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Evolutionary implications of features of aromatic amino acid biosynthesis in the genus *Acinetobacter*

Graham S. Byng[1], Alan Berry[2], and Roy A. Jensen[2]

[1] New Microbiologicals, Biotechnology Group, Miles Laboratories, Inc., Elkhart, IN 46515, USA
[2] Center for Somatic-cell Genetics and Biochemistry, State University of New York at Binghamton, Binghamton, NY 13901, USA

Abstract. Key enzymes of aromatic amino acid biosynthesis were examined in the genus *Acinetobacter*. Members of this genus belong to a suprafamilial assemblage of Gram-negative bacteria (denoted Superfamily B) for which a phylogenetic tree based upon oligonucleotide cataloging of 16S rRNA exists. Since the *Acinetobacter* lineage diverged at an early evolutionary time from other lineages within Superfamily B, an examination of aromatic biosynthesis in members of this genus has supplied important clues for the deduction of major evolutionary events leading to the contemporary aromatic pathways that now exist within Superfamily B. Together with *Escherichia coli*, *Pseudomonas aeruginosa* and *Xanthomonas campestris*, four well-spaced lineages have now been studied in comprehensive detail with respect to comparative enzymological features of aromatic amino acid biosynthesis. *A. calcoaceticus* and *A. lwoffii* both possess two chorismate mutase isozymes: one a monofunctional isozyme (chorismate mutase-F), and the other (chorismate mutase-P) a component of a bifunctional P-protein (chorismate mutase-prephenate dehydratase). While both P-protein activities were feedback inhibited by L-phenylalanine, the chorismate mutase-P activity was additionally inhibited by prephenate. Likewise, chorismate mutase-F was product inhibited by prephenate. Two isozymes of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase were detected. The major isozyme (> 95%) was sensitive to feedback inhibition by L-tyrosine, whereas the minor isozyme was apparently insensitive to allosteric control. Prephenate dehydrogenase and arogenate dehydrogenase activities were both detected, but could not be chromatographically resolved. Available evidence favors the existence of a single dehydrogenase enzyme, exhibiting substrate ambiguity for prephenate and L-arogenate. Dehydrogenase activity with either of the latter substrates was specific for $NADP^+$, NAD^+ being ineffective. Consideration of the phylogeny of Superfamily-B organisms suggests that the stem ancestor of the Superfamily possessed a single dehydrogenase enzyme having ambiguity for both substrate and pyridine nucleotide cofactor. Since all other members of Superfamily B have NAD^+-specific dehydrogenases, specialization for $NADP^+$ must have occurred following the point of *Acinetobacter* divergence, leading to the dichotomy seen in present-day Superfamily-B organisms.

Key words: Phylogeny – Biochemical evolution – Aromatic biosynthesis – *Acinetobacter* – Regulatory enzymes

Offprint requests to: G. S. Byng

The biosynthesis of L-phenylalanine and L-tyrosine proceeds by a complex biochemical pathway that exhibits diversity in the use of certain enzymatic steps, in the regulatory properties of individual enzymes, and in the utilization of either NAD^+ or $NADP^+$ (or both) as cofactors for the one or two dehydrogenases that may participate in L-tyrosine biosynthesis. The expanding base of information on the distribution of these diverse features in nature has been reviewed (Byng et al. 1982). Given an evolutionary tree for a manageable group of organisms, such diversity offers bright prospects for deducing the evolutionary history of aromatic biosynthesis (Jensen 1985). Phylogenetic relationships of prokaryotes (Fox et al. 1980; Stackebrandt and Woese 1981) are now being constructed by oligonucleotide cataloging of 16S rRNA. By comparison of the biosynthetic pathway that exists in each present-day organism with its dendrogram postion derived by rRNA oligonucleotide cataloging, plausible events (gene duplication, etc.) can be postulated for the evolution of the pathway within well-defined sections of the dendrogram (Byng et al. 1983b; Jensen 1985). For example, in considering pseudomonad group I, no evolutionary changes were found with respect to L-tyrosine biosynthetic enzymes (Byng et al. 1980) or to the isozyme pattern of the first enzyme committing carbon to aromatic biosynthesis, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase (Byng et al. 1983a). However, relatively recent evolutionary divergence of this group into fluorescent and non-fluorescent species was shown to be paralleled by changes in L-phenylalanine biosynthesis. Members of the non-fluorescent subgroup lack chorismate mutase-F and arogenate dehydratase, components of one of the two biochemical routes to L-phenylalanine that are characteristic of fluorescent pseudomonads (Byng et al. 1983c). A rationale has been presented in support of the evolutionary loss of the arogenate overflow pathway (Fiske et al. 1983) in non-fluorescent pseudomonads, rather than the gain of this character state in the fluorescent pseudomonads (Byng et al. 1983c).

