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Phylogenetic Distribution of Components of the Overflow Pathway to L-Phenylalanine within the Enteric Lineage of Bacteria[1]

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Abstract. The enteric lineage of prokaryotes (traditional enteric bacteria, *Aeromonas,* and *Alteromonas*) encompasses closely related genera that share many common character states of aromatic amino acid biosynthesis. For example, they uniformly employ the tightly regulated bifunctional P-protein (chorismate mutase : prephenate dehydratase) to form L-phenylalanine via phenylpyruvate. A second, unregulated pathway to phenylalanine, originally termed the overflow pathway in *Pseudomonas aeruginosa,* consists of a monofunctional chorismate mutase (CM–F) and a cyclohexadienyl dehydratase. The evolution of the overflow pathway has been dynamic in the enteric lineage. *Serratia marcescens, Erwinia herbicola, Erwinia amylovora,* and several other *Erwinia* species possess an intact pathway. *Salmonella, Klebsiella,* and *Erwinia carotovora* possess an incomplete overflow pathway, while *Escherichia, Proteus, Aeromonas,* and *Alteromonas* lack it altogether.

Microorganisms vary widely in their mode of L-phenylalanine biosynthesis. In *Bacillus subtilis* and other Gram-positive bacteria, L-phenylalanine biosynthesis occurs solely via phenylpyruvate, prephenate dehydratase being stimulated by remote effectors (hydrophobic amino acids), and feedback inhibited by L-phenylalanine [7, 17, 27, 28]. *Escherichia coli* and *Salmonella typhimurium* also use phenylpyruvate as the immediate precursor of L-phenylalanine, but a bifunctional protein (chorismate mutase : prephenate dehydratase) is utilized, both catalytic activities of this "P-protein" being feedback inhibited by L-phenylalanine [11, 13, 15]. The P-protein is distributed throughout Superfamily B (gamma subdivision [35]) and probably throughout Superfamily A (beta subdivision [34]) as well [1]. Phenylpyruvate was thought to be a universal precursor for phenylalanine synthesis until the discovery of L-arogenate [29, 36]. The arogenate pathway, operating via a specific arogenate dehydratase, is the apparent sole route to phenylalanine in *Pseudomonas diminuta* (a Superfamily C pseudomonad) [37], *Euglena gracilis* [9], and in higher plants [23].

Patel et al. [26] reported results showing *Pseudomonas aeruginosa* to exemplify what has proven to be a common biochemical arrangement whereby dual pathways to phenylalanine (and tyrosine) co-exist (Fig. 1). In *P. aeruginosa, Xanthomonas campestris,* and most other members of Superfamily B, a broad specificity cyclohexadienyl dehydratase (CDT) that utilizes both prephenate and L-arogenate as substrates coexists with the P-protein [10, 26, 32]. The P-protein dehydratase was tightly feedback-inhibited by L-phenylalanine while the CDT enzyme was not regulated in these microorganisms.

All Superfamily B organisms (*Acinetobacter* species, Group I/Group V pseudomonads, *Oceanospirillum* species, and the enteric lineage of bacteria) possess the chorismate mutase-P (CM-P) component of the bifunctional P-protein and either a monofunctional chorismate mutase (CM-F) or the chorismate mutase-T (CM-T) component of the bifunctional T-protein (chorismate mutase : prephenate dehydrogenase) [1, 22]. It was previously assumed that the T-protein evolved following side-by-side fusion of ancestral genes encoding CM-F and prephenate dehydrogenase activities [22]. This demands the absence of CM-F in organisms with an evolved T-protein. However, the simulta-

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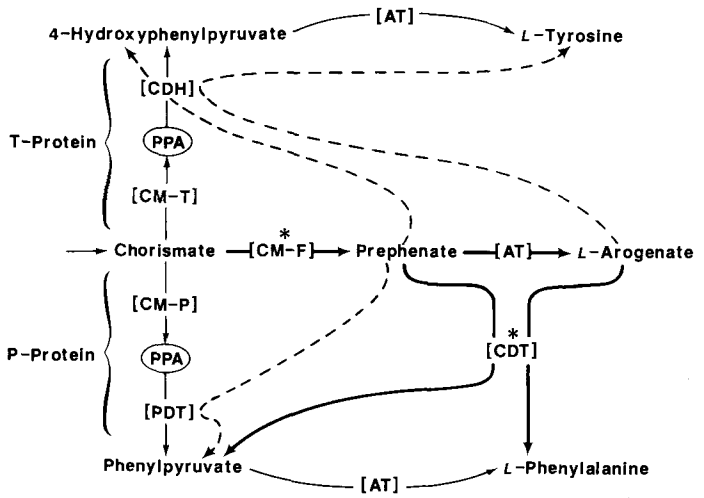

