllactate or *spiro*-arogenate were not found, but the presence of L-arogenate was readily apparent. As much as 30% of the total cyclohexadienyl accumulation in the culture supernatant can be L-arogenate (Table 1). A rough time course of metabolite accumulation was ascertained, and it was found that the time course of L-arogenate accumulation did not parallel that of prephenate accumulation. The initial formation of L-arogenate lagged behind that of prephenate. However, the rate of L-arogenate formation eventually exceeded that of prephenate, as indicated by the declining ratio of prephenate to arogenate as a function of accumulation time.

TABLE 1. Extracellular accumulation of prephenate and L-arogenate by the *S. typhimurium tyrA19* auxotroph

Accumulation time (h)	Accumulated concn (mM) of[a]:		Ratio[b]
	Prephenate	L-Arogenate	
6	0.30	0.07	4.3
9	0.60	0.21	3.0
20	1.00	0.40	2.5

[a] Average of two experiments, each based on an average of six flasks.
[b] Ratio of prephenate to L-arogenate.

The intracellular pools size of prephenate and L-arogenate were determined after 6 h of accumulation in the *tyrA19* mutant. The intracellular ratio of L-arogenate to prephenate was 8.0, a striking contrast to the extracellular ratio (Table 1). Thus, the expandable amino acid (L-arogenate) pool size exceeds the expandable keto acid (prephenate) pool size.

Variations of the accumulation protocol designed to increase the yield or rate of prephenate and/or L-arogenate accumulation in the culture supernatant were ineffective. These variations included provision of shikimate, L-glutamate, L-leucine, and Phe (singly or in combination). These manipulations had also proven to be ineffective in *Neurospora crassa* (19).

Prephenate aminotransferase in crude *Salmonella* extracts. The standard HPLC assay for prephenate aminotransferase in higher plants (9) involves the use of the substrate combination of prephenate and L-glutamate. Although the L-arogenate product coelutes with L-glutamate in the 60% methanol–buffer system used, the L-arogenate product is quantitatively converted to Phe at low pH. The peak area of Phe eluting at 12 min can then be measured without interference from L-glutamate eluting at 3 min. For crude extracts of *S. typhimurium*, the above assay is, however, seriously complicated by the presence of a very active prephenate dehydratase which (i) competes for the prephenate substrate and (ii) generates phenylpyruvate, which can transaminate with L-glutamate to form Phe. Although the HPLC column conditions can be changed (15% methanol–buffer system) to provide separated eluate peaks of L-glutamate and L-arogenate (thus measuring the L-arogenate product directly), analysis of each sample requires about 40 min (Fig. 2B).

This problem can be avoided by using Phe in place of L-glutamate as the amino donor substrate in combination with prephenate. Phe was as good as or better than L-glutamate, and the potentially interfering activity of prephenate dehydratase was prevented through the feedback inhibitory effect (100%) of Phe. Thus, relatively rapid assays (about 14 min with a retention time of about 2.3 min for L-arogenate) can be carried out with crude extracts (Fig. 2A). The specific activity of prephenate aminotransferase when 20 mM prephenate and 10 mM Phe were used was 0.72 nmol min^{-1} mg^{-1}. This is about 25-fold lower than the specific activities characteristic of plant cells (8). In crude extracts of *S. typhimurium*, Tyr, L-aspartate, and L-leucine will substitute for Phe as cosubstrates with prephenate to yield 100, 67, and 18%, respectively, of the activity found with Phe.

Whereas prephenate aminotransferase from higher plants saturates at about 0.8 mM prephenate (8, 9), even 20 mM prephenate was not saturating in *S. typhimurium*. Figure 3 illustrates this with substrate saturation curves obtained by using either Phe or L-glutamate as the amino donor substrate. It was estimated by extrapolation to V_{max} that about 3 M prephenate would be required for saturation.

Crude extracts prepared from wild-type *E. coli* W ATCC 9637 and from *K. pneumoniae* ATCC 25304 exhibited prephenate aminotransferase specific activities of 0.57 and 0.72 nmol min^{-1} mg^{-1}, respectively, when 20 mM prephenate was used in combination with 10 mM Phe. No appreciable differences were apparent in the properties of prephenate aminotransferase and the enzyme obtained from *S. typhimurium*.

