JOURNAL OF BACTERIOLOGY, Oct. 1983, p. 429–433
0021-9193/83/100429-05$02.00/0

Vol. 156, No. 1

A Pair of Regulatory Isozymes for 3-Deoxy-D-*arabino*-Heptulosonate 7-Phosphate Synthase Is Conserved Within Group I Pseudomonads

GRAHAM S. BYNG,* ALAN BERRY, AND ROY A. JENSEN

Center for Somatic-Cell Genetics and Biochemistry, State University of New York at Binghamton, Binghamton, New York 13901

Received 18 April 1983/Accepted 18 July 1983

Two closely related subgroups of group I pseudomonads, which differ from one another in the overall enzymatic makeup of aromatic amino acid biosynthesis, possess in common the recently characterized major (tyrosine-sensitive) and minor (tryptophan-sensitive) isozymes of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase of *Pseudomonas aeruginosa* (17). Since these characterizations were made for strains whose phylogenetic positions have been determined by oligonucleotide cataloging, an initial perception of the evolution of aromatic pathway construction and regulation is emerging.

Until recently, the objective of tracing the evolution of biochemical pathways seemed largely unapproachable for procaryotes because of the seeming impossibility of establishing firm phylogenetic relationships. However, the suitability of oligonucleotide cataloging (7, 14) for construction of definitive phylogenetic trees means that the direction of biochemical pathway changes in evolutionary time can now be determined for any set of strains whose evolutionary relationships with one another are known. A managable example of such a well-defined bacterial group is the group I pseudomonads, which diverge to form two distinct subgroups (13). Subgroup Ia contains only four species at present: *Pseudomonas mendocina*, *P. alcaligenes*, *P. pseudoalcaligenes*, and *P. stutzeri*, all of which are nonfluorescent species. Subgroup Ib, which makes up the vast majority of group I species, contains such well-known fluorescent species as *P. aeruginosa*, *P. putida*, *P. syringae*, and *P. fluorescens*.

The pathway of aromatic biosynthesis is diverse in nature, varying in enzyme steps used, allosteric control patterns, and cofactor specificity for pathway dehydrogenases (2, 3). Subgroups Ia and Ib differ with respect to phenylalanine biosynthesis (16). Subgroup Ib has two separate pathways to L-phenylalanine, one proceeding through phenylpyruvate and the other through L-arogenate. The latter flow route (consisting of an unregulated chorismate mutase, prephenate aminotransferase, and an unregulated arogenate dehydratase) has been shown to behave as an overflow pathway to L-phenylalanine in *P. aeruginosa* (6). During the growth of *P. aeruginosa* on carbon sources (e.g., glucose) which favor formation of initial substrates entering the aromatic pathway, an excess level of L-phenylalanine is generated through the unregulated overflow pathway. When 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase-tyrosine is rendered insensitive to feedback inhibition by mutation, prephenate dehydratase (a later point of allosteric regulation) is effectively bypassed by the presence of the overflow pathway, and considerable L-phenylalanine is excreted. Subgroup Ia lacks the overflow pathway, since member species lack the unregulated chorismate mutase (G. S. Byng, A. Berry, R. J. Whitaker, and R. A. Jensen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983. K250, p. 166.) as well as arogenate dehydratase (16). The direction of evolutionary change is deduced to be that of enzyme loss in subgroup Ia, since another group of organisms (group V pseudomonads) which diverge from group I pseudomonads at a deeper phylogenetic level (2) are identical to subgroup Ib pseudomonads with respect to phenylalanine enzyme arrangement (16).

The regulatory pattern of DAHP synthase has long been recognized as a relatively conservative characteristic of microorganisms (3, 11, 12). In an extensive comparative survey, DAHP synthase activities of all group I pseudomonads were found to be sensitive primarily to feedback inhibition by L-tyrosine (15). However, the DAHP synthase activity of *P. aeruginosa* was recently resolved into two regulatory isozymes (17). A major (>90%) isozyme called DAHP synthase-tyrosine was found to be highly sensitive to feedback inhibition by L-tyrosine, whereas a minor (<10%) isozyme called DAHP synthase-tryptophan was very sensitive to feedback

inhibition by L-tryptophan. The minor isozyme is not readily detected in crude extracts, a circumstance which raised the question of whether the minor regulatory isozyme was characteristic of only subgroup Ib or of the entire assemblage of group I pseudomonads. Hence, a more detailed reevaluation of selected representatives of group I species was carried out, as reported in this paper.