Fig. 1. Post-chorismate enzymes of tyrosine and phenylalanine biosynthesis within the enteric lineage of Superfamily-B bacteria. Within this group both the P-protein and the T-protein are inevitably present. Chorismate mutase-F [CM-F] and cyclohexadienyl dehydratase [CDT], highlighted with asterisk symbols, may be present or absent in any combination. [AT] denotes an unknown number of aminotransferases having overlapping substrate specificities and able to transaminate prephenate, phenylpyruvate, or 4-hydroxyphenylpyruvate in vitro. Five such aminotransferases have been demonstrated [31] in *Pseudomonas aeruginosa,* a Superfamily-B organism that lies outside of the enteric lineage. Prephenate molecules (PPA) formed by chorismate mutase-T [CM-T] are channeled to 4-hydroxyphenylpyruvate via the action of the cyclohexadienyl dehydrogenase (CDH) component of the T-protein. The broad specificity of this previously named prephenate dehydrogenase has recently been demonstrated [4]. Prephenate formed by chorismate mutase-P [CM-P] is channeled to phenylpyruvate through the action of the prephenate dehydratase [PDT] component of the P-protein. Prephenate molecules formed by the monofunctional chorismate mutase species [CM-F] are fated for ultimate transformation to L-tyrosine (mediated by [CDH]) or to L-phenylalanine via either phenylpyruvate (mediated by either [CDT] or [PDT]) or L-arogenate (mediated by [CDT]). For catalytic steps that include and follow [CM-F], feedback-sensitive reactions are indicated by dashed arrows, whereas unregulated steps that define overflow sequences are shown with heavy arrows.

neous presence of CM-F, CM-P, and CM-T in *Serratia marcescens* and some other enteric bacteria has led to more complicated explanations [2, 3].

The distribution of CM-F and CDT within the enteric lineage has been determined. All combinations of presence or absence of these two enzymes occur within the enteric lineage of bacteria, in contrast to the uniformity of other character states of aromatic biosynthesis. These studies and others [16] illustrate how information obtained at the gene–product level (which depends upon sequencing data obtained at the nucleic acid level) provides evolutionary conclusions that cannot be reached by molecular-genetic techniques alone.

Materials and Methods

Organisms and growth conditions. *Klebsiella pneumoniae* ATCC 25304, *Salmonella typhimurium* ATCC 15277, *Erwinia carotovora* ATCC 15713, *E. herbicola* ATCC 33243, *E. amylovora* ATCC 15580, *E. milletiae* ATCC 33261, *E. chrysanthemi* ATCC 11663, *E. tracheiphila* ATCC 33245, *Serratia marcescens* ATCC e13880, *Proteus mirabilis* ATCC 29906, *Aeromonas hydrophila* ATCC 14715, and *Alteromonas putrefaciens* ATCC 8071 were obtained from the American Type Culture Collection (Rockville, Maryland). *Klebsiella pneumoniae* (37°C), *S. typhimurium* (37°C), *E. carotovora* (26°C), *E. herbicola* (26°C), *S. marcescens* (30°C), and *A. hydrophila* (28°C) were grown on M9 medium as described by Winkler and Stuckman [33]. *Proteus mirabilis* (37°C) was grown on M9 medium containing 1 mg/liter each of nicotinamide, *p*-aminobenzoate, calcium pantothenate, and thiamine. *Erwinia amylovora* was grown at 26°C, as described by Miller and Schroth [25]; *E. milletiae, E. chrysanthemi,* and *E. tracheiphila* were grown at 26°C in 0.8% nutrient broth (Difco). *Alteromonas putrefaciens* was grown at 30°C in basal medium containing half-strength artificial sea water and supplemented with 0.2% (wt/vol) glucose as described by Baumann et al. [6]. The organisms were grown to the late exponential phase of growth, harvested by centrifugation, washed twice with 50 m*M* K phosphate buffer (pH 7.0) containing 1 m*M* dithiothreitol (DTT) (buffer A), and stored at −80°C until used.