The group I pseudomonads comprise one small part of a suprafamilial group of Gram-negative bacteria designated Superfamily B (Byng et al. 1983b). From rRNA oligonucleotide cataloging studies (Fox et al. 1980; Stackebrandt and Woese 1981), this Superfamily also includes *Xanthomonas*, *Chromatium*, *Vibrio*, *Acinetobacter*, *Oceanospirillum*, and various well-known enteric bacteria. Expansion of the comparative studies of aromatic biosynthesis throughout this Superfamily (of known phylogeny) in concert with deductive reasoning allows the biochemical changes in aromatic biosynthesis to be traced backwards in

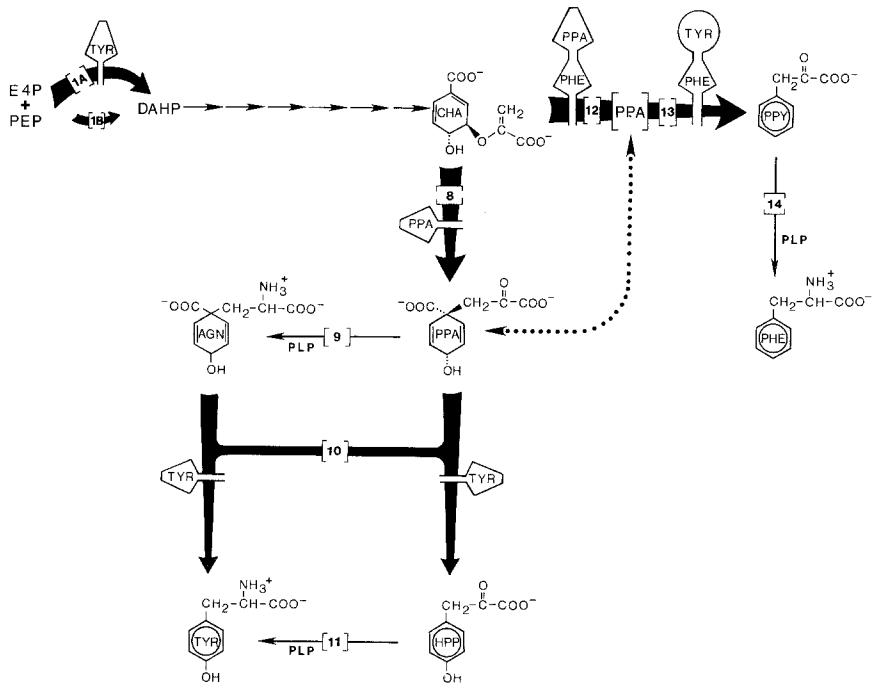

Fig. 1. Route and regulation of phenylalanine and tyrosine biosynthesis in *Acinetobacter calcoaceticus*. Heavy arrows indicate allosteric control points. Effector molecules for each allosteric enzyme are shown, inhibitors being identified within spade-shaped symbols and the activator within a circular symbol. Enzymes 1A and 1B are DAHP synthase-tyr and DAHP synthase-O, respectively. The fractional amounts of each DAHP synthase isozyme are indicated schematically. Enzymes [12] and [13] are chorismate mutase and prephenate dehydratase activities of the bifunctional P-protein. Enzyme [8] is chorismate mutase-F and enzyme [10] is a dehydrogenase capable of utilizing either prephenate or L-arogenate as substrate. Other enzymes: [9], prephenate aminotransferase; [11], 4-hydroxyphenylpyruvate aminotransferase; [14], phenylpyruvate aminotransferase. Abbreviations: *E4P*, D-erythrose-4-phosphate; *PEP*, phosphoenolpyruvate; *DAHP*, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; *CHA*, chorismate; *PPA*, prephenate; *AGN*, L-arogenate; *HPP*, 4-hydroxyphenylpyruvate; *PPY*, phenylpyruvate; *PHE*, L-phenylalanine; *TYR*, L-tyrosine; *PLP*, pyridoxal 5′-phosphate

evolutionary time (Jensen 1985). The genus *Acinetobacter* diverges at an early point in Superfamily B, prior to the separation of group I pseudomonads and enterics (Jensen 1985). As such, details of aromatic biosynthesis in the *Acinetobacter* lineage have already had a significant impact on deductions concerning aromatic-pathway evolution within Superfamily B. The route and regulation of L-phenylalanine and L-tyrosine biosynthesis in present-day *Acinetobacter* species is summarized in Fig. 1.

Materials and methods

Microbiological procedures. Acinetobacter lwoffii ATCC 15309 and *A. calcoaceticus* ATCC 23055 and ATCC 14987 were obtained from the American Type Culture Collection (Rockville, MD, USA). *Acinetobacter* strain C1W was obtained form James R. Stewart, University of Texas at Tyler. All ATCC strains were maintained and grown under conditions recommended in the 1982 ATCC catalog of strains. *Acinetobacter* C1W was grown on Difco nutrient broth, solidified (when necessary) with 1.5% Difco agar. Liquid cultures were grown at 25° C to the late-exponential phase of growth on a New Brunswick rotary shaker at 150 rpm. Cells were harvested by centrifugation, washed twice with cold 50 m*M* potassium phosphate buffer (pH 7.0) and stored at − 80° C until needed.

Preparation of extracts. Crude extracts were prepared and desalted at 4° C in 50 m*M* potassium phosphate buffer (pH 7.0) containing 1.0 m*M* dithiothreitol (DTT), as described by Byng et al. (1983c).