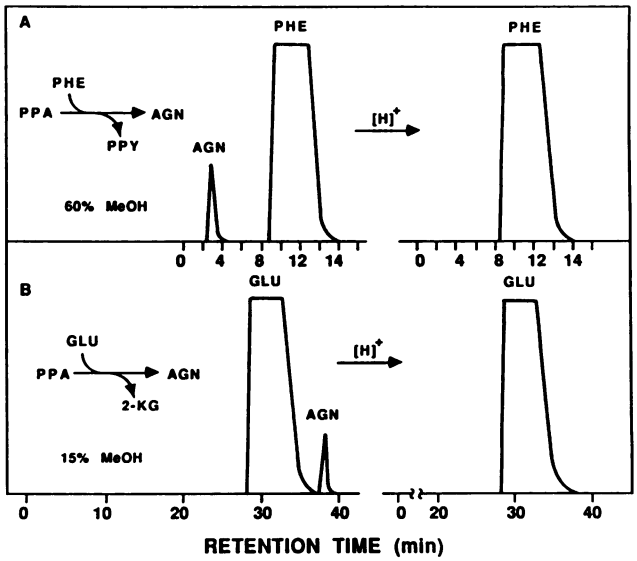

FIG. 2. HPLC identification of the L-arogenate (AGN) product from prephenate aminotransferase reaction mixtures in crude extracts from the *S. typhimurium tyrA19* mutant. (A) HPLC analysis of a prephenate aminotransferase reaction mixture containing the substrate combination shown at the upper left. When a 60% methanol-buffer system was used (see Materials and Methods), AGN eluted with a retention time of about 2.3 min and L-phenylalanine (PHE), the acid-catalyzed product of AGN, eluted with a retention time of about 8.6 min. (B) HPLC analysis of a prephenate aminotransferase reaction mixture containing the substrates shown at the lower left. A 15% methanol-buffer system was used to separate the AGN product from the substrate L-glutamate (GLU). Both AGN and GLU exhibited very late retention times under the conditions used. Both panels demonstrate the disappearance of AGN following acidification ($[H]^+$) of the reaction mixture. PHE, formed by acidification, was not eluted from the column (see Materials and Methods) under the conditions of low methanol used in the buffer system of this procedure.

Fractionation of aminotransferase activities. DEAE-cellulose (DE52) column chromatography was used to determine whether prephenate aminotransferase activity could be attributed to one or more of the aminotransferases of *S. typhimurium* corresponding to those that have been so well characterized in *E. coli* (14, 18). The elution positions of TRD, TRB, and TRA were readily determined by substrate preferences (Fig. 4A). The elution position of alanine-valine aminotransferase (TRC) was also determined, but the data are not shown since this enzyme lacked prephenate aminotransferase activity. TRB has significant activity when assayed as TRD, but does not overlap in substrate specificity with TRA. TRD functions very well when assayed as TRA and exhibits modest activity when assayed as TRB. On the other hand, TRA functions very well when assayed as TRD but exhibits negligible activity as TRB. TRA activity separated into two distinct peaks, perhaps reflecting complexing of a fractional portion of TRA with another enzyme. The substrate recognition properties of the two TRA species

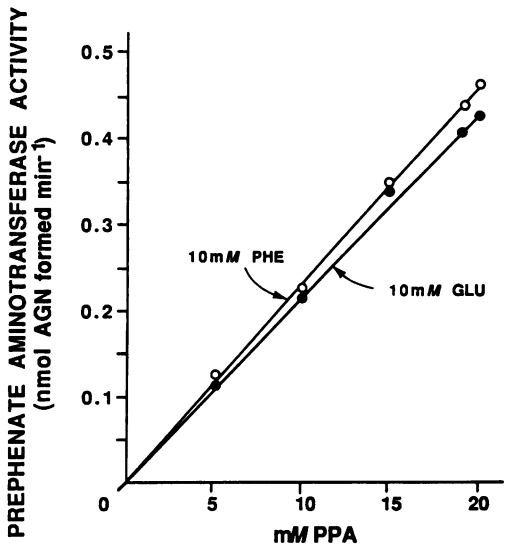

FIG. 3. Relationship of prephenate aminotransferase activity to substrate concentration in crude extract of the *S. typhimurium tyrA19* mutant, with either 10 mM L-phenylalanine (PHE) (○) or 10 mM L-glutamate (GLU) (●) as the amino donor substrate.

