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The dynamic progression of evolved character states for aromatic amino acid biosynthesis in Gram-negative bacteria

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A systematic analysis of the evolution of aromatic amino acid biosynthesis in the Proteobacteria, previously focussed mainly upon the γ subdivision, has now been extended to the β subdivision. Five lineages were studied, represented by *Neisseria gonorrhoeae*, *Nitrosomonas europaea*, *Alcaligenes faecalis*, rRNA Group-III pseudomonads/*Rubrivivax gelatinosus*, and rRNA Group-II pseudomonads/*Rhodocyclus tenuis*. Within the phenylalanine pathway, the bifunctional P-protein (chorismate mutase/prephenate dehydratase) was present in each lineage and must have evolved in a common ancestor of the β and γ subdivisions. Each P-protein was found to be subject to activation by L-tyrosine, and to feedback inhibition by L-phenylalanine. Phenylalanine-inhibited (DS-phe) and tyrosine-inhibited (DS-tyr) isoenzymes of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase probably existed in the common β-subdivision ancestor, with DS-tyr being lost in *N. gonorrhoeae* and *A. faecalis*. The participation of DS-phe in a dissociable multienzyme complex with one or more other common-pathway enzymes is known to exist in *N. gonorrhoeae*. The same complex is indicated by two peaks of DS-phe seen in chromatographic profiles of Group-III pseudomonads and *A. faecalis*. It is concluded that the contemporary DS-phe species present in subdivisions γ and β must have had independent origins. Tyrosine biosynthesis was found to be quite diverse within the β subdivision. *Nit. europaea* possessed an arogenate dehydrogenase which was specific for $NADP^+$. In all other lineages, a broad-specificity cyclohexadienyl dehydrogenase (CDH) was present. In *N. gonorrhoeae* the CDH was specific for NAD^+ while the remaining CDH species could utilize either NAD^+ or $NADP^+$. Only the CDH species within the rRNA Group-II pseudomonad/*R. tenuis* lineage was feedback-inhibited by L-tyrosine, and this correlated with an allosteric pattern where activation of the prephenate dehydratase component of the P-protein by L-tyrosine was relatively poor. However, the CDH enzyme present in *N. gonorrhoeae* and *A. faecalis* was subject to inhibition by 4-hydroxyphenylpyruvate, this being competitive with respect to the cyclohexadienyl substrate. The monofunctional species of chorismate mutase (CM-F) and cyclohexadienyl dehydratase, widely distributed among the γ-subdivision assemblage and recently shown to be periplasmic enzymes, were demonstrated in *Pseudomonas pickettii*, a member of rRNA homology Group-II.

Keywords: biochemical evolution, aromatic amino acid biosynthesis, Proteobacteria phylogeny

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Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; E4P, erythrose 4-phosphate; HPP, 4-hydroxyphenylpyruvate; PEP, phosphoenolpyruvate; P-protein, bifunctional protein with catalytic domains for chorismate mutase (CM-P) and prephenate dehydratase (PDT); CM-F, monofunctional species of chorismate mutase; DS, DAHP synthase; DS-phe, phe-inhibited isoenzyme of DS; DS-tyr, tyr-inhibited isoenzyme of DS; DS-O, allosterically insensitive isoenzyme of DS; CDH, cyclohexadienyl dehydrogenase; CDH-a, CDH assayed with L-arogenate as substrate; CDH-p, CDH assayed with prephenate as substrate; CDT, cyclohexadienyl dehydratase; CDT-a, CDT assayed with L-arogenate as substrate; CDT-p, CDT assayed with prephenate as substrate.

INTRODUCTION

Phylogenetic trees for prokaryotes are being constructed on the basis of the ubiquitous and highly conserved 16S rRNA sequences (Woese, 1987). The extent to which such molecular trees are valid for entire organisms is uncertain. In large part this depends on the frequency of horizontal transmission of genes across the species barrier. Genes encoding proteins which are conditionally dispensable, such as catabolic enzymes or resistance determinants, are often organized on plasmids and thus are the most likely source of chimeric 'impurity'. In contrast, essential genes required for biosynthesis are likely to parallel the vertical line of descent displayed by genes for 16S rRNA. Even here, a case has been made for horizontal transfer of a gene as fundamental as glutamine synthetase (Smith *et al.*, 1992). However, should such discrete incidents of horizontal transfer be confirmed, their very recognition depends upon the generally vertical transmission of most genes.

Enzymes which participate in the biosynthesis of aromatic amino acids exhibit a diverse assemblage of qualitative 'character states' whose distribution can be traced in a phylogenetic progression. The concordance of the phylogenetic tree based upon 16S rRNA and the clustering observed for character states of aromatic amino acid biosynthesis has been amply documented (Byng *et al.*, 1983, 1986; Ahmad *et al.*, 1986; Ahmad & Jensen, 1987).

The study of a suitable diversity of character states in a well-defined phylogenetic progression of organisms has merit because: (i) the order of branching at a fine-tuned hierarchical level (Byng *et al.*, 1986) or even at deep hierarchical levels (Jensen & Ahmad, 1990) can be determined in cases where order of branching is not resolved by 16S rRNA comparisons; (ii) the evolution of the pathway and its regulation can be traced backwards through evolutionary time (Jensen, 1985); (iii) insight into the overall physiological nature of a biochemical pathway can be gained through knowledge of the context of its variation within a cluster of related organisms; and (iv) systematic elucidation of the qualitative character states can form a logical basis for further molecular-genetic comparisons.