DAHP synthase was assayed as described by Calhoun et al. (5), and protein concentrations of extracts were estimated by the method of Bradford (1). All cultures were grown, harvested, and used to prepare crude extracts free of small molecules, as previously described (17). Strains representing three subgroup Ia species (*P. alcaligenes*, *P. mendocina*, and *P. stutzeri*) were compared with *P. aeruginosa* (ATCC 15692) and *P. fluorescens* (ATCC 13525) the latter two chosen as representative species of subgroup Ib. Isozymes of DAHP synthase were fractionated by ion-exchange chromatography with DEAE-cellulose, as illustrated in Fig. 1 for *P. alcaligenes*, *P. fluorescens*, and *P. aeruginosa*. Two distinct isozymes were discerned for all five species, one eluting as a major peak and the other as a minor peak. Except for *P. fluorescens*, each major isozyme was recovered in the wash elute, whereas each minor isozyme eluted in the salt gradient.

Appropriate fractions recovered after DEAE-cellulose chromatography were characterized for sensitivity of DAHP synthase to potential feedback inhibitors (Tables 1 and 2). Each isozyme was assayed in the presence of a 0.5 mM concentration of L-tyrosine, L-tryptophan, phenylpyruvate, chorismate, or L-phenylalanine. Chorismate was prepared by the method of Gibson (8). Neither isozyme from any of the species studied was inhibited by L-phenylalanine. Although phenylpyruvate has been shown to be a weak competitive inhibitor of DAHP synthase-tyrosine in *P. aeruginosa* (17), a fact which may be physiologically significant (10),

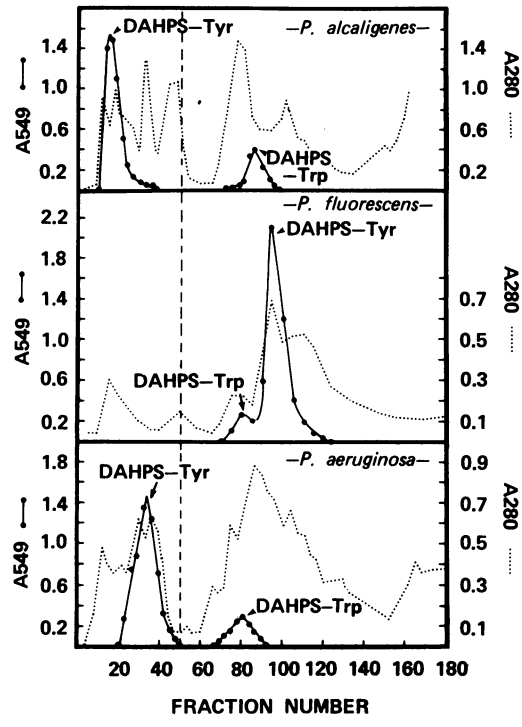

FIG. 1. Elution profiles of DAHP synthase-tyrosine and DAHP synthase-tryptophan isozymes resolved by DEAE-cellulose chromatography. Crude extracts prepared from the three species shown and containing up to 100 mg of protein were applied individually at 4°C to Whatman DE-52 ion-exchange columns (1.5 by 20 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) and 1 mM dithiothreitol. The loaded columns were washed with 100 ml of equilibration buffer, and bound proteins were eluted with 300-ml volumes of KCl applied as a linear gradient between 0 and 0.5 M KCl. Each reservoir also contained the equilibration buffer. The vertical dashed lines indicate the onset point of gradient elution. Fractions of 2.2 ml were collected and assayed for DAHP synthase activity, expressed here as A549. The distribution of protein elution is shown by dotted lines.