TABLE 2. Prephenate aminotransferase activities expressed in aminotransferase mutants of *E. coli*

Strain	Aminotransferase phenotype	Sp act (nmol/min/mg of extract protein) of:			
		TRA	TRD	TRB	PPA-AT[a]
DG27	$AspC^+$ $TyrB^-$ $IlvE^-$	449	402	3	1.2
DG34	$TyrB^+$ $AspC^-$ $IlvE^-$	28	119	27	0.2
DG44	$IlvE^+$ $AspC^-$ $TyrB^-$	0	21	56	0.7
DG30	$AspC^-$ $TyrB^-$ $IlvE^-$	0.4	0.8	0	0.0

[a] PPA-AT, Prephenate aminotransferase.

appeared to be identical. Each of the four aminotransferase activities separated was capable of prephenate transamination, as indicated by the correspondence of peak eluate positions in a comparison of Fig. 4A and B.

Salmonella aminotransferase mutants are not available, but a complete set of *E. coli* mutants has been isolated and characterized (14). Mutant constructions possessing only one of the three aminotransferases were analyzed (Table 2). The $AspC^+$ strain possesses a TRA having good activity as a TRD substitute, but not as a TRB substitute. The $TyrB^+$ strain possesses a TRD having significant activity as both TRA and TRB substitutes. The $IlvE^+$ strain possesses a TRB able to function as a TRD substitute but not as a TRA substitute. Each double mutant possesses prephenate ami-