DEAE-cellulose chromatography. Crude extract containing up to 100 mg of protein was applied at 4° C to a 1.5 × 20 cm DE-52 (Whatman) ion-exchange column equilibrated with 50 m*M* potassium phosphate buffer (pH 7.0) containing 1.0 m*M* DTT. The protein was washed into the column with 100 ml of equilibration buffer and eluted with 300 ml of a linear gradient of KCl (0 − 0.7 *M*). Each reservoir contained the aforementioned phosphate buffer. Fractions of 2.2 ml were collected. For larger samples of up to 450 mg protein, a 2.5 × 20 cm DE-52 column was used with 330 ml of wash buffer and a 900-ml gradient, collecting 6.6 ml fractions.

Gel-filtration. Fractions recovered from DEAE-cellulose containing the specified enzyme were pooled and concentrated by Amicon PM10 filtration. These concentrates were applied separately to an Ultrogel AcA34 gel-filtration column (2.5 × 60 cm) equilibrated in 50 m*M* potassium phosphate buffer (pH 7.0) containing 1.0 m*M* DTT. The column was calibrated for molecular weight determination with the following standards: catalase (210,000), aldolase (158,000), fraction V bovine serum albumin (67,000), hen egg ovalbumin (43,000) and chymotrypsinogen A (25,000). The molecular weight of small proteins was estimated in a similar fashion using a Sephadex G-75 column (2.6 × 75 cm) using albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A (13,700) as markers. Void volumes were determined using blue dextran.

Table 1. Enzyme activities[a] of aromatic amino acid biosynthesis detected in crude extracts

Enzyme assayed	*Acinetobacter calcoaceticus*		*A. lwoffii*	*Acinetobacter*
	ATCC 23055	ATCC 14987	ATCC 15309	C1W
DAHP synthase[b]	56.2	25.2	32.4	87.4
Chorismate mutase	60.2	34.9	44.4	85.2
Prephenate dehydratase[c]	2.2	1.0	1.3	9.8
Arogenate dehydratase	0	0	0	0
Prephenate dehydrogenase[d]	6.3	9.4	6.6	9.3
Arogenate dehydrogenase[d]	3.9	4.3	6.3	2.1

[a] All data are given as specific activities expressed as nanomol of product formed per minute per milligram of protein

[b] Specific activities for DAHP synthase in *A. calcoaceticus* ATCC 23055 and ATCC 14987, as well as *Acinetobacter* C1W, were determined from assays run in the presence of 1.0 m*M* $MnSO_4$. Specific activity for DAHP synthase from *A. lwoffii* ATCC 15309 was determined by assaying in the presence of 1.0 m*M* $CoSO_4$

[c] Prephenate dehydratase activity in crude extract of *Acinetobacter* C1W was barely detectable in the absence of L-tyrosine. Thus, the specific activity value given was obtained by assaying in the presence of 0.5 m*M* L-tyrosine

[d] Assayed with $NADP^+$; NAD^+ did not substitute

Analytical procedure. Chorismate mutase and prephenate dehydratase were assayed as described by Patel et al. (1977) Arogenate dehydratase was assayed by the method of Shapiro et al. (1981). Partially purified preparations of chorismate mutase were shown to be free of aromatic aminotransferase activities (necessary to avoid interference when screening for potential sensitivity to allosteric effectors) using the spectrophotometric assay described by Patel et al. (1977). DAHP synthase was assayed using the method of Srinivasan and Sprinson (1959) as modified by Jensen and Nester (1966). Prephenate dehydrogenase and arogenate dehydrogenase assay procedures have been previously described (Byng et al. 1980). Care was taken to assure that proportionality of velocity was obtained with respect to protein concentration and elapsed reaction time and that saturating substrate concentrations were used. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as the reference protein.

Biochemicals. Protein standards for molecular weight determinations, amino acids, D-erythrose-4-phosphate, phenylpyruvate, phosphoenolpyruvate, DTT, Sephadex G-25, and Sephadex G-75 were obtained from Sigma. Ultrogel AcA34 was obtained from LKB. Chorismate and prephenate were isolated and purified from culture supernatants of *Klebsiella pneumoniae* (Gibson 1964) and *Salmonella typhimurium* (Dayan and Sprinson 1970), respectively. The isolation and preparation of L-arogenate from a multiple auxotroph of *Neurospora crassa* was carried out as previously described (Jensen et al. 1977; Zamir et al. 1980) except that the eluent used in the final desalting purification step (Sephadex G-10) was 10 m*M* sodium phosphate buffer at pH 7.6.