Currently, interpretations about the evolutionary history of many genes is usually limited by the ability to compare a given gene only with others which happen by chance to have been studied by other groups. Better success requires a systematic study of a manageable group of organisms, appropriately spaced on a phylogenetic tree. Such an approach, illustrated by studies of Superfamily B (γ subdivision of Woese) prokaryotes (Jensen, 1985) is now expanded to include Superfamily A (β subdivision) of the Proteobacteria.

METHODS

Biochemicals. Erythrose-4-phosphate (E4P) was prepared according to 'Procedure B' of Ballou (1963) except that the monosodium salt of glucose 6-phosphate was used and the sulfuric acid treatment of the salt was omitted. The concentration of E4P was estimated by using a partially purified preparation of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase from *Neisseria gonorrhoeae* ATCC 27630. L-Arogenate (90% pure) was prepared as described by Zamir *et al.* (1985). Chorismate (85% pure) was prepared by the method of Gibson (1964). Barium prephenate (90% pure), prepared as described (Bonner *et al.*, 1990), was converted to the potassium salt just before use by treatment with 100 mM potassium phosphate buffer (pH 7·0) and removing the barium phosphate by centrifugation. Bradford reagent was purchased from Bio-Rad. Dithiothreitol was from Research Organics. The trisodium salt of phosphoenolpyruvate (PEP), amino acids and 4-hydroxyphenylpyruvate (HPP) were purchased from Sigma.

Bacterial strains, media and growth conditions. The bacterial strains and growth conditions used were as follows (names in parentheses are alternative names used in the 1992 ATCC catalogue of Bacteria & Bacteriophages). *N. gonorrhoeae* ATCC 27628, was grown in a defined medium (Hendry, 1983) at 37 °C in 400 ml batches in 2 l flasks. Lower fluid-to-flask volume ratios resulted in poor or no growth. *Alcaligenes faecalis* ATCC 8750 was grown in the rich medium recommended in the 1992 ATCC catalogue. *Nitrosomonas europeae* (Schmidt strain) (Koops *et al.*, 1991) was grown in continuous culture as described previously (Arciero *et al.*, 1991). *Pseudomonas testosteroni* ATCC 17409 (*Comamonas testosteroni*) and *Pseudomonas pickettii* ATCC 27511 were grown as 1 l cultures contained in 2·8 l fernbach flasks at 26 °C and 30 °C, respectively, in the minimal medium of Guroff & Ito (1963) as modified (Berry *et al.*, 1985), except that proline was omitted. Other pseudomonad organisms were grown as previously described (Byng *et al.*, 1980). Cells were harvested by centrifugation and stored at −80 °C. *Rhodocyclus tenuis* (Imhoff *et al.*, 1984) and *Rubrivivax gelatinosus* (Willems *et al.*, 1991) were grown anaerobically at the American Type Culture Collection facility (Rockville, MD, USA) and shipped to our laboratory on dry ice. *Rubrivivax gelatinosus* has previously carried the designations *Rhodopseudomonas gelatinosa* (Fox *et al.*, 1980) and then *Rhodocyclus gelatinosus* (Imhoff *et al.*, 1984). *Rubrivivax gelatinosus* and *Rhodocyclus tenuis* clearly merit different generic designations (Willems *et al.*, 1991).

Preparation of crude extracts. All crude extracts were prepared in 100 mM potassium phosphate buffer, pH 7·0, containing 1·0 mM DTT (Buffer A). Frozen cells were resuspended by thawing at room temperature in Buffer A with gentle agitation. Generally a buffer-to-cell ratio (v/w) of 2:1 was used, except for *Nit. europaea* where a higher ratio of 6:1 was used to ensure a good suspension in the presence of the encapsulating slime which otherwise caused the highly viscous solution to froth during cell breakage and resulted in considerable loss of activities. Cells were broken in a French pressure cell at 1000 p.s.i. (6900 kPa). The suspensions were centrifuged at 150000 ***g*** for 37 min, and the supernatant was used as crude extract. Crude extracts were desalted by passage through an Econo Pac 10DG (Bio-Rad) desalting column equilibrated with Buffer A. For column chromatography the extract was similarly desalted except that the equilibration buffer was 20 mM potassium phosphate, pH 7·0, containing 1·0 mM DTT (Buffer B). All operations were conducted at 4 °C. Protein concentrations were estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

Enzyme assays. DAHP synthase (DS) was assayed as described (Jensen & Nester, 1966). Standard reaction mixtures contained 1 mM each of E4P, PEP and $MnSO_4$. Chorismate mutase (CM)

and prephenate dehydratase (PDT) were assayed according to Patel *et al.* (1977), standard reactions containing 1 mM substrate. Arogenate dehydratase was measured by HPLC (Fischer & Jensen, 1987a). Arogenate dehydrogenase (Bonner & Jensen, 1987) and prephenate dehydrogenase (Fischer & Jensen, 1987b) were assayed as described previously.

In this study the elution profiles of various pairs of enzyme activities are coincident and concluded to be catalytic capabilities of a single protein. Thus, the P-protein exhibits separate catalytic domains for CM (denoted CM-P) and PDT. CDH can function as either prephenate dehydrogenase (CDH-p) or arogenate dehydrogenase (CDH-a), and CDT can function as either prephenate dehydratase (CDT-p) or arogenate dehydratase (CDT-a). In most cases one or more additional fractionation steps have been done, showing persistent coelution (data not shown). With the arbitrary conditions of assay that are necessarily used for screening fractions in order to generate elution profiles of the various enzymes assayed, the ratio of the two activities corresponding to the P-protein, CDH or CDT were not necessarily constant when measured in fractions across a given peak. For example, in cases such as the *A. faecalis* P-protein where the ratio of CM-P to PDT activity is high, PDT but not CM-P is saturated with substrate in peak fractions. This creates disproportional conditions of assay for CM-P in some samples. In cases where PDT activity of a given P-protein requires L-tyrosine as an activator, the magnitude of activation can vary with enzyme concentration in different fractions, whereas CM-P activity is affected less or not at all by these variables. However, fractions assayed across a given peak for co-eluting activities yielded calculated ratios which were within 95% when matched assay conditions of proportionality were rigorously established for each sample under comparison.