Preparation of cell extracts and enzyme assay. Cell pellets were suspended in buffer A, disrupted by sonication, and centrifuged at 150,000 *g* for 1 hr. The resulting supernatant was passed through a Sephadex G-25 column (1.5 × 20.0 cm) equilibrated either in buffer A or 20 m*M* K phosphate (pH 7.0) containing 1 m*M* DTT (buffer B). The protein-containing fractions were pooled and are termed crude extract.

Chorismate mutase was assayed by the method of Cotton and Gibson [11]. The reaction mixture in a final volume of 0.2 ml contained buffer A, 1 m*M* K chorismate, and suitable amount of enzyme. Incubation at 37°C was carried out for 20 min, and then 0.1 ml of 1 *N* HCl was added and incubated at 37°C for 15 min to

convert the prephenate formed to phenylpyruvate. Phenylpyruvate was measured at 320 nm after the addition of 0.7 ml of 2.5 *N* NaOH. An extinction coefficient of 17,500 was used for calculations [11].

Prephenate dehydratase was assayed by the method of Cotton and Gibson [11]. The reaction mixture in a final volume of 0.2 ml contained buffer A, 1 m*M* K prephenate, and a suitable amount of enzyme. After incubation at 37°C for 20 min, 0.8 ml of 2.5 *N* NaOH was added, and the absorbance was read at 320 nm.

Arogenate dehydratase activity was assayed by measurement of phenylalanine formation as described by Zamir et al. [37]. The reaction mixture in a final volume of 0.2 ml contained buffer A, 1 m*M* K arogenate, and a suitable amount of enzyme. After incubation at 37°C for 20 min, 0.05 ml of 0.5 *N* NaOH was added, and the phenylalanine formed was estimated by high performance liquid chromatography (HPLC) as described by Lindroth and Mopper [24].

Prephenate dehydrogenase was assayed as described by Patel et al. [26]. The reaction mixture in a final volume of 0.2 ml contained buffer A, 0.5 m*M* nicotinamide adenine dinucleotide (NAD^+), and 1 m*M* K prephenate. The reaction was started by the addition of substrate, and the continuous formation of NADH was followed spectrophotofluorometrically (excitation at 340 nm, emission at 460 nm).

Protein in the crude extract was estimated by the method of Bradford [8] with bovine serum albumin as the standard protein.

DE52 column chromatography. Approximately 100 mg of crude extract protein was applied to a DEAE–cellulose (DE52) column (1.5 × 20.0 cm) equilibrated in buffer A or buffer B as indicated. The column was washed with two bed volumes of the equilibration buffer, and then the bound proteins were eluted with 300 ml of a linear gradient of KCl (0.0–0.35 *M*) contained in the equilibration buffer. Fractions of 2.2 ml collected were assayed for absorbance at 280 nm and for chorismate mutase, prephenate dehydratase, prephenate dehydrogenase, and arogenate dehydratase activities. Fractions containing the enzyme activity were tested for sensitivity to inhibition by aromatic amino acids.

Hydroxylapatite chromatography. Appropriate fractions eluted from the DE52 column were pooled, concentrated to 4 ml by ultrafiltration on a PM-10 membrane (Amicon Corp.), and dialyzed to 10 m*M* K phosphate (pH 7.0) containing 1 m*M* DTT (buffer C). The concentrated sample was applied to a bio-gel hydroxylapatite (1.0 × 9.0 cm) column equilibrated with buffer C. After washing of the column, the bound proteins were eluted with a 200-ml linear gradient of 0.01 to 0.3 *M* K phosphate (pH 7.0) containing 1 m*M* DTT. Fractions of 2.2 ml were collected and assayed for absorbance at 280 nm and enzyme activities.

Biochemicals and chemicals. Amino acids, bovine serum albumin, NAD^+, DTT, and Sephadex G-25 were obtained from Sigma Chemical Co. (St. Louis, Missouri). DE52 was purchased from Whatman, Inc. (New Jersey). Hydroxylapatite (HTP) was obtained from Bio-Rad Laboratories (Richmond, California). Prephenate was prepared as the barium salt from culture supernatants of a tyrosine auxotroph of *S. typhimurium* [14] and was converted to the potassium salt before use. Chorismate was isolated from the accumulation medium of a triple auxotroph (62-1) of *K. pneumoniae* and purified as the free acid [20]. L-Arogenate was prepared from a triple auxotroph of *Neurospora crassa* (ATCC 36373) as previously described [36]. All other chemicals were standard reagent grade.