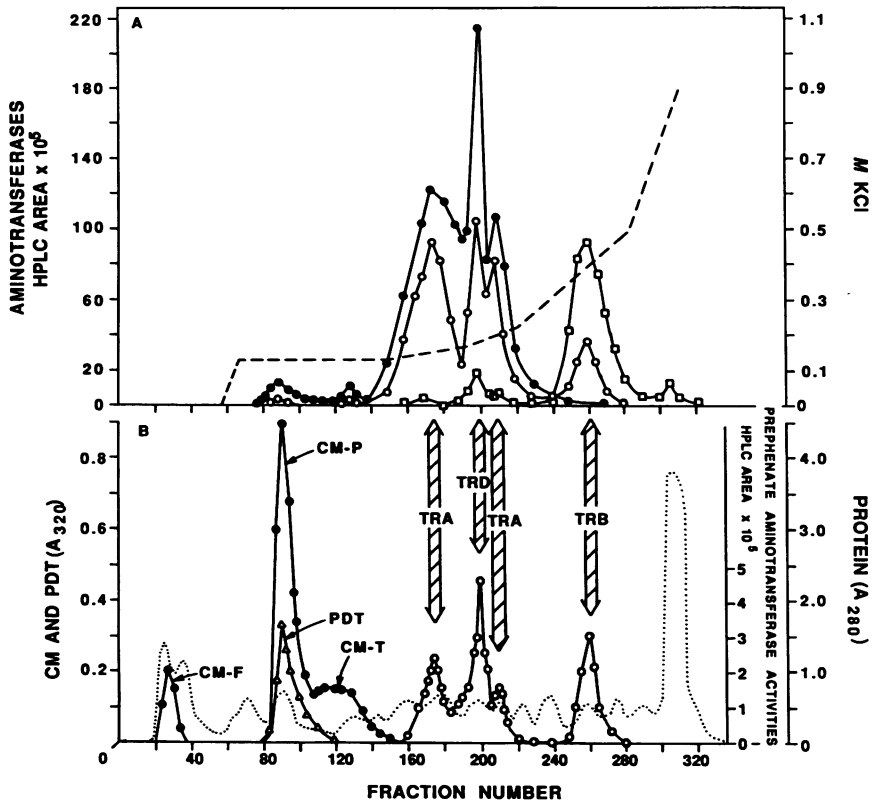

FIG. 4. Elution profiles of aromatic pathway enzymes following DEAE-cellulose column chromatography of crude extracts from the *S. typhimurium tyrA19* mutant. (A) Four aminotransferase peaks were located in the KCl gradient. TRA activity (●) was recovered in two bands peaking at fractions 176 and 210. TRD activity (○) peaked at fraction 198. TRB activity (□) peaked at fraction 260. (B) CM-F eluted in the wash fractions, while both CM-P and residual CM-T eluted in the gradient. Prephenate dehydratase (PDT) coeluted with CM-P, as expected for a bifunctional protein. The profile of prephenate aminotransferase activity (○) exhibited four peaks at fractions 176, 198, 210, and 260, exactly coincident with elution positions of TRA, TRD, and TRB shown in panel A.

notransferase activity, indicating the ability of TRA, TRD, and TRB to function as prephenate aminotransferase. The triple mutant DG30, in which TRA, TRD, and TRB are absent, exhibited total abolition of prephenate aminotransferase activity.

Figure 4B also shows the elution positions of other chorismate- and prephenate-utilizing enzymes in the *S. typhimurium tyrA19* mutant. The monofunctional CM-F eluted in the wash fractions. The two activities of the bifunctional chorismate mutase:prephenate dehydratase (CM-P:PDT) coeluted as expected. Although the *tyrA19* strain possesses a deficient bifunctional T protein (chorismate mutase:cyclohexadienyl dehydrogenase), a portion of the chorismate mutase (CM-T) activity is retained. Cyclohexadienyl dehydrogenase activity was not detected, consistent with the phenotype of tyrosine auxotrophy. A DE52 column fractionation was also carried out with wild-type *S. typhimurium* (ATCC 15277), using an extract prepared from a culture grown in minimal medium. Similar results (data not shown) were obtained to those from the *tyrA19* mutant (Fig. 4), except for the presence of a cyclohexadienyl dehydrogenase band at fraction 120. Its profile coincided with a larger peak of CM-T than that shown in Fig. 4, as expected.

Pathway construction in *Klebsiella* spp. Mutant 62-1 of *K. pneumoniae* (ATCC 25306) is the primary biotechnological source of chorismate. It lacks CM-P and CM-T isozymes of chorismate mutase owing to the mutant deficiencies imposed in the genes specifying the bifunctional P protein as well as the bifunctional T protein (1). Unlike *Salmonella* spp., the monofunctional CM-F isozyme of chorismate mutase has not been detected in *Klebsiella* spp. (5). Mutant 62-1 exhibits an absolute growth requirement for tyrosine, but is somewhat leaky for the phenylalanine requirement (Fig. 5). Although the prephenate dehydratase component of the P protein is absent via mutation, a very active prephenate dehydratase activity elutes in the wash fractions following DE52 chromatography. This is a CDT which can utilize prephenate (specific activity, 410 nmol min^{-1} mg^{-1}) or prephenyllactate (specific activity, 25 nmol min^{-1} mg^{-1}), but (unusually for CDT enzymes) not L-arogenate as a substrate. Since CDT would appear to provide sufficient prephenate dehydratase to accommodate normal production of Phe, the growth-limiting step in mutant 62-1 cultured in the presence of L-tyrosine and L-tryptophan must be conversion of chorismate to prephenate. Whether this is due to unstable or low levels of CM-F, CM-T or CM-P, or whether nonenzymatic (thermal) conversion of chorismate to prephenate might account for leaky growth is presently unknown.