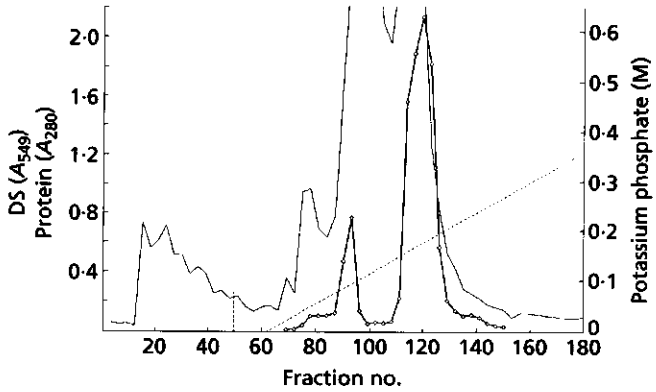

Fig. 1. Elution profile of DS activity from an hydroxylapatite chromatography matrix following application of crude extract from *N. gonorrhoeae* ATCC 27628. A portion (85 mg) of crude extract prepared as described in Methods in Buffer B was loaded on a 1·5 × 20 cm column equilibrated with Buffer B and the unbound protein removed by washing with at least 2 bed-vols of Buffer B. Bound protein was eluted by application of a linear gradient of 20–300 mM potassium phosphate (·····) in a total volume of 300 ml. DS activity (○) is plotted as A_{549}. An A_{549} value of 1·0 corresponds to 48 nmol of DAHP formed. Protein elution (——) is followed as A_{280}. The vertical dashed line denotes the point of gradient application. Both peaks of activity were inhibited 70% by 0·5 mM phenylalanine under standard assay conditions.

RESULTS

Phylogenetic tree for the β subdivision lineages studied

16S rRNA sequence data were available for a large proportion of the organisms examined in this study. Exceptions were *Pseudomonas acidovorans*, *Pseudomonas facilis*, *Pseudomonas saccharophila*, and *Pseudomonas solanacearum*, which can be placed approximately from earlier RNA/DNA hybridization studies (Palleroni *et al.*, 1973) and *Rho. tenuis* which can be placed from earlier 16S rRNA catalogue studies (Woese *et al.*, 1984). A phylogenetic tree based on the 16S rRNA sequence data is available on line from the ribosomal database project (Larsen *et al.*, 1993).

The *N. gonorrhoeae* lineage

A fairly detailed background of biochemical work exists describing enzymological features of aromatic amino acid biosynthesis in *N. gonorrhoeae* (Berry *et al.*, 1987). The basis for growth inhibition by L-phenylalanine observed in some clinical isolates has been evaluated (Bhatnagar *et al.*, 1989). Key character states found were the presence of a bifunctional P-protein (chorismate mutase/prephenate dehydratase) inhibited by L-phenylalanine and strongly activated by L-tyrosine; a cyclohexadienyl dehydrogenase (CDH) that is specific for NAD^+, exhibits a high preference for prephenate as substrate, and is insensitive to feedback inhibition by L-tyrosine; and a single species of DS inhibited by L-phenylalanine (DS-phe). In contrast to the single peak of DS-phe obtained by Berry *et al.* (1987), use of different chromatographic conditions resolved two peaks of DS-phe (Fig. 1). This has recently been attributed to a dissociable complex of DS-phe with shikimate dehydrogenase and possibly a number of other common-pathway enzymes (P. Subramaniam, unpublished data).

Although CDH was insensitive to feedback inhibition by L-tyrosine, it was reported to be sensitive to inhibition by HPP (Berry *et al.*, 1987). This inhibition (at 2·5 and 5 mM HPP) was strictly competitive with respect to prephenate (data not shown). The K_m for prephenate was 0·44 mM. Whether this inhibition is physiologically significant is unknown.

The *A. faecalis* lineage

A. faecalis was cultured, crude extract was prepared and DEAE-cellulose chromatography was carried out as described in the legend of Fig. 2. Chromatographic profiles obtained for DS, CDH, CM and PDT are shown. Like *N. gonorrhoeae*, two peaks of DS-phe were obtained. Profiles for prephenate dehydrogenase and arogenate dehydrogenase were coincident, indicating the presence of a broad-specificity CDH. $NADP^+$ was the preferred cosubstrate, NAD^+ being about 15% as effective. As shown in Fig. 2, prephenate was distinctly preferred to L-arogenate as substrate. L-Tyrosine did not exert feedback inhibition, but HPP was inhibitory (46% inhibition by 1 mM HPP using 50 μM prephenate, and 60% inhibition by 1 mM HPP using 1·1 mM L-arogenate). CM and PDT