Results

Phenylalanine-branch enzymes in *Serratia marcescens*. The elution profiles following DE52 chromatography of the crude extract of the enzymes participating in the biosynthesis of L-phenylalanine from *S. marcescens* are shown in Fig. 2. Three peaks of chorismate mutase were resolved. One peak of activity washed through the column and was not associated with any other enzyme activity of aromatic biosynthesis (top panel). This monofunctional species of chorismate mutase has been denoted CM-F. Of the two chorismate mutase species eluting in the gradient fractions, the leading peak co-eluted with the prephenate dehydratase peak of activity (top panel), whereas the trailing peak co-eluted with prephenate dehydrogenase activity (data not shown). Hence, the latter two isozymes are CM-P and CM-T components of the bifunctional P-protein and bifunctional T-protein, respectively.

Two peaks of prephenate dehydratase were resolved (bottom panel, Fig. 2). One species co-eluted with CM-P and is a component of the P-protein. The second peak of dehydratase activity could utilize L-arogenate as an alternative substrate, thus being CDT.

Enzyme patterns in Erwinia species. Three chorismate mutase isozymes were resolved following DE52 chromatography of the crude extract from *E. herbicola* (Fig. 3). Although CM-F co-eluted with CDT (see bottom panel), the two enzymes separated completely following a second purification step of hydroxylapatite chromatography (data not shown). Two peaks of chorismate mutase activity eluted in the gradient fractions (Fig. 3). Isozymes CM-P and CM-T were recognized by the coincidence of their elution profiles with those of prephenate dehydratase and prephenate dehydrogenase, respectively.

Two peaks of prephenate dehydratase activity were separated. The dehydratase species that washed through the column utilized L-arogenate as an alternative substrate, thus being CDT. The second peak of activity was found to be the prephenate-specific dehydratase component of the bifunctional P-protein.

Similar results were obtained from *Erwinia amylovora*, *E. milletiae*, and *E. chrysanthemi* (data not shown). *E. carotovora* differed from the other *Erwinia* species in having three chorismate mutase isozymes, as shown in Fig. 3 (top panel), but only a single peak of prephenate dehydratase activity was

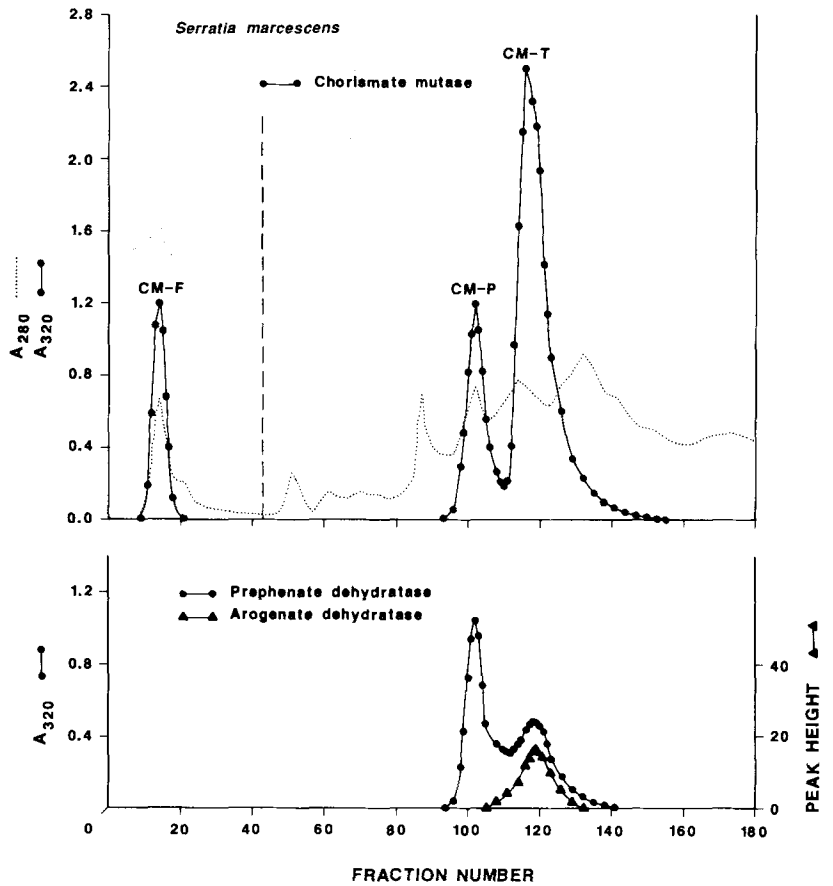

Fig. 2. Elution profiles of chorismate mutase isozymes (top panel) and of prephenate dehydratase and arogenate dehydratase activities (bottom panel) following DE52-column chromatography of crude extract prepared from *Serratia marcescens*. The enzyme eluting at fraction 118 is CDT. The DE52 column was equilibrated and developed in buffer B. The vertical dashed line indicates the onset point of gradient elution. Chorismate mutase and prephenate dehydratase activities are expressed as phenylpyruvate absorbance in base at 320 nm. CDT activity is expressed as peak height of L-phenylalanine formed (calculated from HPLC data). The distribution of proteins (as monitored by absorbance at 280 nm) is shown by the broken line (top panel).

