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Variable Enzymological Patterning in Tyrosine Biosynthesis as a Means of Determining Natural Relatedness Among the *Pseudomonadaceae*

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Enzymes of tyrosine biosynthesis (prephenate dehydrogenase and arogenate dehydrogenase) were characterized in 90 species currently classified within the genera *Pseudomonas*, *Xanthomonas*, and *Alcaligenes*. Variation in cofactor specificity and regulatory properties of the dehydrogenase proteins allowed the separation of five groups. Taxa defined by enzymological patterning corresponded strikingly with the five ribosomal ribonucleic acid (rRNA) homology groups established via rRNA-deoxyribonucleic acid hybridization. rRNA homology groups I, IV, and V all lack activity for arogenate/nicotinamide adenine dinucleotide phosphate (NADP) dehydrogenase and separated on this criterion from groups II and III, which have the activity. Group II species possess arogenate dehydrogenase enzyme (reactive with either NAD or NADP) sensitive to feedback inhibition by tyrosine, thereby separating from group III species whose corresponding enzyme was totally insensitive to feedback inhibition. The presence of prephenate/NADP dehydrogenase in group IV defined its separation from groups I and V, which lack this enzyme activity. Group I species possess an arogenate/NAD dehydrogenase that was highly sensitive to inhibition by tyrosine and a prephenate/NAD dehydrogenase of relative insensitivity to tyrosine inhibition. The opposite pattern of sensitivity/insensitivity was seen in group V species. These dehydrogenase characterizations are highly reliable for the keying of a given species to one of the five rRNA homology groups. If necessary, other confirmatory assays can be included using other aromatic pathway enzymes. These results further document the validity and utility of the approach of comparative enzymology and allostery for classification of microorganisms.

The focal point of previous studies, whereby the approach of comparative enzymology has been employed as a tool of microbial classification, has been diversity in allosteric control patterns. The most extensive application of this approach has been with 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase (19, 20) although successes have been achieved with other enzymes as well (6). Each distinctive control pattern for DAHP synthetase was found to be a highly conserved trait among member species of a given genus (20). It was previously thought that a given allosteric control pattern for DAHP synthetase operated in the context of identical biochemical routing. The discovery of different pathways in nature for phenylalanine and tyrosine biosynthesis utilizing the newly found intermediate arogenate (13, 17, 30, 38) opens the possibility that variant allosteric patterning of DAHP synthetase reflects correlative variation in the biochemical pathway itself.

There is an expanding basis for optimism that other biochemical diversity in major metabolic pathways will provide highly conservative characteristics for definition of natural relationships. Major lines of evolution in fungi were interpreted based upon the observed distribution of two pathways for lysine biosynthesis (23, 40). Variant pathways to amino acids uncovered by recent studies were reviewed by Jensen (17). It is instructive that new pathway variations are emerging from results obtained with microorganisms that have received relatively little attention in the past. Where alternative biochemical routes occur in nature, each enzyme system has been suggested to be a conserved unit derived from an ancient evolutionary event, thus providing a basis for degree of relatedness or divergence among examined species (21). Since a complex biochemical pathway maximizes the number of interactive and interdependent features, it seems likely (20) that pathway complexity equates with enhanced conservatism in resistance to evolutionary change. The shikimate

pathway is an excellent example of a large multibranched pathway containing a large number of potentially variable characters. The useful array of variations available includes different biosynthetic routes for the formation of phenylalanine and tyrosine, different patterns of cofactor specificity, and different regulatory patterns for allosteric enzymes positioned at the multiple metabolic branch points which serve aromatic biosynthesis.

In the case of tyrosine biosynthesis, the textbook route (as established and extensively documented in eubacteria [5, 7]) utilizes the intermediate 4-hydroxyphenylpyruvate. Cyanobacteria were the first group of organisms shown to depend upon a different sequence utilizing arogenate as a precursor (38). This has been followed with the demonstration of the obligatory use of the arogenate route for tyrosine biosynthesis in coryneform bacteria (13, 14). *Pseudomonas aeruginosa* expresses both pathways to tyrosine simultaneously (30). The presence of dual enzyme sequences to tyrosine has been proposed as an explanation of the inability to isolate tightly blocked tyrosine auxotrophs (reluctant auxotrophy) in this organism (31). The two enzyme sequences for tyrosine biosynthesis are shown in Fig. 1. The extensive enzymological analysis of *P. aeruginosa* (4, 18, 30, 31, 39) provides a comprehensive background for a comparative enzymological analysis of related pseudomonad organisms.