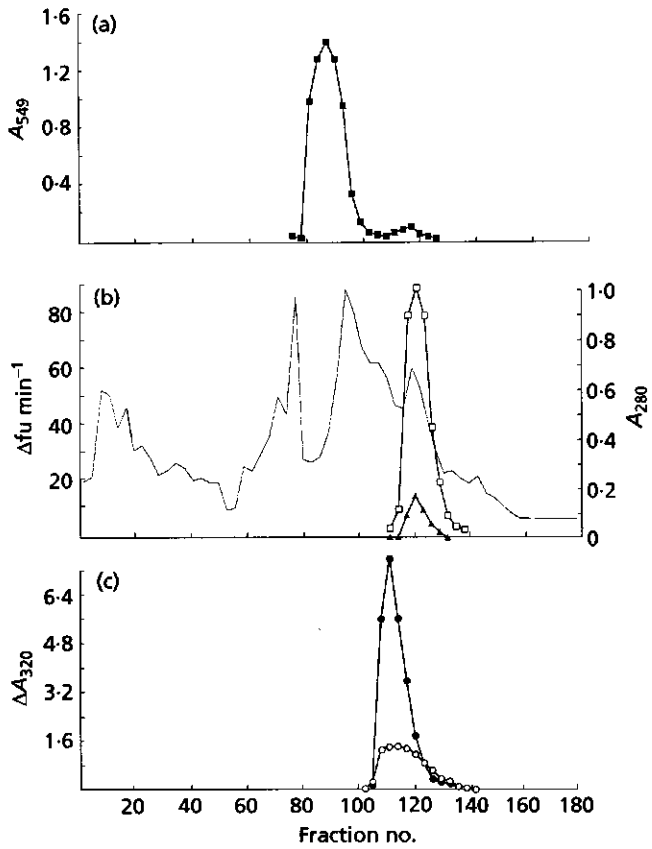

Fig. 2. Chromatographic separation of enzymes from *A. faecalis*. Crude extract in Buffer B (100 mg protein) was loaded on a 1·5 × 20 cm DEAE-cellulose column equilibrated in Buffer B. After washing the column with 2 vols of equilibration buffer, bound protein was eluted by application of a linear gradient of 0–500 mM KCl in Buffer B in a total volume of 300 ml. (a) Elution position of DS (■); (b) the CDH-p (□) and CDH-a (▲) activities of CDH measured as change in fluorescence (Δfu) min^{-1} and protein measured as A_{280} (——); (c) CM-P (●) and PDT (○) components of the P-protein. The leading and trailing peaks of DS were inhibited 66% and 52%, respectively, by L-phenylalanine.

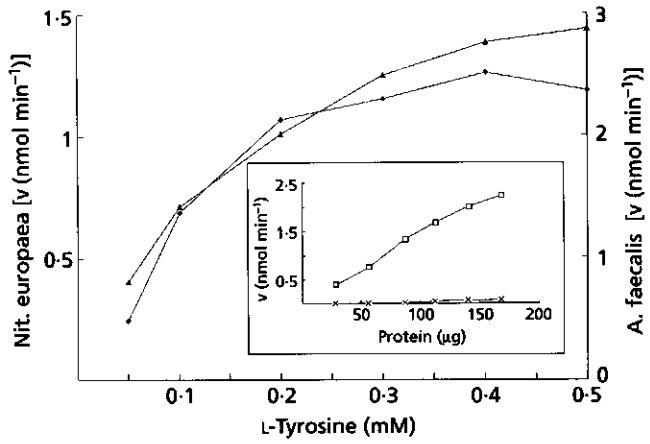

Fig. 3. Activation of P-protein PDT by L-tyrosine. The activities of PDT from *A. faecalis* (▲) and *Nit. europeae* (◆) (1 mM prephenate) are shown as a function of increasing L-tyrosine concentrations. A partially purified (DEAE-cellulose fractions, Fig. 2) preparation from *A. faecalis* and crude extract from *Nit. europaea* were used. Inset: the proportional effect of increasing amount of protein on the activity of PDT from *Nit. europaea* at a maximally activating concentration (0·5 mM) of tyrosine (□). No activity was seen up to 84 μg protein in the absence of tyrosine (×).

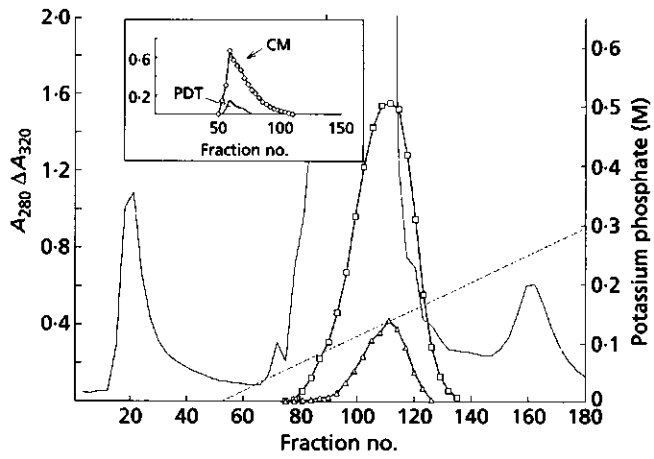

Fig. 4. CM-P (□) and PDT (△) activities of *Nit. europeae* (measured as ΔA_{320}) following hydroxylapatite chromatography. A portion (90 mg) of crude extract was chromatographed as described in Fig. 1, except that the gradient boundaries were between 20 and 400 mM L-tyrosine, which was required for activity. ——, Protein (A_{280}); ·····, potassium phosphate. The inset shows that the two activities were also coincident after DEAE-cellulose chromatography run as described in Fig. 2 (35 mg crude extract; 0–400 mM KCl gradient).

profiles were coincident, indicating the existence of the ubiquitous bifunctional P-protein. PDT catalysis exhibited an absolute requirement for L-tyrosine, a potent activator molecule.