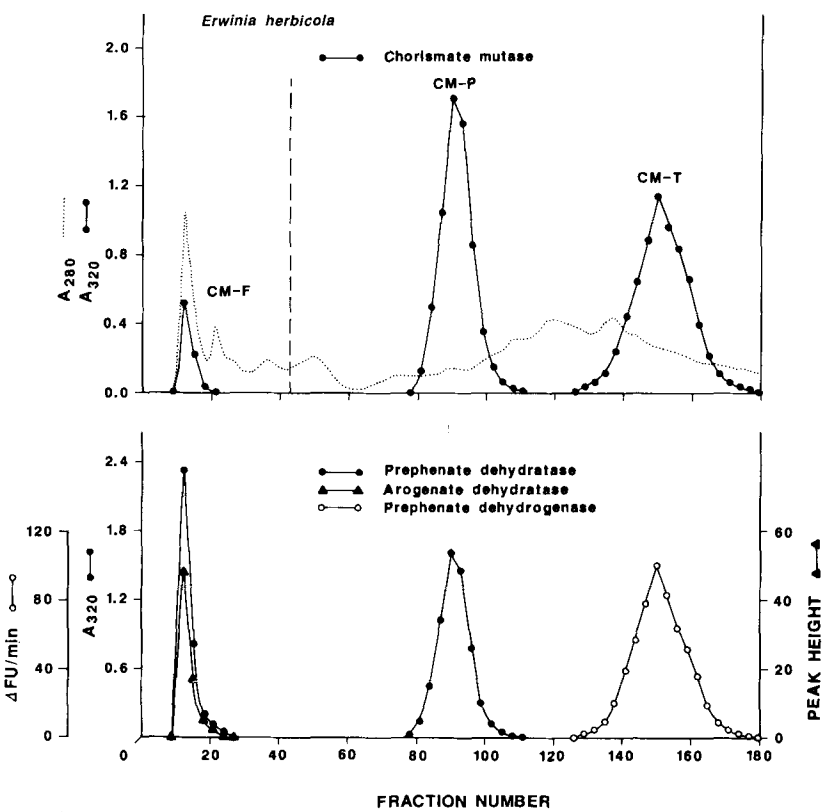

Fig. 3. Elution profiles of chorismate mutase isozymes (top panel) and of prephenate dehydratase, arogenate dehydratase, and prephenate dehydrogenase activities (bottom panel) following DE52-column chromatography of crude extract prepared from *Erwinia herbicola*. The DE52 column was equilibrated and developed in buffer A. The broad-specificity dehydratase eluting in the wash fractions is CDT. Prephenate dehydrogenase activity shown is the property of a broad-specificity cyclohexadienyl dehydrogenase (CDH) (activity with L-arogenate not shown). The symbols used and other details are the same as in Fig. 2.