Members of the current *Pseudomonas* genus are inhabitants of a wide range of environments and include at least one mammalian pathogen (*P. mallei*) and numerous plant pathogenic species. The genus is heterogeneous, and various species have been differentiated on the basis of a variety of phenotypic observations and growth responses (36). The proximities of relationship among a number of named *Pseudomonas* species have been deduced by results of nucleic acid hybridization analyses. These studies have included DNA-DNA hybridization (26, 28, 33) and rRNA-DNA hybridization, two techniques that discriminate at different hierarchical levels of classification. The rRNA-DNA hybridization method (Table 1) separates five pseudomonad groupings (29). Within each rRNA-DNA group, member species can be distinguished from one another by DNA-DNA hybridization experiments.

rRNA homology group I corresponds to the large DNA homology cluster which includes the fluorescent nomen species (*P. fluorescens*, *P. aeruginosa*, *P. cichorii*, and *P. syringae*) as well as the nonfluorescent species *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, and *P. pseudoalcaligenes* (26). rRNA homology group II consists of a DNA homology group comprised of animal pathogens (*P. pseudomallei* and *P. mallei*) and the phytopathogens (*P. cepacia* [*multivorans*], *P. marginata* [*allicola*], and *P. caryophilli*) (2). *P. pickettii* and *P. solanacearum* were separable from other member species of group II via DNA homology discrimination (34). rRNA-DNA homology group III contains three distinct DNA homology groups. These comprise *P. acidovor-*

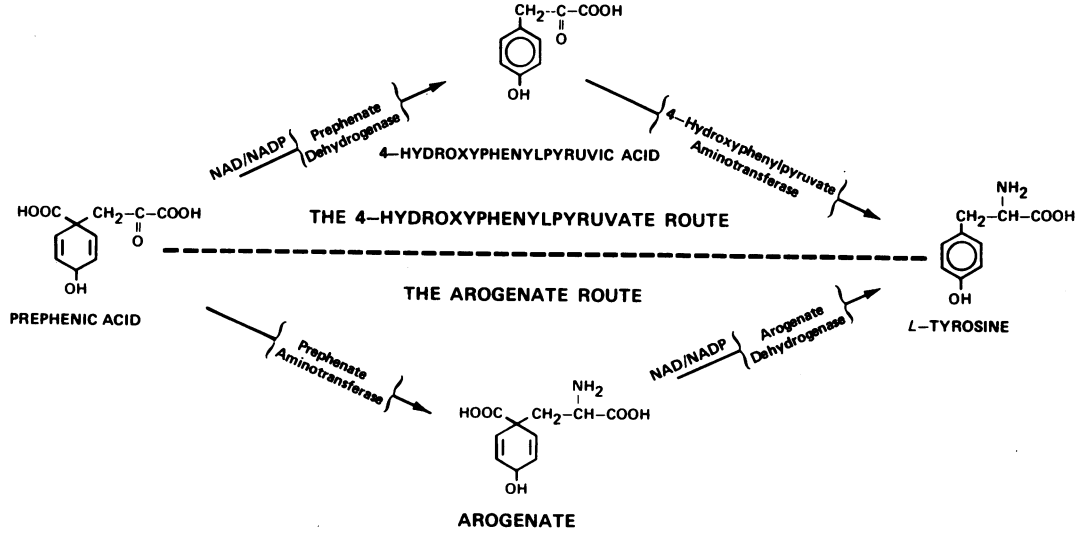

FIG. 1. *Enzymatic steps of the 4-hydroxyphenylpyruvate (top) and the arogenate branchlets (bottom) leading to* L*-tyrosine biosynthesis. The aminotransferase reactions shown also require an amino-donor substrate (such as* L*-glutamate) and pyridoxal-5'-phosphate.*