The activation response of PDT to progressively increasing concentrations of L-tyrosine during assays carried out at a fixed prephenate concentration is illustrated in Fig. 3.

The *Nit. europaea* lineage

As expected, CM and PDT profiles were coincident following hydroxyapatite and DEAE-cellulose chromatography (Fig. 4), indicating the presence of a bifunctional P-protein. The PDT component exhibited a near-absolute requirement for L-tyrosine in order to obtain measurable activity (Fig. 3). An arogenate-specific dehydrogenase, exhibiting absolute specificity for NADP$^+$, was readily apparent in crude extracts. L-Tyrosine was totally ineffective as an inhibitor of arogenate dehydrogenase activity. We were unable to determine the number and allosteric classes of DS present in *Nit. europaea* due to an unknown compound which interfered with the thiobarbituric assay for DAHP.

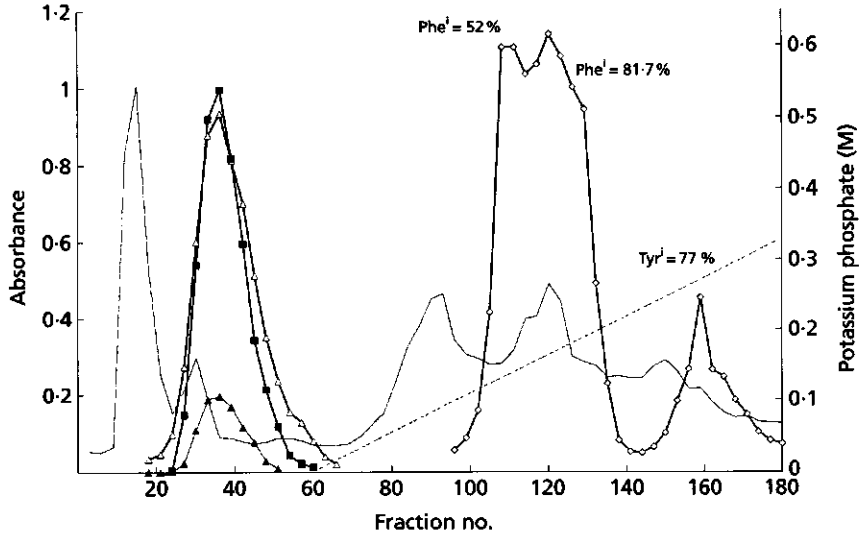

Fig. 5. Activity profiles of enzymes from *P. testosteroni* ATCC 17409 eluted from DEAE-cellulose. Crude extracts were chromatographed on DEAE-cellulose as in Fig. 2, except that the gradient applied was 0–400 mM KCl in a total volume of 350 ml. L-Phenylalanine and L-tyrosine inhibition of DS, Phe^i and Tyr^i, are shown at peak fractions assayed. PDT activity is shown with and without L-tyrosine in the reaction mixture. ——, Protein (A_{280}); ◇, DS (A_{549}); △, CM (A_{320}); ▲, PDT−Tyr (A_{320}); ■, PDT+Tyr (A_{320}); ·····, potassium phosphate.

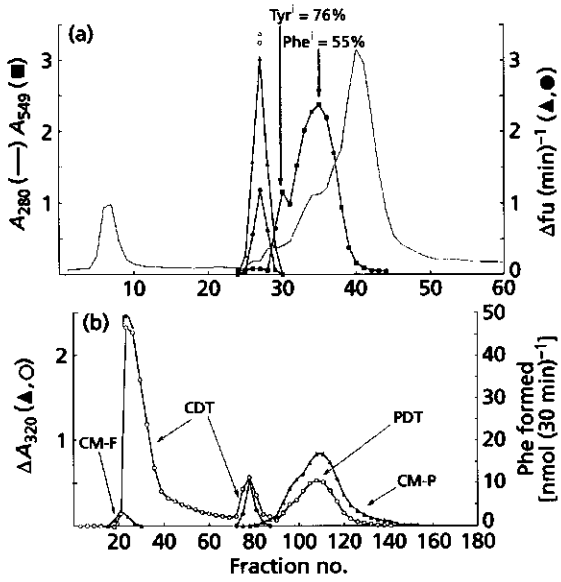

Fig. 6. Activity profiles of enzymes of *P. pickettii* ATCC 27511 following hydroxylapatite chromatography of crude extracts. Columns were run as described in Fig. 1, except that the gradient applied was 20–400 mM potassium phosphate in a total of 340 ml. (a) Activity profiles of DS (■) and CDH. The inhibition of DS by L-phenylalanine (Phe^i) and L-tyrosine (Tyr^i) is shown in the fractions indicated. The prephenate dehydrogenase (CDH-p, ▲) and arogenate dehydrogenase (CDH-a, ●) activity profiles of CDH with NAD^+ as cosubstrate are shown in full. Under the latter conditions CDH-a was inhibited (77%) by 0·5 mM L-tyrosine, while CDH-p was unaffected. However, at 40 μM prephenate (PPA) the ratio $V_{NADP,\,1mM}/V_{NAD,\,1mM} = 2$ and CDH-p activity is inhibited > 90% by L-tyrosine. Profiles were also obtained using $NADP^+$ as a cosubstrate in combination with prephenate or arogenate (CDH-p/NADP, △ and CDH-a/NADP, ○). For simplicity of presentation, only the data points at the peak fraction are shown. ——, Protein. (b) Activity profiles for CM (▲), PDT and CDT. Separate CM-F and CM-P peaks were resolved. CDT activity was measured with prephenate (CDT-p, ○) or L-arogenate (CDT-a, ●) as alternative substrates. For the leading peak of CDT the activity determined with arogenate is shown only at the peak position.