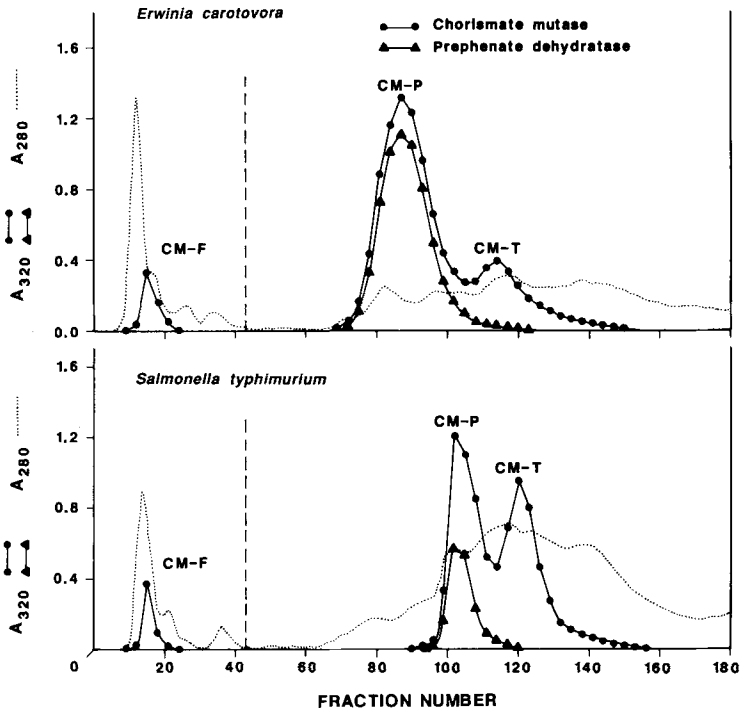

Fig. 4. Elution profiles of chorismate mutase and prephenate dehydratase activities following DE52-column chromatography of crude extract prepared from *Erwinia carotovora* (top panel) and *Salmonella typhimurium* (bottom panel). The DE52 column was equilibrated and developed in buffer A (top panel) and in buffer B (bottom panel). Four times more column eluate was used for the assay of CM-F than for assay of CM-P and CM-T in *S. typhimurium* (bottom panel). See Fig. 2 for other details.

recovered, this proving to be the P-protein dehydratase (Fig. 4, top panel). CDT activity was not detected in either crude extract or in the DE52 column fractions from *E. carotovora*.

Enzyme patterns in *Salmonella typhimurium*. Three peaks of chorismate mutase activity eluted in column fractions following DE52 chromatography of the crude extract from *S. typhimurium* (Fig. 4, bottom panel). The CM-P and CM-T activities eluting in the gradient fractions were associated with prephenate dehydratase activity (Fig. 4, bottom panel) and prephenate dehydrogenase activity (data not shown), respectively, as has already been reported [15]. However, the activity that washed through the column without retardation was not associated with any other activity of aromatic biosynthesis, and this CM-F isozyme has not been previously reported.

Enzyme patterns in *Proteus mirabilis* and *Aeromonas hydrophila*. As shown in Fig. 5, only two peaks of activity for chorismate mutase eluted in column fractions following DE52 chromatography of the crude extracts prepared from *P. mirabilis* (top panel) and from *A. hydrophila* (bottom panel). In each case, one isozyme of chorismate mutase (CM-P) eluted with exact coincidence with prephenate dehydratase activity, whereas the second isozyme (CM-T) co-eluted with prephenate dehydrogenase (data not shown). The prephenate dehydratase enzyme could not use L-arogenate as an alternative substrate. CDT activity was not detected in either the crude extracts or DE52-column fractions from *P. mirabilis* or *A. hydrophila*. Similar results were obtained from *A. putrefaciens* (data not shown).

Regulation of mutase and dehydratase activities. The sensitivities of chorismate mutase isozymes to inhibition by aromatic amino acids are shown in Table 1. With each organism studied, CM-F (if present) and CM-T were insensitive to allosteric effects in the presence of individual aromatic amino acids or any combination thereof. In contrast, the CM-P species was inhibited by L-phenylalanine.

The sensitivity of the dehydratase enzymes to inhibition by aromatic amino acids is shown in Table 2. Prephenate dehydratase-P was tightly regulated by L-phenylalanine. In contrast, CDT was not inhibited by L-phenylalanine or any other aromatic amino acid (whether assayed with prephenate or L-arogenate as a substrate). Effects of L-tyrosine and/or L-tryptophan, commonly found even with monofunctional prephenate dehydratase [7, 17, 27, 28] are of interest for further study.

Discussion

Dynamic evolution of phenylalanine biosynthesis. Two aromatic-pathway character states within Superfamily-B Gram-negative bacteria have evolved only recently, these being acquisition of the 3-de-