TABLE 1. *Nucleic acid hybridization groupings in pseudomonad taxa*

Species	rRNA-DNA group	DNA-DNA group
P. aeruginosa	I	I
P. fluorescens		
P. putida		
P. syringae		
P. cichorii		
P. stutzeri		
P. mendocina		
P. alcaligenes		
P. cepacia	II	IIa
P. marginata		
P. pseudomallei		
P. mallei		
P. caryophilli		
P. pickettii	II	IIb
P. solanacearum		
P. acidovorans	III	IIIa
P. testosteroni		
P. delafieldii	III	IIIb
P. facilis		
P. saccharophila	III	IIIc
P. diminuta	IV	IV
P. vesiculare		
P. maltophilia	V	V
X. campestris		

ans/P. testosteroni, P. facilis/P. delafieldii, and *P. saccharophila* (33). The final two rRNA homology groups contain *P. diminuta/P. vesiculare* (1) and *P. maltophilia/Xanthomonas campestris* (29).

The enzymological patterning discerned in governance of aromatic biosynthesis among *Pseudomonas* species separates groupings that parallel those defined by rRNA homology to a remarkable extent, as shown by data presented in this communication.

MATERIALS AND METHODS

Authenticity of microbial species. Credible interpretation of data in microbial systematics is enormously vulnerable in two respects. Firstly, a given stock must be generally available for distribution and must be maintained indefinitely under conditions that maximize unaltered preservation of properties. Secondly, even minor contamination of cultures used for enzymological characterizations is unacceptable. A small fractional impurity in a given culture could introduce disproportional qualitative impact owing to the huge variation in specific activities among microbial species. We regard the organization of our studies as a model effort with respect to the rigorous address of these concerns. A collaborative arrangement with the American Type Culture Collection (ATCC) was negotiated through a subcontract agreement. All strains examined are from the ATCC collection and are referred to by name and ATCC catalog number. A compilation of the species studied is given in Table 2. Cultures were grown and harvested at the ATCC facility, and the expertise of ATCC personnel in documentation of authenticity and purity of cultures was utilized. Cultures were grown in a rotary shaker at 1,000 rpm in the medium and at the ambient temperature recommended in the ATCC catalog of strains (14th ed., 1980). Harvest was at mid- or late-exponential phase of growth. Cell pellets obtained by centrifugation were shipped to New York in dry ice packages, and after arrival the cell pellets were maintained at −80°C prior to preparation of extracts for enzymological determination.

Preparation of extracts. Cell suspensions were sonicated in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, followed by high-speed centrifugation at 150,000 × *g* for 1 h. Centrifugation at lower speeds (30,000 × *g* for 1 h) was usually unsatisfactory because such extracts possess an oxidase activity which then masks dehydrogenases of low activity. Apparently, this oxidase is membrane bound or particulate, or both, as it is completely eliminated after high-speed centrifugation. Extracts were subsequently passed through a Sephadex G-25 column to remove small molecules prior to enzyme assay.

Prephenate and arogenate dehydrogenase. All enzyme assays were carried out under conditions of proportionality, with respect to protein concentration and time at saturating substrate concentrations. The reaction mixture (200 μl) contained 0.5 mM NAD or NADP, enzyme, and 0.4 mM prephenate or arogenate in 50 μM potassium phosphate buffer, pH 7.5. Trizma·HCl (Sigma Chemical Co.) was an unsatisfactory buffer for dehydrogenase assays because it contained oxidizable contaminants. The continuous formation of NADH or NADPH was determined spectrophotofluorimetrically with an Aminco-Bowman spectrophotofluorimeter (excitation wavelength, 340 nm; emission wavelength, 460 nm). As an extra control, arogenate preparations were quantitatively converted to phenylalanine by use of 0.5 N HCl, incubated for 10 min at 37°C, and reneutralized with NaOH. Extracts giving positive arogenate dehydrogenase activity were then tested with acid-converted arogenate as substrate. Occasionally, a few strains showed a slight background activity due to a trace contaminant in the arogenate preparation.

Other analytical techniques. Protein concentrations were estimated by the method of Bradford (3) as described in Bio-Rad technical bulletin 1051. The method of Calhoun et al. (4) was used to assay DAHP synthetase.

Biochemical and chemicals. Amino acids, NAD, NADP, Trizma, and Sephadex G-25 were obtained from Sigma Chemical Co. Barium prephenate was prepared from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (9) and was converted to the potassium salt with excess K_2SO_4 prior to use. Arogenate was prepared from the culture supernatants of a triple auxotroph of *Neurospora crassa* (22). The purification and isolation procedure was modified as described by Zamir et al. (41).