rRNA Group-III pseudomonads

Comprehensive data encompassing a large number of Group-III pseudomonads have been obtained for DS (Whitaker *et al.*, 1981a), CDH (Byng *et al.*, 1980), and the bifunctional P-protein (Whitaker *et al.*, 1981b). The latter data have shown these to be a cohesive group sharing the same distinctive character states for aromatic biosynthesis. DS-phe and DS-tyr isoenzymes of DS have been resolved and characterized in *P. acidovorans* ATCC 11299a (Berry *et al.*, 1985). The partial purification of DS isoenzymes and the bifunctional P-protein is shown for *P. testosteroni* ATCC 17409 in Fig. 5. DS-phe and DS-tyr were separated. DS-phe exhibited two forms, similar to results obtained with *P. acidovorans* ATCC 11299a (Berry *et al.*, 1985), as well as with *N. gonorrhoeae* and *A. faecalis* in this study. The activation of PDT by L-tyrosine is illustrated by comparison of the profiles obtained with and without tyrosine. CDH exhibited relatively good activity with either prephenate or L-arogenate, as well as with either NAD^+ or $NADP^+$ (Byng *et al.*, 1980). It was unaffected by L-tyrosine.

rRNA Group-II pseudomonads

Group-II pseudomonads have also been shown to be a cohesive group with respect to character states involving DS (Whitaker *et al.*, 1981a), CDH, (Byng *et al.*, 1980; Friedrich *et al.*, 1976a, b) and the bifunctional P-protein (Friedrich *et al.*, 1976a, b; Whitaker *et al.*, 1981b). While *Alcaligenes eutrophus* appears to possess a single DS that is subject to cumulative feedback inhibition by both phenylalanine and tyrosine (Friedrich & Schlegel, 1975), additive effects of L-phenylalanine and L-tyrosine in crude extracts of other Group-II pseudomonads had been interpreted as strong evidence for the presence of DS-phe and DS-tyr isoenzymes of DS (Whitaker *et al.*, 1981a). Confirmation of this was obtained by the partial separation of such isoenzymes in *P. pickettii* following hydroxyapatite chromatography as shown in Fig. 6(a). Fraction 87

Table 1. Comparative sensitivities of aromatic-pathway dehydrogenases to feedback inhibition

Organism	Enzyme*	Percentage inhibition† (0·5 mM tyrosine)
Rho. tenuis	CDH	100
Group II pseudomonads		
P. cepacia	CDH	59
P. pickettii	CDH	77
P. solanacearum	CDH	68
A. eutrophus	CDH	62
Rubrivivax gelatinosus 17011	CDH	0
Rubrivivax gelatinosus 17013	CDH	0
Group III pseudomonads		
P. acidovorans	CDH	0
P. testosteroni	CDH	0
P. facilis	CDH	0
P. saccharophila	CDH	0
A. faecalis	CDH	0
N. gonorrhoeae	CDH	0
Nit. europaea	ADH	0

* Specific activities (nmol product formed min^{-1} mg^{-1} at 37 °C) using L-arogenate as substrate in crude extracts varied between 0·2 (*Rubrivivax gelatinosus* ATCC 17013) and 21·6 (*A. eutrophus*). ADH, arogenate dehydrogenase.

† Inhibition measured using 0·5 mM L-arogenate as substrate.

Table 2. Relative CDH activities with alternative substrates

Organism	Activity ratios	
	NAD:NADP	PPA:AGN‡
Rho. tenuis and Group-II pseudomonads*	0·76	0·95
Rubrivivax gelatinosus and Group-III pseudomonads†	1·09	2·70
A. faecalis	0·18	6·12
N. gonorrhoeae	NAD-specific	12·31
Nit. europaea	NADP-specific	AGN-specific

* Mean of four Group-II pseudomonads (see Table 1).

† Mean of four Group-III pseudomonads (see Table 1).

‡ PPA, Prephenate; AGN, L-arogenate.

exhibited 76% inhibition by 0·5 mM L-tyrosine, while fraction 105 exhibited 55% inhibition by 0·5 mM L-phenylalanine. Relative activities of CDH assayed with its alternative substrates, prephenate (CDH-p) or L-arogenate (CDH-a) and NAD^+ or $NADP^+$, are illustrated. As shown with other tyrosine-sensitive CDH proteins (Xia & Jensen, 1990), L-tyrosine inhibits competitively with respect to the cyclohexadienyl substrate. Typically,

Table 3. Stimulation of prephenate dehydratase by L-tyrosine

Organism	Ratio*
Rho. tenuis	1·1
Group II pseudomonads	
P. cepacia	3·0
P. solanocearum	1·1
A. eutrophus	2·7
P. pickettii	6·5
Rubrivivax gelatinosus 17011	NA
Rubrivivax gelatinosus 17013	NA
Group III pseudomonads	
P. acidovorans	3·2
P. testosteroni	4·7
P. facilis	4·3
P. saccharophila	19·7
N. gonorrhoeae	21·2
A. faecalis	NA
Nit. europaea	NA

* Ratio of specific activities obtained plus L-tyrosine: those obtained minus L-tyrosine. NA, no activity was obtained in the absence of 0·5 mM L-tyrosine. Crude extract specific activities in the presence of L-tyrosine ranged between 2·6 for *Rho. tenuis* and 25·2 for *P. acidovorans*.

the affinity for prephenate exceeds that for L-arogenate, and therefore tyrosine inhibition is more readily apparent under standard assay conditions when L-arogenate is used as substrate. Fig. 6(b) illustrates the coincident profiles of CM-P and PDT, indicating the presence of a bifunctional P-protein. L-Tyrosine was a relatively modest activator of PDT (6·5-fold).