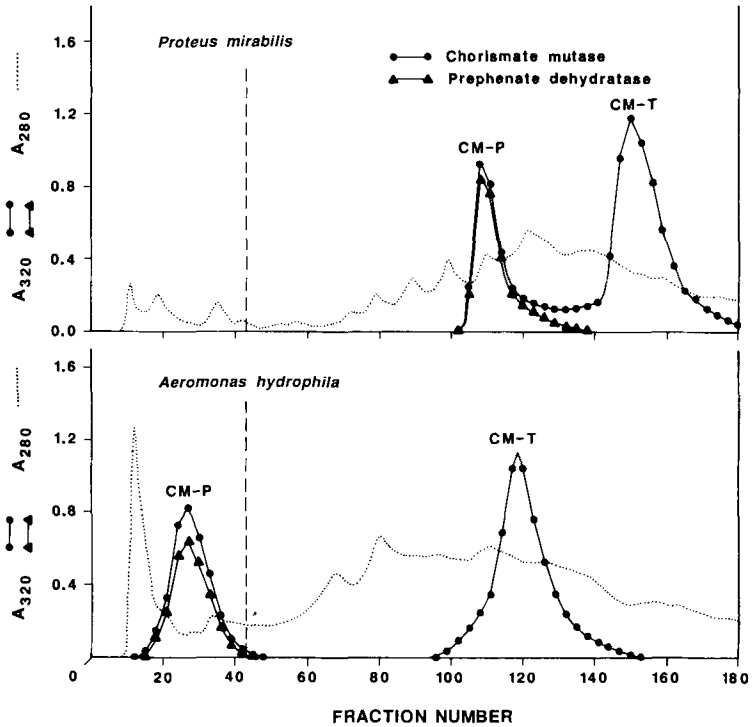

Fig. 5. Elution profiles of chorismate mutase and prephenate dehydratase activities following DE52-column chromatography of crude extracts prepared from *Proteus mirabilis* (top panel) and *Aeromonas hydrophila* (bottom panel). The DE52 column was equilibrated and developed in buffer B (top panel) and buffer A (bottom panel). See legend of Fig. 2 for explanation of symbols and other details.

Table 1. Inhibition of chorismate mutase isozymes[a] by aromatic amino acids

Organism	% Inhibition[b] of chorismate mutase-P by			
	L-Phenyl-alanine	L-Tyro-sine	L-Trypto-phan	Aro
Serratia marcescens	50	0	0	50
Erwinia herbicola	22	0	2	23
Erwinia carotovora	38	1	2	35
Proteus mirabilis	47	0	0	46
Aeromonas hydrophila	49	2	0	47

[a] Chorismate mutase isozymes were recovered from DE52 column fractions (refer to Figs. 2–5). Chorismate mutase-F was not detected in *P. mirabilis* and *A. hydrophila*. Neither chorismate mutase-F nor chorismate mutase-T was inhibited by any of the aromatic acids or combinations thereof (data not shown).
[b] The final concentration of aromatic amino acids added individually or in combination (Aro) was 0.5 m*M*.

Table 2. Inhibition of prephenate dehydratase by aromatic amino acids

Organism	% Inhibition[a] of prephenate dehydratase-P[b] by			
	L-Phenyl-alanine	L-Tyro-sine	L-Trypto-phan	Aro
Serratia marcescens	86	+10	5	85
Erwinia herbicola	55	29	21	61
Erwinia carotovora	74	43	54	89
Proteus mirabilis	80	+ 8	+5	76
Aeromonas hydrophila	85	+15	+4	79

[a] The final concentration of aromatic amino acids, individually or in combination (Aro) was 0.5 m*M*. The + symbol denotes activation.
[b] Enzyme activities were recovered from DE52 column fractions (refer to column profiles shown in Figs. 2–5). CDT activity was not inhibited by aromatic amino acids (data not shown).

oxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase-phe isozyme [5] and of the bifunctional T-protein [3]. This distinctive phylogenetic cluster containing traditional enteric genera plus *Aeromonas* and *Alteromonas* is referred to as the enteric lineage. Within this lineage, character states of aromatic biosynthesis are uniform except for the overflow pathway of phenylalanine biosynthesis (Fig. 1). The organisms studied can be grouped into four classes, which represent all possible permutations of presence or absence of CM-F and CDT (Fig. 6).

Alteromonas, Aeromonas, Proteus, and *Escherichia* all lack both CM-F and CDT. Figure 6 depicts the conclusion that these were initially lost