TABLE 2. *Subgroup status of species examined*

Named species	ATCC no.	Group[a]	Named species	ATCC no.	Group[a]
Pseudomonas acidovorans	17476	III	*Pseudomonas piscicida*	15251	*
P. agarici	25941	I	*P. putida*	12633	I
P. agarici	25943	I	*P. putida*	795	I
P. alboprecipitans	19860	III	*P. putrefaciens*	8071	*
P. alcaligenes	14909	I	*P. pyrocinia*	15958	II
P. alkanolytica	21034	?	*P. rubescens*	12099	*
P. amyloderamosa	21262	?	*P. saccharophila*	15946	III
P. andropogonis	23060	III	*P. solanacearum*	10692	II
P. angulata	13453	I	*P. stutzeri*	17591	I
P. antirrhini	19871	I	*P. stutzeri*	17588	I
P. asplenii	10855	I	*P. synxantha*	9890	I
P. atlantica	19262	*	*P. syringae*	12273	I
P. aureofaciens	13986	I	*P. syringae*	9005	I
P. caryocyanae	19373	I	*P. syringae*	9004	I
P. caryophilii	25418	II	*P. syringae*	8727	I
P. cepacia	10856	II	*P. syringae*	19322	I
P. cepacia	17460	II	*P. syringae*	19872	I
P. cepacia	25609	II	*P. syringae*	10205	I
P. chlororaphis	17810	I	*P. syringae*	19866	I
P. cichorii	10857	I	*P. syringae*	13523	I
P. cichorii	14120	I	*P. syringae*	19306	I
P. delafieldii	17505	III	*P. syringae*	10862	I
P. diminuta	11568	IV	*P. taetrolens*	4683	I
P. facilis	11228	III	*P. testosteroni*	17510	III
P. floridana	25616	*	*P. vesiculare*	11426	IV
P. fluorescens	13525	I			
P. fragi	4973	I			
P. gardneri	19865	V			
P. geniculata	19374	V			
P. hibiscicola	19867	?			
P. huttiensis	14670	II	*Xanthomonas albilineans*	12785	V
P. maltophilia	17806	V	*X. ampelina*	29074	?
P. maltophilia	17445	V	*X. axonopodis*	19312	V
P. maltophilia	13637	V	*X. campestris*	9924	V
P. marginalis	10844	I	*X. campestris*	12612	V
P. marginata	10248	II	*X. campestris*	8721	V
P. marginata	25417	II	*X. campestris*	9563	V
P. marginata	10854	II			
P. marginata	19302	II			
P. marina	25374	?			
P. mendocina	25411	I			
P. methanolica	21074	II			
P. nautica	27132	?	*Alcaligenes eutrophus*	17697	[II]
P. palleronii	17724	III	*A. faecalis*	15173	[III]
P. palleronii	17728	III	*A. faecalis*	15554	[III]
P. panici	19875	I	*A. faecalis*	8750	[III]
P. pavonacea	951	*	*A. paradoxus*	17713	[III]
P. perfectomarinus	11405	?			
P. perlurida	490	?			
P. pickettii	27511	II			

[a] Assigned group from analysis of tyrosine biosynthesis. Some species have been assigned to a given subgroup for the first time, and these should be considered provisional until confirmed by rRNA homology data. Asterisks denote species not in accord with the generic description of *Pseudomonas*. Named species of *Alcaligenes* are given bracketed subgroup designations to indicate the tentative status of subgroup assignment until rRNA homology data are available.

RESULTS

Dehydrogenase characterizations. Prephenate and arogenate dehydrogenase activities were assayed in extracts of all species studied. Determinations were made of the presence of one or both dehydrogenase activities, whether NAD or NADP, or both, functioned as cofactor, and of the degree of feedback inhibition of each

activity by tyrosine. Although the studies of Palleroni et al. (29) which defined the five rRNA homology groups shown in Table 1 were not as comprehensive as this study (Table 2), all species studied in common faithfully segregated into the same subgroups.