Results

Enzymological patterning. Key enzymes of aromatic amino acid biosynthesis were compared in crude extracts of two *Acinetobacter calcoaceticus* strains, one strain of *A. lwoffii*, and *Acinetobacter* strain C1W. Activities of DAHP synthase and chorismate mutase, allosteric enzymes common to both phenylalanine and tyrosine biosynthesis, were detected (see Table 1). DAHP synthase activities in crude extracts were tested for stimulation by divalent cations. In both *A. calcoaceticus* strains as well as *Acinetobacter* C1W, Mn^{2+} promoted the greatest stimulation of DAHP synthase activity, whereas Co^{2+} stimulated activity to a lesser extent. The reverse results were found in *A. lwoffii*. Mg^{2+} had no effect on DAHP synthase from any strain. DAHP synthase activity was inhibited only by L-tyrosine, greater than 92% inhibition being achieved at 0.1 m*M* L-tyrosine in all four cases. For phenylalanine synthesis, prephenate dehydratase was detected, but not arogenate dehydratase. Dual pathways for tyrosine biosynthesis were implicated by the presence of both arogenate dehydrogenase and prephenate dehydrogenase activities. Dehydrogenase activity with either prephenate or L-arogenate required $NADP^+$, a cofactor requirement that could not be satisfied by NAD^+. Dehydrogenase activity was sensitive to feedback inhibition by L-tyrosine when approximately K_m levels (see below) of either cyclohexadienyl substrate were used.

Characterization of partially purified enzymes. A. calcoaceticus 23055 was chosen for in-depth study, and a crude extract of this strain was fractionated by chromatography over DEAE-cellulose. The elution positions of key enzymes of aromatic biosynthesis are shown in Fig. 2. Two peaks of activity were recovered for DAHP synthase and for chorismate mutase. Only a residual trace of prephenate dehydratase activity was recovered, eluting exactly coincident with the gradient peak of chorismate mutase. The addition of L-tyrosine has been shown to be essential for prephenate dehydratase activity in some organisms (Fazel and Jensen 1980). However, addition of up to 0.5 m*M* L-tyrosine did not stimulate this residual prephenate dehydratase activity. Addition of 0.1 m*M* L-tyrosine and/or 20% glycerol to the sonication buffer (prior to cell disruption) and to all subsequent column buffers did not stabilize this enzyme. Prephenate dehydrogenase activity and arogenate dehydrogenase activity were found to co-elute in the gradient.

i) Chorismate mutase. The peak of activity denoted chorismate mutase-F in Fig. 2 was separate form prephenate dehydrogenase, and therefore *Acinetobacter* species lack the bifunctional T-protein (chorismate mutase-prephenate dehydrogenase) that is found in enteric bacteria (Cotton and Gibson 1965; Dayan and Sprinson 1971; Koch et al. 1970, 1971). Chorismate mutase-F was found to be product inhibited by prephenate but was not inhibited by L-phenylalanine, L-tyrosine, or L-tryptophan. This monofunctional chorismate mutase resembles chorismate mutase-F of subgroup Ib pseudomonads (Byng et al. 1983a) and of the xanthomonad lineage (Whitaker et al. 1985) within

Superfamily B. Chorismate mutase-F of *A. calcoaceticus* 23055 eluted from Sephadex G-75 at a molecular-weight position of 23,000. Other properties of this enzyme are given in Table 2. In addition, chorismate mutase-F from *A. calcoaceticus* 14987 was examined and was found to be very similar to chorismate mutase-F from *A. calcoaceticus* 23055, the former enzyme having a molecular weight of 21,000, a K_m of 0.08 m*M*, and a K_i (for competitive inhibition by prephenate) of 0.123 m*M*.

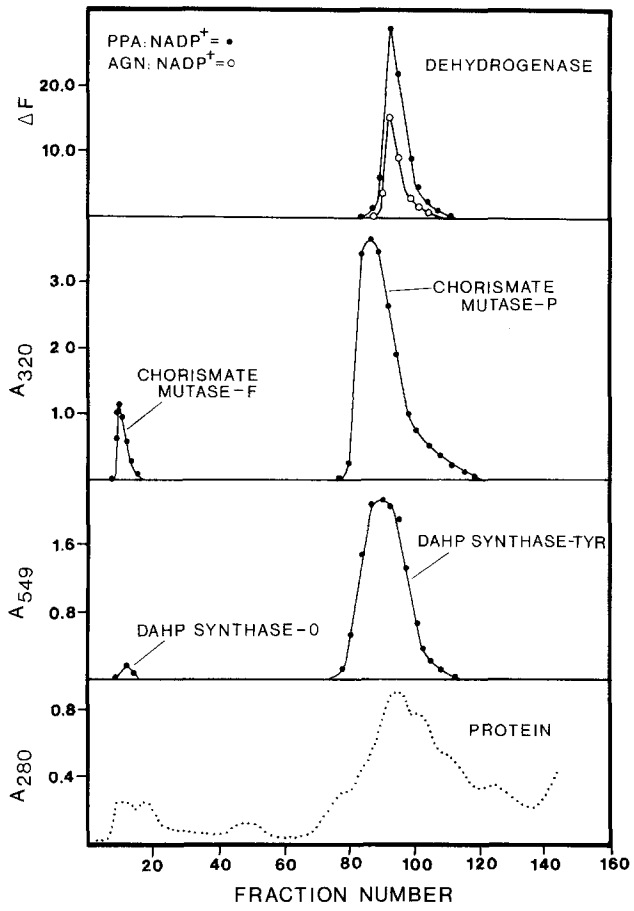

Fig. 2. Elution of key aromatic-pathway enzymes from a crude extract of *A. calcoaceticus* 23055 following DEAE-cellulose (DE-52) fractionation. The first 50 fractions were wash eluate recovered before application of the salt gradient. The following specific activities (given as nanomol of product formed per minute per milligram of protein) of peak eluate fractions were obtained: DAHP synthase-tyr (31.5), DAHP synthase-O (36.9), chorismate mutase-P (56.8), chorismate mutase-F (15.9), prephenate (PPA) dehydrogenase (16.1), arogenate (AGN) dehydrogenase (8.9). Dehydrogenase activities were measured using $NADP^+$ as cofactor