A monofunctional species of chorismate mutase (CM-F) was found in the wash eluate. Two peaks of CDT, each capable of using prephenate or L-arogenate as alternative substrates, were located. Each CDT species was unaffected by L-phenylalanine or L-tyrosine. Chromatographic isoforms of CDT have been noted before in *P. aeruginosa* (Patel *et al.*, 1977). In the γ subdivision, CM-F and CDT have been shown to be periplasmic (Xia *et al.*, 1993; Zhao *et al.*, 1993a; and P. Subramanian, unpublished data).

Tyrosine pathway diversity

The dehydrogenase function within the tyrosine pathway of β-subdivision organisms has undergone particularly dynamic evolutionary changes (Table 1). In addition to the qualitative change in specificity in *Nitrosomonas* whereby narrowed specificity to accept only L-arogenate is observed, CDH within the *Rho. tenuis*/Group II pseudomonad lineage acquired a singular sensitivity to feedback inhibition by L-tyrosine. A narrowed but different specificity for pyridine nucleotide cosubstrate occurred in the *Neisseria* (NAD^+) and *Nitrosomonas* ($NADP^+$) lineages (Table 2). In addition, dehydrogenases having broad specificity for either the cyclohexadienyl

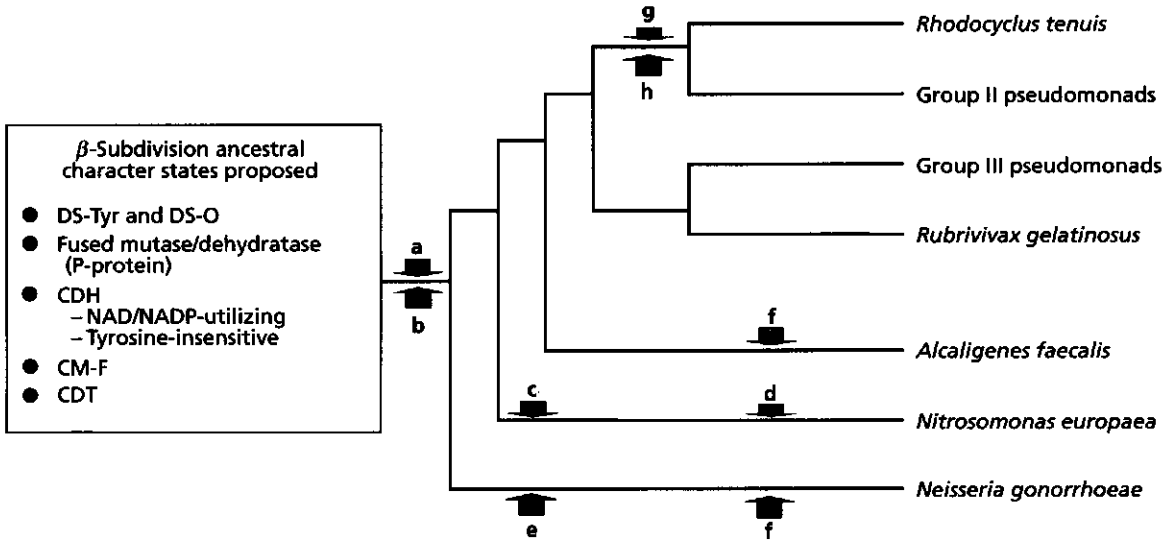

Fig. 7. **Dendrogram showing the major lineages considered in this study. No attempt has been made to give a quantitative scale of evolutionary time on the horizontal axis, but the order of branching is accurate. Consult the ribosomal database (Larsen *et al.*, 1993) for the quantitative portrayal. The ancestral character states deduced are shown on the left, and evolutionary events postulated are indicated by arrows on the dendrogram. Evolutionary events deduced: a, DS-O acquires a Phe-binding domain; b, CM-F and CDT are lost (cryptic?); c, CDH becomes $NADP^+$-specific; d, CDH becomes arogenate-specific; e, CDH becomes NAD^+-specific; f, DS-Tyr is lost; g, CM-F and CDT are restored; h, CDH acquires Tyr-binding domain for feedback inhibition.**

substrate or the pyridine nucleotide cosubstrate exhibited considerable variation in relative activities observed with the alternative substrates at saturating concentrations.

Phenylalanine pathway diversity

The bifunctional P-protein is uniformly present throughout the β subdivision. However, the PDT catalytic component is highly variable in the extent of its activation by L-tyrosine (Table 3). Enzyme from organisms such as *A. faecalis* and *Nit. europaea* exhibit an absolute catalytic requirement for tyrosine. In organisms such as *N. gonorrhoeae*, the factor of activation is well over an order of magnitude, in contrast to more modest effects in most of the other organisms.

DISCUSSION

The β and γ subdivisions of the Proteobacteria are sister lineages whose common ancestor diverged from the α lineage. Since the bifunctional P-protein is present throughout the β and γ subdivisions but not in the α subdivision (Ahmad & Jensen, 1988a), the presumed gene fusion responsible for its origin arose after the α subdivision diverged from the common ancestor. At the β/γ superfamily levels, two main character-state differences stand out. The tyrosine pathway CDH is uniformly sensitive to feedback inhibition by L-tyrosine in the γ subdivision, but is typically insensitive in the β subdivision. Secondly, the DS-phe isoenzyme of DS is the most prevalent within the β subdivision, in contrast to its limited presence in only a tiny cluster of γ subdivision members.