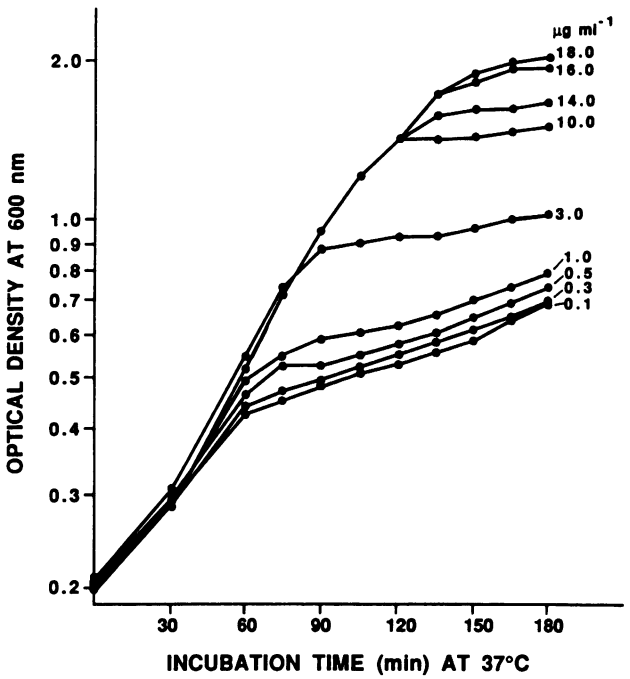

FIG. 5. Leaky growth requirement of *K. pneumoniae* 62-1 for L-phenylalanine. Replicate flasks were inoculated from a common culture of exponentially growing cells (A_{600} = 1.6) containing 50 μg of each aromatic amino acid ml^{-1} to a starting turbidity of about 0.2 at 600 nm. Each flask contained 25 μg of L-tyrosine and L-tryptophan ml^{-1} but differed in the yield-limiting concentration of L-phenylalanine present (numbers to the right of the family of growth curves). Culture turbidity was monitored with a Bausch & Lomb Spectronic 20, using 125-ml Erlenmeyer flasks fitted with sidearm tubes.

DISCUSSION

The ancestral pathway specifying aromatic biosynthesis in enteric bacteria (and still retained by organisms such as *Serratia* and *Erwinia* spp.) deploys multiple flow routes that lead from chorismate to Phe or to Tyr (Fig. 1). CM-F, unlike CM-P and CM-T, generates prephenate molecules that do not have fates fixed by channeling. The fate of prephenate molecules formed by CM-F is either conversion to Tyr via L-arogenate or conversion to Phe via either phenylpyruvate or L-arogenate. In *S. typhimurium* the absence of CDT leaves CM-F as a potential initial step to Tyr only. In the wild type this flow route undoubtedly operates at a very low level or not at all, primarily because the abilities of TRA, TRB, and TRD to catalyze the prephenate aminotransferase reaction are poor owing to low affinity for prephenate. Any prephenate molecules generated by CM-F are likely to be scavenged by the prephenate dehydratase or prephenate dehydrogenase components of the P protein or T protein, respectively.

In the *tyrA19* mutant the potential of CM-F and TRA, TRB, and/or TRD to form L-arogenate is dramatically unmasked. The absence of cyclohexadienyl dehydrogenase (by mutation) and the total feedback inhibition of prephenate dehydratase by exogenous PHE prevent scavenging of prephenate by these enzymes. The unphysiologically high levels of accumulated prephenate would markedly enhance its transamination by TRA, TRB, and/or TRD. The intracellular absence of 4-hydroxyphenylpyruvate or phenylpyruvate (normal keto acid substrates) might maximize the ability of TRD to generate L-arogenate in vivo. Another factor creating bias for TRD transamination of prephenate in vivo is that the protocol of starvation for Tyr which is used in the accumulation protocol derepresses TRD, the gene product of *tyrB*.