The chorismate mutase activity which eluted in the salt gradient is denoted chorismate mutase-P. This activity was subject to noncompetitive inhibition by L-phenylalanine as well as product inhibition by prephenate. Chorismate mutase-P was found to have a molecular weight of 79,000. Other characteristics are given in Table 2. Chorismate mutase-P in some other bacteria, including all members of Superfamily B studied so far, is a member activity of a bifunctional enzyme along with prephenate dehydratase (P-protein). Although only a small fraction of the original prephenate dehydratase activity (Table 1) was recovered following ion-exchange chromatography, this activity did co-elute exactly with chorismate mutase-P, suggesting the existence of bifunctional P-protein in *Acinetobacter*. Indeed, when crude extracts of *A. calcoaceticus* strain 14987 or *Acinetobacter* strain C1W were subjected to the same chromatographic procedure used with *A. calcoaceticus* 23055, nearly indentical profiles of all aromatic-pathway enzymes were obtained with the exception that prephenate dehydratase activities were much higher. The latter activities were stable and co-eluted exactly with chorismate mutase-P. These prephenate dehydratases both were sharply activated by L-tyrosine. Further studies with *A. calcoaceticus* 14987 revealed that prephenate dehydratase and chorismate mutase-P do in fact exist as a bifunctional protein. Details have been reported separately (Berry et al. 1985a).

ii) DAHP synthase. Two isozymes of DAHP synthase were recovered (Fig. 2); a major peak of activity (denoted DAHP synthase-tyr) eluted in the KCl gradient, and a minor peak of activity (DAHP synthase-O) eluted in the wash fractions. Each isozyme was tested for stimulation by divalent cations. DAHP synthase-tyr was stimulated maximally by Co^{2+} (33.7-fold at 1.0 m*M* cation), whereas Mn^{2+} had a lesser effect. DAHP synthase-O was also stimulated by Mn^{2+} and Co^{2+} (1.75-fold and 1.3-fold, respectively, at 1.0 m*M* cation).

DAHP synthase-tyr was subject to strong feedback inhibition by L-tyrosine. The properties of this enzyme are summarized in Table 2. Other potential effectors such as L-phenylalanine, prephenate, L-tryptophan, chorismate, or L-

Table 2. Properties of partially purified enzymes of aromatic biosynthesis from *A. calcoaceticus* ATCC 23055

Enzyme	Molecular weight	Substrate$_1$	K_{m_1}	Substrate$_2$	K_{m_2}	Effector(s)	K_{i_1}	K_{i_2}	Inhibition kinetics[a]
DAHP synthase-tyr	43,000	E4P	0.286	PEP	1.00	TYR	0.017	0.011	NC_1 NC_2
DAHP synthase-O	44,700	Not done	Not done	Not done	Not done	None			
CHA mutase-F	23,000	CHA	0.057			PPA	0.140		C
CHA mutase-P	79,000	CHA	0.800			PHE/PPA	1.53/1.32		NC/C
PPA dehydrogenase	210,000	PPA	0.033	$NADP^+$	0.005	TYR	0.009	0.038	C_1 NC_2
AGN dehydrogenase	210,000	AGN	0.313	$NADP^+$	0.005	TYR	0.009	0.035	C_1 NC_2

K_m and K_i data are expressed in m*M*. Subscripts refer to first and second substrates. For example, K_{m_2} is the K_i value for the specified inhibitor when the substrate listed under substrate$_2$ was varied. Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; E4P, D-erythrose-4-phosphate; PEP, phosphoenolpyruvate; TYR, L-tyrosine; CHA, chorismate; PPA, prephenate; PHE, L-phenylalanine; AGN, L-arogenate; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate

[a] Inhibition type: NC, noncompetitive; C, competitive

arogenate did not affect activity. Phenylpyruvate did not inhibit DAHP synthase, but was found to inhibit the chemical assay of DAHP. *Pseudomonas aeruginosa* was previously observed to possess a DAHP synthase-tyr isozyme subject to apparent inhibition by high concentrations of phenylpyruvate (Jensen et al. 1973, Whitaker et al. 1982). However, this is also due to inhibition of the chemical assay by phenylpyruvate (unpublished observations). The apparent substrate affinities of *A. calcoaceticus* 23055 DAHP synthase-tyr for phosphoenolpyruvate (1.0 m*M*) and erythrose-4-phosphate (0.286 m*M*), were derived from Lineweaver-Burk plots. In the latter plots, deviation from linearity occurred at high concentrations of both substrates (greater than 1.0 m*M*). Therefore, the K_m values reported were extrapolated from the linear portions of the plots.