Within the β subdivision the Group-II pseudomonad/*Rho. tenuis* lineage is perhaps most reminiscent of γ subdivision members in that CDH is sensitive to feedback inhibition by L-tyrosine and the monofunctional CM-F and CDT enzyme species, widely distributed in the γ subdivision (Ahmad & Jensen, 1988b), have been found.

A unique character state in the β subdivision is provided by *Nit. europaea*, the first example of an arogenate dehydrogenase in a Gram-negative bacterium. The exclusive use of L-arogenate for tyrosine biosynthesis and phenylpyruvate for phenylalanine biosynthesis is a pattern found in cyanobacteria and coryneform bacteria (Ahmad & Jensen, 1988a). In each case where this pathway arrangement exists, the focal point of regulation by L-tyrosine (allosteric activator) and L-phenylalanine (feedback inhibitor) is at PDT. Thus, an appropriate flow of precursor to tyrosine is indirectly controlled by the effect of tyrosine upon flow of precursor to the competing phenylalanine pathway.

Fig. 7 presents a possible scenario of evolutionary events in the β subdivision which led from ancestral character states deduced according to principles of maximal parsimony, and in consideration of ancestral character states previously deduced for the γ subdivision (Ahmad & Jensen, 1988a; Jensen, 1985). CDH, functioning in L-tyrosine biosynthesis, originally existed as a form that was insensitive to allosteric control. This type of CDH has been fully characterized in *Zymomonas mobilis* (Zhao *et al.*, 1993b), a member of the α subdivision, and probably resembles an ancient ancestral species in Gram-negative bacteria. CDH acquired allosteric control in the Group-II pseudomonad/*Rho. tenuis* lineage. It appears that CDH

allostery must have evolved independently on two occasions, once in the γ subdivision and once in the β subdivision. The evolutionary acquisition of direct allosteric control by L-tyrosine of its own pathway branch seems to correlate with a weakened activating effect of L-tyrosine upon PDT of the opposing phenylalanine branch.

Specificity of the ancestral CDH for pyridine nucleotide cosubstrate was broad and remained so in three lineages, but became specific for NAD^+ in *N. gonorrhoeae* and specific for $NADP^+$ in *Nit. europaea*. The narrowing of cofactor specificity for tyrosine-pathway dehydrogenases appears to be a relatively recent evolutionary event (Jensen, 1992). The specificity for the cyclohexadienyl substrate of CDH (prephenate or L-arogenate) varies quantitatively from lineage to lineage, but narrowed to specificity for L-arogenate in *Nit. europaea*. The presence and levels of CM-F and CDT are quite variable in subdivision γ (Ahmad & Jensen, 1988b). These periplasmic enzymes may have been lost from all but the Group-II pseudomonad/*Rho. tenuis* lineage, although more data are needed to establish this.

DS-tyr and the feedback-insensitive DS-O were suggested to comprise ancestral character states in the γ subdivision (Ahmad *et al.*, 1986), and these are therefore logical ancestral character states for the sister β subdivision as well (Fig. 7). If so, then DS-O may have acquired an allosteric domain for phenylalanine (thus becoming DS-phe) prior to divergence of the five β lineages studied. It appears that DS-phe must have evolved twice, once very recently within the γ subdivision (Ahmad *et al.*, 1987) and once much longer ago within the β subdivision. In both *N. gonorrhoeae* and *A. faecalis*, the DS-tyr isoenzyme was lost, leaving DS-phe as the sole species of DS in operation. Within the β subdivision DS-phe has often been noted to fractionate into two chromatographic peaks. In *N. gonorrhoeae* this reflects the participation of DS-phe in a dissociable complex with shikimate dehydrogenase and perhaps other common-pathway enzymes. It seems likely that the observation of a similar double peak in *A. faecalis*, *P. testosteroni*, *P. acidovorans* (Berry *et al.*, 1985), and *A. eutrophus* (Friedrich & Schlegel, 1975) may reflect a widespread occurrence of this protein complex throughout the β subdivision.

Considerable effort has been expended to evaluate the character states in the three lineages represented by *A. faecalis*, *Nit. europaea* and *N. gonorrhoeae*. However, the differences illustrated in Fig. 7 between these lineages include evolutionary events which could be traced more definitively if additional organisms belonging to each lineage were analysed. Thus, the order of events **c** and **d** or events **e** and **f** (Fig. 7) could be readily deduced if other representatives of these lineages branching off at well-spaced intervals were studied.

The independent acquisition of a given character state (e.g. sensitivity of CDH to feedback inhibition) in different phylogenetic clusters is perfectly consistent with a vertical mode of transmission of character states during evolution. Specialization for NAD^+ or $NADP^+$ in the various tyrosine-pathway dehydrogenases has been shown to occur many times as separate events within the Proteobacteria, starting with an ancestral dehydrogenase of broad specificity for the pyridine nucleotide cofactor (Jensen, 1992). On the other hand, the necessity to conclude that successive evolutionary events occurred of acquisition, loss and reacquisition of character states such as CM-F and CDT might prove to exemplify instances of horizontal gene transfer. The latter might be consistent with the periplasmic location of CM-F (Xia *et al.*, 1993) and CDT (Zhao *et al.*, 1993a) and the apparent redundancy of these enzymes (Patel *et al.*, 1977) with respect to a primary biosynthetic pathway present in the cytoplasm.

A systematic evaluation of enzymological character states such as that summarized here provides a basis for the choice of appropriately spaced organisms (which can differ for each character state depending upon how dynamic the pace of evolutionary change has been for that character state) for molecular-genetic analysis of proteins and protein domains.

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