TABLE 1. Characteristics of the DAHP synthase-tyrosine isozyme isolated from group I pseudomonads

Subgroup	*Pseudomonas* spp.	% Inhibition[a] by:			Sp act[b]	% Total activity[c]
		Tyr	Trp	CHA		
Ib	*P. fluorescens* ATCC 13525	95	0	9	102	93
Ib	*P. aeruginosa* ATCC 15692	94	0	2	63	94
Ia	*P. alcaligenes* ATCC 14909	91	0	0	118	82
Ia	*P. stutzeri* ATCC 17588	96	0	0	110	99
Ia	*P. mendocina* ATCC 25411	96	1	6	307	93

[a] The final concentration of L-tyrosine (Tyr), L-tryptophan (Trp), or potassium chorismate (CHA) was 0.5 mM.

[b] Nanomoles of DAHP formed per minute per milligram of protein at 37°C.

[c] Fraction of total DAHP synthase activity recovered as DAHP synthase-tyrosine.

TABLE 2. Characteristics of the DAHP synthase-tryptophan isozyme isolated from group I pseudomonads

Subgroup	*Pseudomonas* spp.	% Inhibition[a] by:			Sp act[b]	% Total activity[c]
		Tyr	Trp	CHA		
Ib	*P. fluorescens* ATCC 13525	0	81	43	20	7
Ib	*P. aeruginosa* ATCC 15692	0	70	40	38	6
Ia	*P. alcaligenes* ATCC 14909	21	97	56	31	18
Ia	*P. stutzeri*[d] ATCC 17588				6	1
Ia	*P. mendocina* ATCC 25411	33	94		9	7

[a] The final concentration of L-tyrosine (Tyr), L-tryptophan (Trp), or potassium chorismate (CHA) was 0.5 mM.

[b] Nanomoles of DAHP formed per minute per milligram of protein at 37°C.

[c] Fraction of total DAHP synthase activity recovered as DAHP synthase-tryptophan.

[d] A minor isozyme of DAHP synthase was recovered from DEAE-cellulose, but activity was too low for determinations of inhibitor sensitivities.