The minor isozyme, DAHP synthase-O, was not affected by the addition of L-tyrosine, L-phenylalanine, L-tryptophan (or combinations of these possible effectors), chorismate, prephenate or L-arogenate (up to 0.5 m*M* each). Phosphate ion was found to be inhibitory, with 50% inhibition occurring at about 50 m*M* potassium phosphate, pH 7.0. However, replacement of the phosphate buffer with PIPES [piperazine-N,N′-bis(2-ethane sulfonic acid)] or MOPS [3-(N-morpholino) propane sulfonic acid] buffer at the same molarity and pH resulted in instability of the enzyme. The initial low activity of this minor isozyme, coupled with phosphate inhibition and enzyme instability made further characterization difficult.

When crude extracts of *A. calcoaceticus* 14987 or *Acinetobacter* C1W were fractionated on DEAE-cellulose columns, two isozymes of DAHP synthase were found that exhibited elution patterns and general regulatory properties identical to the DAHP synthase isozymes from *A. calcoaceticus* 23055 (data not shown).

iii) Dehydrogenase activities. Prephenate dehydrogenase and arogenate dehydrogenase activities co-eluted from a DEAE-cellulose column (Fig. 2). Both activities also co-eluted from a gel-filtration column at an identical molecular-weight position of 210,000. Arogenate dehydrogenase and prephenate dehydrogenase activities both were specific for $NADP^+$ as cofactor. The characterization of both activities is summarized in Table 2. An almost ten-fold difference in the K_m values for prephenate and L-arogenate were observed. However, both activities displayed the same affinity for cofactor, and both were inhibited by L-tyrosine (competitively with respect to substrate and noncompetitively with respect to cofactor). When the substrate for each dehydrogenase activity was used at approximately K_m level, identical sigmoid inhibition curves for L-tyrosine were obtained (Fig. 3). The K_i for L-tyrosine inhibition with respect to either substrate was identical (9 μM). Likewise, the K_i values for L-tyrosine inhibition with respect to $NADP^+$ were similar, regardless of whether prephenate or L-arogenate was used as substrate (see Table 2).

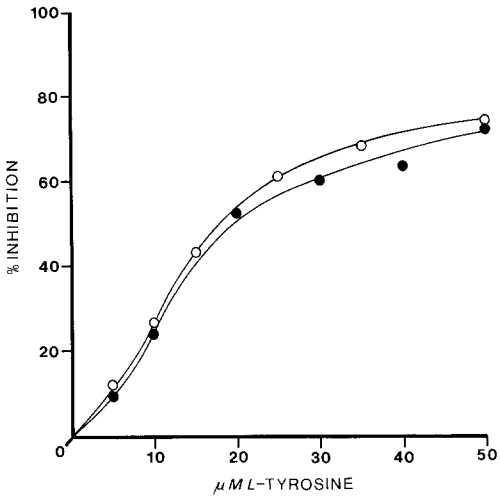

Fig. 3. Inhibition curves showing the effect of L-tyrosine upon prephenate dehydrogenase (○) and arogenate dehydrogenase (●) activities from *A. calcoaceticus* 23055. Assays were conducted using preparations obtained following chromatography of a crude extract on DEAE-cellulose. The concentrations of substrates used were: L-arogenate, 0,5 m*M*; prephenate, 0.05 m*M*, $NADP^+$, 0.5 m*M*

Discussion

Within the Superfamily-B assemblage of prokaryotes, comparative characterizations of aromatic-pathway enzymes have been completed for a large number of organisms that occupy established positions on a phylogenetic tree. Extensive, in-depth characterizations of the entire pathway, however, are only feasible for selected organisms representing major lineage sections of the dendrogram. Such a background of comprehensive data are available (see Jensen 1985) for *Escherichia coli*, *Xanthomonas campestris*, *Pseudomonas aeruginosa*, and now *Acinetobacter calcoaceticus*. Figure 4 shows a summary scheme of major evolutionary events postulated for Superfamily-B members. The order of evolutionary events depicted has been established through the study of many other organisms that branch off from the major dendrogram lineages at various evolutionary intervals.

L-Phenylalanine biosynthesis. Every member of Superfamily B thus far studied (Byng et al. 1983c; Calhoun et al. 1973; Cotton and Gibson 1965; Davidson et al. 1972; Schmit and Zalkin 1969; Whitaker et al. 1985) possesses a bifunctional P-protein (chorismate mutase-prephenate dehydratase), and *A. calcoaceticus* is no exception (Berry et al. 1985a). However, *A. calcoaceticus* lacks arogenate dehydratase and thus relies exclusively on the phenylpyruvate route for L-phenylalanine synthesis.