(i) Unassigned species. Only four species studied lacked both prephenate dehydrogenase and arogenate dehydrogenase activities: *P. amyloderamosa* (21262), *P. perlurida* (490), *P. rubescens* (12099), and *P. hibiscicola* (19867). Most other species possessed both dehydrogenase activities. A few marine pseudomonads (*P. atlantica* [19262], *P. marina* [25374], and *P. piscicida* [15251]) only expressed prephenate dehydrogenase activity. It should be noted that *P. atlantica* and *P. piscicida* possess other characters that are discrepant with the generic description of *Pseudomonas* (16); e.g., in both of the latter cases the guanine plus cytosine content of the DNA is 43 to 45 mol%. Additional named *Pseudomonas* species investigated which may not conform to the current description of *Pseudomonas* are indicated in Table 2.

(ii) Cofactor specificity. Three qualitative patterns of cofactor specificity were realized: NAD dependent, NADP dependent, and reactive with either NAD or NADP. Table 3 contains dehydrogenase activities obtained in the presence of either cofactor for representative species that were also included in the rRNA homology studies (29). No taxonomic significance was related to the variation in absolute values of specific activity obtained for either enzyme in different organisms.

Either dehydrogenase activity in group I species exhibited an absolute requirement for NAD. *P. maltophilia* and species of *Xanthomonas* (group V) also possessed NAD-specific dehydrogenases for tyrosine biosynthesis. *X. axonopodis* (19312) possessed only prephenate/NAD dehydrogenase activity. The majority of species listed under groups II and III possessed tyrosine biosynthetic dehydrogenase enzymes which could

TABLE 3. *Prephenate dehydrogenase and arogenate dehydrogenase activities*[a] *in pseudomonad groups*

Group	ATCC No.	Species	Prephenate dehydrogenase		Arogenate dehydrogenase	
			NAD	NADP	NAD	NADP
I	12633	*P. putida*	3.7	0	2.1	0
	13525	*P. fluorescens*	5.4	0	3.4	0
	17810	*P. chlororaphis*	4.6	0	2.6	0
	13986	*P. aureofaciens*	6.2	0	3.9	0
	19304	*P. syringae*	3.2	0	1.2	0
	10857	*P. cichorii*	2.2	0	1.7	0
	17591	*P. stutzeri*	+	0	+	0
	25411	*P. mendocina*	6.0	0	0.9	0
	14909	*P. alcaligenes*	3.5	0	2.9	0
IV	11568	*P. diminuta*	7.8	2.4	0	0
	11426	*P. vesiculare*	11.2	0.9	2.3	0
V	17806	*P. maltophilia*	+	0	+	0
	12785	*X. albilineans*	0.9	0	0.3	0
	19312	*X. axonopodis*	0.4	0	0	0
	9925	*X. campestris*	0.2	0	0.1	0
II	25418	*P. caryophilli*	7.6	5.1	1.7	1.6
	10856	*P. capacia*	10.1	11.6	17.8	9.8
	10854	*P. marginata (allicola)*	7.5	4.2	4.2	7.5
	25417	*P. marginata*	8.6	6.3	4.3	6.3
	10692	*P. solanacearum*	7.7	7.1	6.4	10.9
III	17476	*P. acidovorans*	8.2	6.8	5.3	4.8
	17510	*P. testosteroni*	3.8	5.6	0	1.6
	17724	*P. palleronii*	5.6	4.5	0	1.5
	15946	*P. saccharophila*	32.0	27.0	3.5	4.8
	17505	*P. delafieldii*	15.1	15.1	4.8	8.5
	11228	*P. facilis*	3.7	4.4	7.3	2.9

[a] Expressed as nanomoles of product per minute per milligram of protein. Organisms scored + showed detectable activity; however, since high endogenous activities were found without addition of substrate, specific activities were not calculated.

utilize either prephenate or arogenate as substrate and either NAD or NADP as cofactor. These two groups could not be distinguished on the sole basis of substrate/cofactor specificity profiles of the aromatic dehydrogenases. Group IV species possessed prephenate dehydrogenase activity with either NAD or NADP as cofactor. *P. vesiculare* exhibited arogenate/NAD activity, in contrast to *P. diminuta*, which lacked arogenate dehydrogenase activity regardless of the cofactor used.