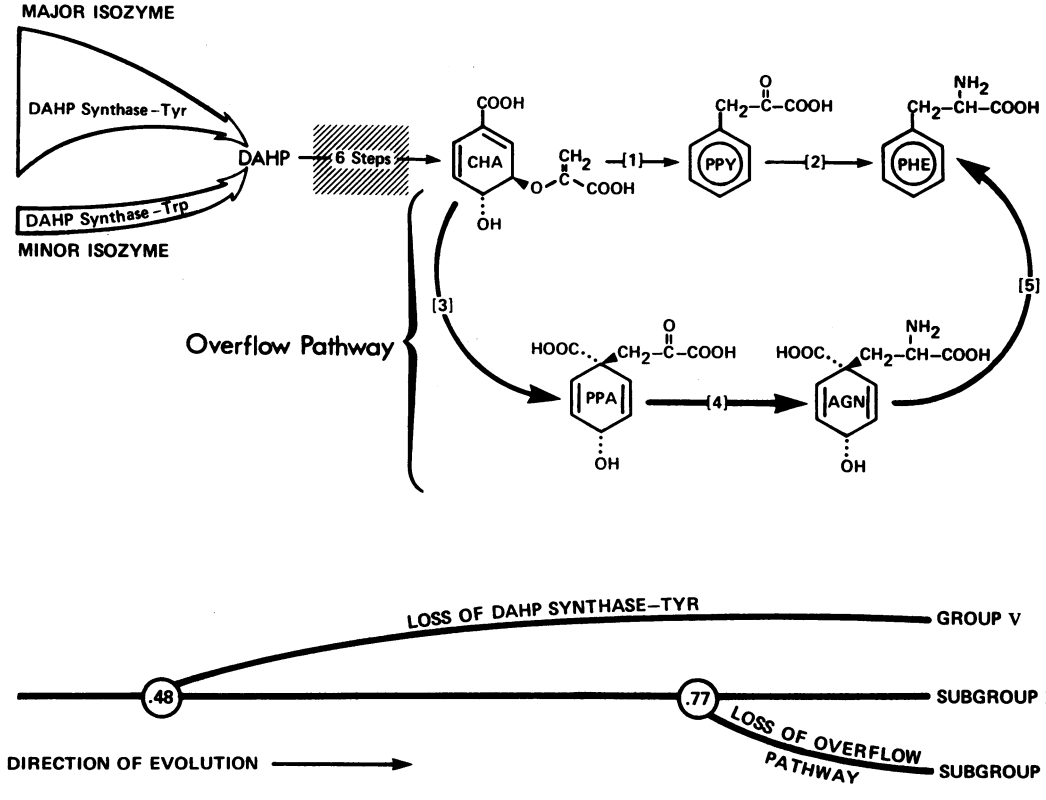

FIG. 2. Evolution of biochemical pathway for aromatic biosynthesis in pseudomonad groups I and V. An abbreviated scheme showing the relevant sections of the pathway is illustrated at the top of the figure. The isozymes of DAHP synthase and the dual routing to L-phenylalanine are suggested as the ancestral arrangement existing at the evolutionary time shown at the far left of the dendogram drawn at the bottom of the figure. This ancestral arrangement is retained unchanged in subgroup Ib. Enzyme denotations are: [1], the multifunctional "P" protein (6) consisting of chorismate (CHA) mutase and prephenate (PPA) dehydratase; [2], phenylpyruvate (PPY) aminotransferase; [3], chorismate mutase; [4], prephenate aminotransferase; and [5], arogenate (AGN) dehydratase. Enzymes [3] through [5] constitute an unregulated overflow pathway to L-phenylalanine, which is lost in subgroup Ia. The circled numbers in the dendogram are S_{AB} (similarity coefficient) values obtained by oligonucleotide cataloging (7, 14).

this sensitivity is not readily detected at the saturating substrate concentrations used. Table 1 shows that each major isozyme (recovered as >80% of total DAHP synthase activity) was feedback-inhibited by L-tyrosine but not by L-tryptophan or chorismate. Therefore, each major isozyme can be designated as DAHP synthase-tyrosine.

Table 2 shows the corresponding analysis for the minor isozymes, the results of which identify these as DAHP synthase-tryptophan. L-Tryptophan was a potent inhibitor of the minor isozymes. DAHP synthase-tryptophan from *P. fluorescens* and *P. alcaligenes* also showed the same sensitivity to inhibition by chorismate that was established in *P. aeruginosa* (17). The subgroup Ia species studied both possess DAHP synthase-tryptophan isozymes, which show low but significant sensitivity to inhibition by L-tyrosine. Perhaps this reflects the ancestral origin of these isozymes from a common gene (9).

Figure 2 is a representation of feasible interpretations that can be made about the evolution of aromatic amino acid biosynthesis in one small portion of the procaryotic phylogenetic tree. The groups considered include pseudomonads from group I and V, related to one another by a similarity coefficient, S_{AB} (7) of 0.48. Subgroups Ia and Ib diverge from one another at an S_{AB} of 0.77. The logical ancestral pathway of aromatic biosynthesis common to contemporary organisms that are the end products of this phylogeny is shown at the top of Fig. 1. Details relevant to L-tyrosine synthesis are not shown, since the dual pathways to L-tyrosine present in *P. aeruginosa* are conserved to a phylogenetic depth which includes pseudomonad groups I and V. (However, note that even relatively subtle differences in tyrosine branchlet regulation can enable us to differentiate reliably (4) between group I and group V pseudomonads.)

We suggest that the group V lineage lost an ancestral DAHP synthase-tyrosine (rather than the group I lineage having acquired it) because the enteric lineage which branches off (7) at a deeper phylogenetic level (S_{AB} = 0.42) possesses (9) tyrosine-sensitive and tryptophan-sensitive isozymes of DAHP synthase (in addition to a phenylalanine-sensitive isozyme). Although the single DAHP synthase of group V organisms has been characterized by its feedback sensitivity to chorismate (15), it is also sensitive to feedback inhibition by L-tryptophan. It differs from DAHP synthase-tryptophan isozymes only in having a quantitatively greater relative sensitivity to chorismate inhibition than to L-tryptophan inhibition (R. J. Whitaker, G. S. Byng, and R. A. Jensen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, R260, p. 175).

Since group V pseudomonads (mainly species of *Xanthomonas*) possess the overflow pathway to L-phenylalanine that exists in the subgroup Ib lineage, it follows that contemporary subgroup Ia species probably pursued an evolutionary direction in which the overflow pathway was lost.

This study was supported by grant DEB 78-12099 from the National Science Foundation.