It has been postulated (Jensen 1985) that dual pathways to phenylalanine (vial phenypyruvate and L-arogenate) existed in the ancestral stem of Superfamily-B organisms. This pathway arrangement has been retained in organisms such as the present-day *P. aeruginosa* (Fiske et al. 1983; Patel et al. 1977). Along the *Acinetobacter* lineage, one evolutionary event was the loss of arogenate dehydratase. Established of dendrogram positions of other organisms along the *Acinetobacter* lineage should allow a more accurate determination of the point at which the loss of arogenate dehydratase occurred.

Chorismate mutase-F. In *E. coli* and other closely related enterics, the bifunctional T-protein (chorismate mutase-prephenate dehydrogenase) presumably arose by fusion of ancestral genes for prephenate dehydrogenase and chorismate mutase-F (Jensen 1985). The putative Superfamily-B

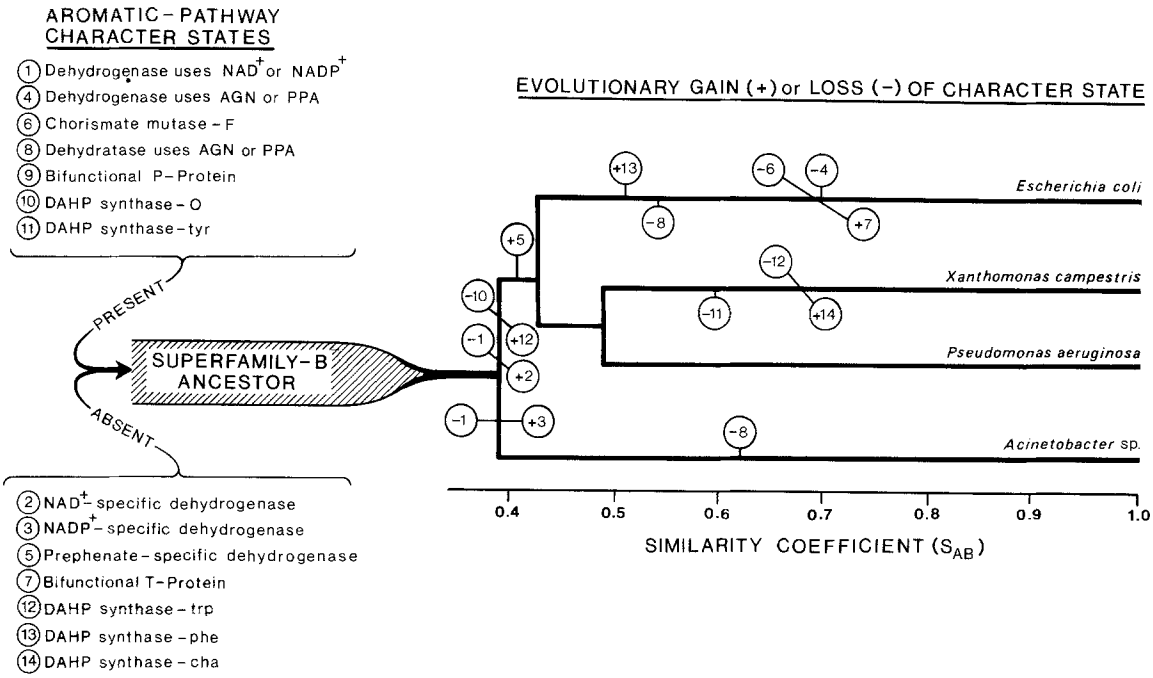

Fig. 4. Evolutionary changes in aromatic biosynthesis and regulation deduced within four major lineages of Superfamily-B prokaryotes. A full rationale for evolutionary conclusions was developed by Jensen (1985). S_{AB} values given along the abscissa scale were obtained by Woese and co-workers using oligonucleotide cataloging. The evolutionary order of events indicated by the positions of character-state alterations in many cases depends upon data from other organisms not shown (see Jensen 1985). In a number of instances loss of one character state is tied to gain of another character state and is assumed to have been a single event. For example, $-1 \rightarrow +3$: specialization of a dehydrogenase broadly specific for nucleotide cofactor to become specific for $NADP^+$; or $-12 \rightarrow +14$: marked decrease in sensitivity of DAHP synthase to inhibition by L-tryptophan accompanied by greatly increased sensitivity to inhibition by chorismate. Consult Fig. 1 for abbreviations and for relationships of enzymes shown to the overall pathway of aromatic amino acid biosynthesis

ancestor (in which the latter fusion event had not yet occurred) would have employed chorismate mutase-F to form prephenate molecules fated for either (i) transformation to L-tyrosine via 4-hydroxyphenylpyruvate, or (ii) transformation to L-phenylalanine (or L-tyrosine) via L-arogenate. The lack of arogenate dehydratase in *Acinetobacter* restricts the function of chorismate mutase-F entirely to L-tyrosine biosynthesis.