In summary, inspection of Table 3 shows that the presence of arogenate/NADP dehydrogenase in groups II and III separated them from groups I, IV, and V, which lack arogenate/NADP activity. The presence of prephenate/NADP activity in group IV separated it from groups I and V, which lack it.

(iii) Are the dehydrogenases separate gene products? In *P. aeruginosa*, gel filtration, DEAE-cellulose chromatography, or hydroxylapatite chromatography did not separate prephenate dehydrogenase activity from arogenate dehydrogenase activity (30). However, arogenate dehydrogenase activity was sensitive to *p*-chloromercuribenzoate whereas prephenate dehydrogenase was not. This information and the inability to isolate tyrosine auxotrophs in *P. aeruginosa* suggest the existence of two gene products (30). Similarly, we have since found that all dehydrogenases for tyrosine biosynthesis from *P. cepacia* (17460) (i.e., activity with both substrates and both cofactors) coelute on DEAE-cellulose chromatography. However, arogenate dehydrogenase activity with either NAD or NADP as cofactor was sensitive to *p*-chloromercuribenzoate whereas prephenate dehydrogenase was not inhibited (data not shown). Again, this suggests that there may be separate gene products in *P. cepacia*, each with a specific substrate requirement but ambiguous in terms of cofactor specificity.

Tyrosine inhibition of prephenate dehydrogenase and arogenate dehydrogenase. Dehydrogenase substrate/cofactor specificity profiles are a successful means of isolating species belonging to groups II and III. These groups were distinguished from one another on the basis of the sensitivity of dehydrogenase activity to tyrosine inhibition (see Table 4). Group II species, in general, showed little or no feedback inhibition of prephenate/NAD or prephenate/NADP dehydrogenase activities by tyrosine. On the other hand, inhibition of arogenate/NAD or arogenate/NADP dehydrogenase was found. Group III species lacked allosteric sensitivity for all four dehydrogenase activities. Some species investigated in this study have not yet been analyzed by nucleic acid hybridization tests, but the dehydrogenase substrate/cofactor specificity profiles obtained placed them cleanly in groups II or III (see bottom of Table 4). Confirmation of these group placements by rRNA-DNA hybridization experiments would be desirable. DNA-DNA hybridization has suggested that *P. solanacearum* is not closely related to other *Pseudomonas* species (27). However, on the basis of rRNA-DNA hybridization this species appears to be distantly related to *P. cepacia* and *P. marginata* (29). The presence in this species of a tyrosine-related arogenate dehydrogenase activity agrees with the suggestion (29) of the placement of *P. solanacearum* in group II.

P. delafieldii (first species in group III, Table 4) and *P. marginata* (last species in group II, Table 4) exhibited similar data. They differed from all other species shown in the sensitivity of arogenate/NAD dehydrogenase to feedback inhibition (like group II) and in the insensitivity of arogenate/NADP dehydrogenase to feedback inhibition (like group III). rRNA homology and our overall enzymological patterning data identified *P. delafieldii* and *P. marginata* (10845) in the group II and III clusters, respectively. However, both rRNA homology and enzymological patterning placed these group clusters relatively near each other. Hence, these two species are excellent candidates for intermediate species in a continuum of species that may eventually be established.

Comparative dehydrogenase allostery to distinguish between groups I and V (and group IV). Among groups I, IV, and V, species within group IV are alone in possession of prephenate/NADP dehydrogenase (Table 3). Confirmation of group IV identity may be obtained through observation of the total insensitivity of dehydrogenases from group IV species to inhibition by tyrosine (Table 5). Although *X. ampelina* (which does not conform to expectations for a group V species) does fit the criteria listed above for species within group IV, other enzymological patterns ruled out its classification within group IV (R. J. Whitaker et al., submitted for publication). *X. ampelina* has also been shown to be at variance with other generic characters of *Xanthomonas* species in group V. Xanthomonads have been shown to contain brominated aryl-polyene pigments, termed xanthomonadins. *X. ampelina* produces different pigments of currently unknown structure (37). The questionable status of *X. ampelina* is further supported by the dissimilarity reported for rRNA cistrons of *X. ampelina* in comparison with those of other named *Xanthomonas* species (11).