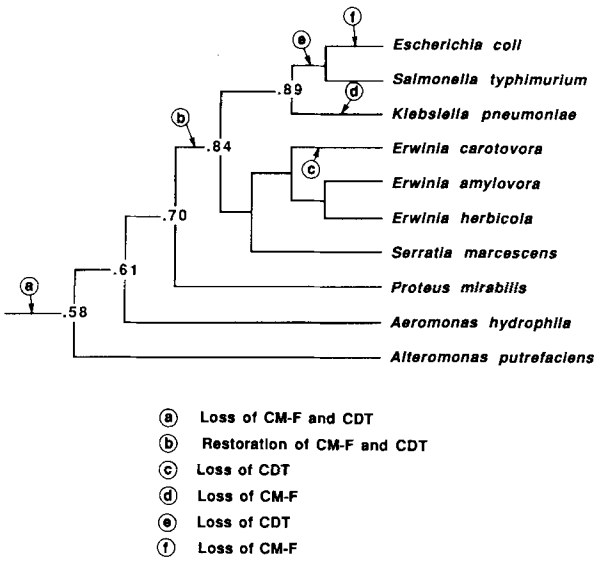

Fig. 6. Dendrograms depicting hypothetical evolutionary events of phenylalanine biosynthesis within the enteric lineage. The enteric lineage is a phylogenetic cluster within Superfamily B defined by the presence of the DAHP synthase-phe isozyme and by the presence of the bifunctional T-protein [3, 5]. The order of phylogenetic branching shown is based upon results of oligonucleotide catalogs (numbers shown are S_{AB} values, ref. [19]). A plausible arrangement of *Erwinia* species, based upon our data and consistent with other results [30], is shown. The common ancestor of the entire assemblage shown is assumed to have possessed genes for CM-F and CDT of the overflow pathway, since these are widespread within the larger Superfamily-B cluster [22]. Events of evolutionary loss or gain are indicated by circled letters explained at the bottom of the figure.

(event *a*) and then regained (event *b*). Thus, *Escherichia coli* is pictured with a genealogical history of having lost CM-F and CDT twice. The scenario shown is slightly more probable than the less parsimonious conclusion that the set of character states were lost as independent events in all four lineages. One wonders if joint loss and gain of genes encoding CM-F and CDT implicate linked cistrons. Joint loss of CM-F and CDT has been noted elsewhere in Superfamily B [2]. It will be interesting to see whether genes for CM-F and CDT are linked in contemporary Superfamily-B organisms such as *Pseudomonas aeruginosa* or *Serratia marcescens*.

The dual pathways to phenylalanine and to tyrosine originally demonstrated in *P. aeruginosa* [26] are present in *S. marcescens* and all species of *Erwinia* (except for *E. carotovora*). The loss of CDT as a single event in a common ancestor of both *S. typhimurium*/*E. coli* and *E. carotovora* would place *E. carotovora* nearer than *K. pneumoniae* to *S. typhimurium*/*E. coli*. Such a phylogenetic placement is highly unlikely on a variety of criteria, a good illustration being the presence of the *trp*(G)D fusion in the *Escherichia*/*Salmonella*/*Klebsiella* cluster, but not in *E. carotovora* [12]. It is of interest that auxotrophic mutants for phenylalanine were readily isolated from *E. carotovora* [21], a result that would not be expected of other *Erwinia* species possessing dual pathways to phenylalanine.

Although *Salmonella* and *Klebsiella* are closely related to *Escherichia*, the former differ from *E. coli* in their possession of a portion of the ancestral overflow pathway. The presence of the CM-F species in *Salmonella* has been overlooked in other studies [15]. This activity probably contributes significantly to the excellent accumulation of prephenate by a tyrosine auxotroph [14]. *Klebsiella* lacks CM-F but possesses CDT. This CDT is in fact synonymous with an unregulated species of prephenate dehydratase originally denoted prephenate dehydratase-A by Cotton and Gibson [11]. It differs from other CDT enzymes in not being able to utilize L-arogenate as a substrate. However, its ability to utilize prephenyllactate (prephenate having a lactyl sidechain) as an alternative substrate indicates it to be a cyclohexadienyl dehydratase, since other CDT enzymes (but not prephenate dehydratase) utilize prephenyllactate (S. Ahmad, unpublished data).

Physiological impact of CM-F and CDT. The overflow pathway has been studied in *P. aeruginosa* [18]. It appears to function primarily under conditions of carbon-source nutrition and early-pathway deregulation that produce elevated intracellular levels of chorismate. Although prephenate molecules generated by CM-F can potentially generate tyrosine as well as phenylalanine (see Fig. 1), overflow to phenylalanine is the consequence of CDH, but not CDT, being tightly feedback inhibited.

The presence of CM-F in the absence of CDT (e.g., *Salmonella*) or the presence of CDT in the absence of CM-F (e.g., *Klebsiella*) presumably is of little utility to the wild-type organism. However, under conditions of either CM-P or CM-T deficiency, one would expect compensatory catalysis (suppression) by CM-F. Likewise, under conditions of prephenate dehydratase deficiency, one would expect compensatory catalysis by CDT.

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