L-Tyrosine biosynthesis. In contrast to the presence of the P-protein for L-phenylalanine synthesis throughout Superfamily B [and possibly Superfamily A (Berry et al. 1985b)], the bifunctional T-protein is not widely distributed, being a recent evolutionary acquisition of *E. coli* and related enterics. *Acinetobacter* and many other Superfamily-B organisms possess dual pathways to L-tyrosine (via 4-hydroxyphenylpyruvate and L-arogenate). *Acinetobacter* apparently has retained the single dehydrogenase (having substrate ambiguity for prephenate and L-arogenate) proposed to have existed in the ancestral stem organism of Superfamily B (Byng et al. 1983b; Jensen 1985). The existence of a single dehydrogenase protein is consistent with the findings that prephenate dehydrogenase and arogenate dehydrogenase activities co-eluted from several chromatographic columns, that both activities exhibited identical apparent K_m values for $NADP^+$, and that both activities exhibited identical apparent K_i values for L-tyrosine (feedback inhibitor). In *P. aeruginosa*, a Group I pseudomonad, the two dehydrogenases also could not be separated following various chromatographic steps, but apparently are separate proteins. Thus, dehydrogenase-deficient mutants were not isolated (Patel et al. 1978), and K_i values for L-tyrosine varied depending on the substrate utilized.

Evolution of cofactor specificity of aromatic-pathway dehydrogenases. The divergence of *Acinetobacter* is at one of the earliest known branch points in the Superfamily-B lineage. *Acinetobacter* species differ from all other Superfamily-B members in their requirement for $NADP^+$ for function of prephenate/arogenate dehydrogenase. These dehydrogenase activities in other Superfamily-B lineages require NAD^+ (Byng et al. 1982, 1983b, Jensen 1985). The simplest interpretation of this dichotomy is that the dehydrogenase from the ancestral Superfamily-B organism possessed a cofactor site able to accomodate either of the pyridine nucleotides. That this cofactor ambiguity probably existed in an even earlier ancestor common to the evolutionary divergence of all three superfamilies of Gram-negative bacteria is depicted in Fig. 4. All members of Superfamily A (and some members of Superfamily C) thus far studied exhibit cofactor ambiguity for aromatic-pathway dehydrogenases (Jensen 1985). In Superfamily B, specialization for $NADP^+$ (*Acinetobacter* lineage), or for NAD^+ (all other lineages) must have occurred at an early evolutionary time.

Evolution of DAHP synthase isozymes. With the exception of *Xanthomonas* (Whitaker et al. 1985) and closely-related organisms (unpublished data), all members of Superfamily B thus far studied possess an L-tyrosine-inhibited DAHP

synthase isozyme [see Jensen (1985) for review]. The major DAHP synthase activity of the isozyme pair contained within *Acinetobacter* is a tyrosine-sensitive isozyme. This is probably the most ancient species of DAHP synthase since members of pseudomonad Group III (a Superfamily-A group) also possess an L-tyrosine sensitive DAHP synthase isozyme (Berry et al. 1985b).

The minor isozyme of DAHP synthase in *Acinetobacter* (DAHP synthase-O) was found to be insensitive to all possible allosteric effectors tested. The only other Superfamily-B member in which DAHP synthase-O has been detected is *Oceanospirillum minutulum* (S. Ahmad et al., in preparation). The pseudomonad Group I lineage also possesses a pair of DAHP synthase isozymes (Byng et al. 1983a), differing from *Acinetobacter* only in that the minor isozyme species is feedback inhibiting by L-tryptophan (DAHP synthase-trp). It is possible that DAHP synthase-O from *Acinetobacter* has a fragile allosteric site for L-tryptophan that is readily desensitized in vitro. A second possibility is that DAHP synthase-O of *Acinetobacter* is a conserved feature of an ancestral state which predated the evolution of allosteric sensitivity to L-tryptophan. A possible origin for the allosteric sensitivity to L-tryptophan of DAHP synthase-trp in *P. aeruginosa* has been proposed (Whitaker et al. 1982) whereby a gene encoding a catalytic subunit of DAHP synthase fused with a duplicate of *trpA* (which encodes the L-tryptophan-regulated aminase subunit of anthranilate synthase). In this scenario the unregulated DAHP synthase-O isozyme in *A. calcoaceticus* may reflect the survival of a lineage in which the putative fusion event never occurred.

Heterogeneity within the Acinetobacter lineage. DNA hybridization studies have demonstrated only weak homology between certain *Acinetobacter* strains (Johnson et al. 1970). At a deeper level, rRNA oligonucleotide cataloging of *A. calcoaceticus* ATCC 14987 and ATCC 23055 shows them to be related by a similarity coefficient (S_{AB}) of only 0.66 (Fox and Woese, personal communication). This approximates the evolutionary divergence of the most distantly related genera within the assemblage of Gram-negative enterics. The fluorescent and nonfluorescent subgroups of Group I pseudomonads are related by an S_{AB} value of 0.77. Since we saw substantial evolutionary changes in the aromatic pathway that paralleled the divergence of these pseudomonad subgroups (Byng et al. 1983c), one might expect to see pathway differences in the even more heterogeneous (on the criterion of S_{AB} value) *Acinetobacter* line of descent. We did not. This illustrates that the evolution of metabolic pathways can be conservative across wide phylogenetic spans in some cases, but can be dynamic across more narrow spans in other cases. It indicates the value, indeed the necessity, of having an established phylogenetic tree available if biochemical pathways are to be traced backwards through evolutionary time.

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