Prephenate can be regarded as a structural analog of 2-ketoglutarate, viewed across the top of the ring (Fig. 1). Since TRA, TRB, and TRD all transaminate prephenate much more nearly in proportion to their activities with 2-ketoglutarate than with 4-hydroxyphenylpyruvate, it appears that prephenate is accepted as a keto acid analog of 2-ketoglutarate, rather than as an analog of 4-hydroxyphenylpyruvate. Since *Salmonella* spp. do not coaccumulate *spiro*-arogenate or prephenyllactate, the formation of these in *Neurospora* spp. is probably enzymatic. Unless isolation of the latter cyclohexadienyl molecules is desired, the *Sal-*

monella system is preferable for isolation of prephenate or L-arogenate because of technical problems that potentially exist if prephenate or L-arogenate preparations are contaminated with *spiro*-arogenate or prephenyllactate. For obvious microbiological reasons, the bacterial system is a more efficient source of L-arogenate than is the fungal system. The fortuitous presence of the CM-F isozyme of chorismate mutase in the *tyrA19* mutant, an evolutionary remnant discarded by *E. coli*, probably contributes to the excellent accumulation of prephenate in *S. typhimurium*.

The singular accumulation of prephenate, without conversion to L-arogenate, might be desirable. If so, introduction of multiple deficiencies for *ilvE*, *tyrB*, and *aspC* in the *tyrA19* background would apparently be needed to abolish L-arogenate formation entirely. On the other hand, if enhanced L-arogenate production is desired, introduction of an efficient plant-type prephenate aminotransferase (8, 9) into the *tyrA19* background should vastly increase the ratio of L-arogenate to prephenate in the accumulation medium.

K. pneumoniae lacks CM-F, and therefore the catalytic potential of CDT is functionally isolated in the wild type, owing to the channeling of prephenate molecules generated by CM-P and CM-T. The *K. pneumoniae* CDT is unusual in its inability to use L-arogenate as a cyclohexadienyl substrate (5). Mutant 62-1 has a high potential to form Phe, provided an intracellular source of prephenate becomes available. It is quite possible that the well-known high frequency of reversion for the Phe requirement of mutant 62-1 in the presence of Tyr and Trp is due to restored expression of a cryptic gene for CM-F. It can also be appreciated now that it was no accident that in the sequential imposition of mutant blocks in mutant 62-1 (11), the tyrosine block preceded the phenylalanine block. In *Klebsiella* spp. the *pheA* deficiency will be masked by the presence of CDT, functioning in concert with the CM-T component of the bifunctional T protein. CM-T, in contrast to the dehydrogenase component, is virtually insensitive to feedback inhibition by L-tyrosine. When the tyrosine deficiency was imposed by Cotton and Gibson (11) via joint loss of the CM-T and dehydrogenase components of the T protein, a genetic background was created whereby a subsequently imposed *pheA* deficiency no longer exhibited a silent phenotype.