TABLE 4. *Comparative allostery of prephenate dehydrogenase and arogenate dehydrogenase in groups II and III*

Group	ATCC no.	Species	% Inhibition[a]			
			Prephenate dehydrogenase		Arogenate dehydrogenase	
			NAD	NADP	NAD	NADP
II	25418	*P. caryophilli*	0	0	45	82
	25609	*P. cepacia*	0	0	60	79
	17460	*P. cepacia*	46	0	10	77
	10692	*P. solanacearum*	28	0	63	68
	25417	*P. marginata*	0	0	61	64
	10856	*P. cepacia*	27	12	26	59
	19302	*P. marginata*	0	0	77	57
	27511	*P. pickettii*	17	13	53	46
	10248	*P. marginata*	0	0	75	43
	10845	*P. marginata*	0	0	48	0
III	17505	*P. delafieldii*	0	0	62	0
	11228	*P. facilis*	0	0	0	0
	17476	*P. acidovorans*	0	0	0	0
	17510	*P. testosteroni*	0	0		0
	17724	*P. palleronii*	0	0		0
	15946	*P. saccharophila*	0	0	0	0
II or III[b]	19860	*P. alboprecipitans*	0	0		0[III][c]
	23060	*P. andropogonis*	0	0	0	0[III]
	25616	*P. floridana*	0	0	0	82[II]
	14670	*P. huttiensis*	0	0	17	75[II]
	21070	*P. methanolica*	0	25	76	79[II]
	15958	*P. pyrocinia*	64	0	46	72[II]

[a] Percent inhibition in the presence of 0.5 mM L-tyrosine.

[b] These species possess a dehydrogenase substrate/cofactor specificity profile similar to group II or III. However, rRNA-DNA hybridization data are not yet available.

[c] Groups to which these species can be assigned based upon this analysis.

Group I encompasses the fluorescent nomen species, the fluorescent phytopathogenic pseudomonads, as well as certain nonfluorescent species (*P. stutzeri*, *P. alcaligenes*, and *P. mendocina*). In Table 5 the phytopathogenic species of group I are segregated together at the bottom of the listing for more convenient comparison with the phytopathogens (mainly xanthomonads) which fall into group V. All group I species displayed a consistent pattern of dehydrogenase allostery in which prephenate/NAD dehydrogenase was relatively insensitive to tyrosine inhibition whereas arogenate/NAD dehydrogenase was very sensitive to feedback inhibition. Exactly the opposite pattern of allostery was found with group V species where prephenate dehydrogenase was most sensitive to feedback inhibition. Group V species identified by dehydrogenase patterning are easily confirmed by their unique allosteric pattern of control for DAHP synthetase (Whitaker et al., submitted for publication).

At present, the 8th edition of *Bergey's Manual of Determinative Bacteriology* recognizes only two species encompassing the fluorescent phytopathogenic pseudomonads. These are *P. syringae* and *P. cichorii*, species shown to fall into the *P. fluorescens* DNA-homology (group I) cluster (26). The majority of organisms are assembled under the name *P. syringae*. However, there may be clustering of strains around certain nomen species on the basis of nutritional or physiological characteristics (24, 25, 35) and also by extensive DNA-DNA hybridization experiments (32). Like fluorescent phytopathogenic pseudomonads, named xanthomonads have emerged as a large number of species based upon pathogenicity known for particular hosts. However, if the host range has not been clearly defined, the same organism may in fact be placed in completely different species, depending on the plant from which it was isolated. For the *Xanthomonas* genus, five taxospecies were recognized by Dye and Lelliott (12). The majority of

TABLE 5. *Comparison of sensitivities of prephenate/NAD dehydrogenase and arogenate/NAD dehydrogenase to tyrosine inhibition in groups I, IV, and V species*