LITERATURE CITED

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
2. **Byng, G. S., J. L. Johnson, R. J. Whitaker, R. L. Gherna, and R. A. Jensen.** 1983. The evolutionary pattern of aromatic amino acid biosynthesis and the emerging phylogeny of pseudomonad bacteria. J. Mol. Evol. **19**:272–282.
3. **Byng, G. S., J. F. Kane, and R. A. Jensen.** 1982. Diversity in the routing and regulation of complex biochemical pathways as indicators of microbial relatedness. Crit. Rev. Microbiol. **9**:227–252.
4. **Byng, G. S., R. J. Whitaker, R. L. Gherna, and R. A. Jensen.** 1980. Variable enzymological patterning in tyrosine biosynthesis as a means of determining natural relatedness among the *Pseudomonadaceae*. J. Bacteriol. **144**:247–257.
5. **Calhoun, D. H., D. L. Pierson, and R. A. Jensen.** 1973. Channel-shuttle mechanism for the regulation of phenylalanine and tyrosine synthesis at a metabolic branch point in *Pseudomonas aeruginosa*. J. Bacteriol. **113**:241–251.
6. **Fiske, M. J., R. J. Whitaker, and R. A. Jensen.** 1983. Hidden overflow pathway to L-phenylalanine in *Pseudomonas aeruginosa*. J. Bacteriol. **154**:623–631.
7. **Fox, G. E., E. Stackebrandt, R. B. Hespell, J. Gibson, J. Maniloff, T. A. Dyer, R. S. Wolfe, W. E. Balch, R. S. Tanner, L. J. Magrum, L. B. Zablen, R. Blakemore, R. Gupta, L. Bonen, B. J. Lewis, D. A. Stahl, K. R. Leuhrsen, K. N. Chen, C. R. Woese.** 1980. The phylogeny of prokaryotes. Science **209**:457–463.
8. **Gibson, F.** 1964. Chorismic acid: purification and some chemical and physical studies. Biochem. J. **90**:256–261.
9. **Jensen, R. A., and G. S. Byng.** 1981. The partitioning of biochemical pathways with isozyme systems p. 143–175. *In* M. C. Ratazzi, J. G. Scandalios, and G. S. Whitt (ed.), Isozymes: Current Topics in Biological and Medical Research, vol 5. A. R. Liss, Inc., New York.
10. **Jensen, R. A., D. H. Calhoun, and S. L. Stenmark.** 1973. Allosteric inhibition of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase by tyrosine, tryptophan and phenylpyruvate in *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **293**:256–258.
11. **Jensen, R. A., D. S. Nasser, and E. W. Nester.** 1967. Comparative control of a branch-point enzyme in microorganisms. J. Bacteriol. **94**:1582–1593.
12. **Jensen, R. A., and J. L. Rebello.** 1970. Comparative allostery of microbial enzymes at metabolic branchpoints; evolutionary implications. Dev. Ind. Microbiol. **11**:105–121.
13. **Palleroni, N. J., R. Kunizawa, R. Contopoulou, and M. Doudoroff.** 1973. Nucleic acid homologies in the genus *Pseudomonas*. Int. J. Syst. Bacteriol. **23**:333–339.
14. **Stackebrandt E., and C. R. Woese.** 1981. The evolution of prokaryotes, p. 1–32. *In* M. J. Carlile, J. F. Collins, B. E. B. Moseley (ed.), Molecular and cellular aspects of microbial evolution. 32nd Symposium of the Society for General Microbiology, Cambridge University Press, Cambridge.
15. **Whitaker, R. J., G. S. Byng, R. L. Gherna, and R. A. Jensen.** 1981. Comparative allostery of 3-deoxy-D-*ara-*

bino-heptulosonate 7-phosphate synthetase as an indicator of taxonomic relatedness in pseudomonad genera. J. Bacteriol. **145**:752–759.

16. **Whitaker, R. J., G. S. Byng, R. L. Gherna, and R. A. Jensen.** 1981. Diverse enzymological patterns of phenylalanine biosynthesis in pseudomonads are conserved in parallel with deoxyribonucleic acid homology groupings. J. Bacteriol. **147**:526–534.
17. **Whitaker, R. J., M. J. Fiske, and R. A. Jensen.** 1982. *Pseudomonas aeruginosa* possesses two novel regulatory isozymes of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase. J. Biol. Chem. **257**:12789–12794.