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LITERATURE CITED

1. **Ahmad, S., and R. A. Jensen.** 1986. The evolutionary history of two bifunctional proteins that emerged in the purple bacteria. Trends Biochem. Sci. **11**:108–112.
2. **Ahmad, S., and R. A. Jensen.** 1987. The prephenate dehydrogenase component of the bifunctional T-protein in enteric bacteria can utilize L-arogenate. FEBS Lett. **216**:133–139.
3. **Ahmad, S., and R. A. Jensen.** 1988. New prospects for deducing the evolutionary history of metabolic pathways in prokaryotes: aromatic biosynthesis as a case-in-point. Origins Life **18**:41–57.
4. **Ahmad, S., and R. A. Jensen.** 1988. The stable evolutionary fixation of a bifunctional tyrosine-pathway protein in enteric bacteria. FEMS Microbiol. Lett. **52**:109–116.
5. **Ahmad, S., and R. A. Jensen.** 1988. Phylogenetic distribution of components of the overflow pathway to L-phenylalanine within the enteric lineage of bacteria. Curr. Microbiol. **16**:295–302.
6. **Ahmad, S., and R. A. Jensen.** 1988. The phylogenetic origin of the bifunctional tyrosine-pathway protein in the enteric lineage of bacteria. Mol. Biol. Evol. **5**:282–297.
7. **Berry, A., and R. A. Jensen.** 1988. Biochemical evidence for phylogenetic branching patterns: Biochemical-pathway characteristics can indicate points of divergence among prokaryotes. BioScience **38**:99–103.
8. **Bonner, C. A., and R. A. Jensen.** 1985. Novel features of prephenate aminotransferase from cell cultures of *Nicotiana silvestris*. Arch. Biochem. Biophys. **238**:237–246.
9. **Bonner, C. A., and R. A. Jensen.** 1987. Prephenate aminotransferase. Methods Enzymol. **142**:479–487.
10. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
11. **Cotton, R. G. H., and F. Gibson.** 1965. The biosynthesis of phenylalanine and tyrosine enzymes converting chorismic acid into prephenic acid and their relationships to prephenate dehydratase and prephenate dehydrogenase. Biochim. Biophys. Acta **100**:76–88.
12. **Dayan, J., and D. B. Sprinson.** 1970. Preparation of prephenic acid. Methods Enzymol. **18**:559–561.
13. **Fischer, R., and R. A. Jensen.** 1987. Prephenate dehydratase (monofunctional). Methods Enzymol. **142**:507–512.
14. **Gelfand, D. H., and R. A. Steinberg.** 1977. *Escherichia coli* mutants deficient in the aspartate and aromatic amino acid aminotransferases. J. Bacteriol. **130**:429–440.
15. **Gibson, F.** 1964. Chorismic acid. Purification and some chemical and physical studies. Biochem. J. **90**:256–261.
16. **Jensen, R. A.** 1985. Biochemical pathways can be traced backward through evolutionary time. Mol. Biol. Evol. **2**:92–108.
17. **Jensen, R. A.** 1986. Tyrosine and phenylalanine biosynthesis: relationship between alternative pathways, regulation and subcellular location. Recent Adv. Phytochem. **20**:57–82.
18. **Jensen, R. A., and D. H. Calhoun.** 1981. Intracellular roles of microbial aminotransferases: overlap enzymes across different biochemical pathways. Crit. Rev. Microbiol. **8**:229–266.
19. **Jensen, R. A., L. O. Zamir, M. St. Pierre, N. Patel, and D. L. Pierson.** 1977. Isolation and preparation of pretyrosine, accumulated as a dead-end metabolite by *Neurospora crassa*. J. Bacteriol. **132**:896–903.
20. **Koch, G. L. E., D. C. Shaw, and F. Gibson.** 1970. Tyrosine biosynthesis in *Aerobacter aerogenes*: purification and properties of chorismate mutase-prephenate dehydrogenase. Biochim. Biophys. Acta **212**:375–386.
21. **Koch, G. L. E., D. C. Shaw, and F. Gibson.** 1971. The purification and properties of chorismate mutase-prephenate dehydrogenase. Biochim. Biophys. Acta **229**:795–804.
22. **Nishioka, M., M. Demerec, and A. Eisenstark.** 1967. Genetic analysis of aromatic mutants of *Salmonella typhimurium*. Genetics **56**:341–351.
23. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–102.
24. **Weiss, V., and J. M. Edwards.** 1980. The biosynthesis of aromatic compounds. John Wiley & Sons, Inc., New York.
25. **Zamir, L. O., R. A. Jensen, B. Arison, A. Douglas, G. Albers-Schonberg, and J. R. Bowen.** 1980. Structure of arogenate (pretyrosine), an amino acid intermediate of aromatic biosynthesis. J. Am. Chem. Soc. **102**:4499–4504.
26. **Zamir, L. O., E. Jung, and R. A. Jensen.** 1983. Co-accumulation of prephenate, L-arogenate, and spiro-arogenate of *Neurospora*. J. Biol. Chem. **258**:6492–6496.
27. **Zamir, L. O., R. Tiberio, K. A. Devor, F. Sauriol, S. Ahmad, and R. A. Jensen.** 1988. Structure of D-prephenyllactate: a carboxycyclohexadienyl metabolite from *Neurospora crassa*. J. Biol. Chem. **263**:17284–17290.
28. **Zamir, L. O., R. Tiberio, M. Fiske, A. Berry, and R. A. Jensen.** 1985. Enzymatic and nonenzymatic dehydration reactions of L-arogenate. Biochemistry **24**:1607–1612.