ATCC no.	Strain[a]	% Inhibition[b]			
		Prephenate dehydrogenase		Arogenate dehydrogenase	
		0.1 mM Tyrosine	0.5 mM Tyrosine	0.1 mM Tyrostine	0.5 mM Tyrosine
	Expected for group IV		0		0
11426	*P. vesiculare*		0		0
11426	*P. vesiculare* (NADP = cofactor)		0		—
11568	*P. diminuta*		0		—
11568	*P. diminuta* (NADP = cofactor)		0		—
	Expected for group I	<50			>50
12633	*P. putida*	0	19	64	100
13525	*P. fluorescens*	25	100		90
17810	*P. chlororaphis*	45	76	79	100
13986	*P. aureofaciens*	25	100	82	87
19304	*P. syringae* (*phaseolicola*)	0	25	92	100
10857	*P. cichorii*		50	82	100
17591	*P. stutzeri*[c]	?	?	?	?
25411	*P. mendocina*		38		
14909	*P. alcaligenes*	69	100	100	100
19871	*P. antirrhini*	34	60	100	100
14120	*P. cichorii*	0	60	100	100
10857	*P. cichorii*	0	50	82	100
10844	*P. marginalis*	25	50	50	77
12273	*P. syringae*	40	95	100	
19304	*P. syringae* (*phaseolicola*)	0	25	92	100
9005	*P. syringae* (*coronafaciens*)	0	41	—	—
8727	*P. syringae* (*glycinae*)		0	84	92
19866	*P. syringae* (*helianthi*)	0	50	44	66
19872	*P. syringae* (*mori*)		0	75	100
19322	*P. syringae* (*morsprunorum*)		0	62	100
13523	*P. syringae* (*savastoni* subsp. *nerii*)		0	0	50
9004	*P. syringae* (*atrofaciens*)		0	0	50
10862	*P. syringae* (tomato)	0	34	—	—
	Expected for group V	>50			<50
12785	*X. albilineans*	42	58		0
19312	*X. axonopodis*	86	100		—
9924	*X. campestris* (*malvacereum*)	50	100		34
12612	*X. campestris* (*hyacinthi*)	66	100		0
8721	*X. campestris* (*pelargonii*)	100	100		0
9536	*X. campestris* (*phaseoli*)	82	100		0
19865	*P. gardneri*	100	100		0
29074	*X. ampelina*		0		0

[a] One named *Pseudomonas* species (*gardneri*) grouped with all named species of *Xanthomonas* except for *X. ampelina* (bottom line) which separates from other group I and V species.

[b] Where activity in the absence of inhibitor was absent or too low to obtain meaningful inhibition data, a dash appears.

[c] Activity was qualitatively present, but high endogenous activity complicated the analysis of tyrosine inhibition.

Xanthomonas species are merged into *X. campestris*, as most can only be differentiated on the grounds of plant host reactions. Group V contains two named *Pseudomonas* species (*P. maltophilia*, Table 3; *P. gardneri* Table 5). The placement of the latter species in group V with species of *Xanthomonas* is very clear when these data on dehydrogenase patterning are consid-

ered together with the allosteric analysis of DAHP synthetase (Whitaker et al., submitted for publication).

Placement of named *Alcaligenes* species within groups II and III? An inspection of Table 6 reveals that *A. eutrophus* possesses dehydrogenase patterning similar to group II species (Table 4), whereas strains of *A. faecalis* and *A. paradoxus* possess dehydrogenase patterning similar to group III species (Table 4). *A. eutrophus* was not found to be related to any group II species at the level of DNA homology (33), but the latter analysis discriminates with finer tuning than rRNA homology. Unfortunately, no rRNA homology data with named species of *Alcaligenes* have been reported.

The current separation of genera such as *Alcaligenes* or *Gluconobacter* from *Pseudomonas* is based on very few characters. In the case of *Alcaligenes*, the major differentiating characteristic is the presence of peritrichous or degenerate peritrichous flagella. However, members of this genus are reported to share many phenotypic characters with pseudomonads (8). It is additionally suggestive that *A. paradoxus* was reported to be readily confused with *P. palleronii* and *P. flava* (8), both group III species.

DISCUSSION

The analysis of tyrosine biosynthetic enzymes has been shown in this report to be a reliable means of distinguishing subgroups within the *Pseudomonadaceae*. Each of the groupings derived by this approach of enzymological patterning corresponds to rRNA-DNA hybridization groups. Enzymological patterning does not usually distinguish between species clusters delineated by DNA-DNA hybridization (32), a method that accommodates classification at a lower hierarchical level of discrimination than does DNA-rRNA hybridization. The total information obtained via independent approaches of rRNA homology and enzymological patterning supports a rationale favoring the elevation of each of the five subgroups to the rank of genus.

The properties of prephenate dehydrogenase and arogenate dehydrogenase alone are nearly always sufficient to discriminate the five subgroups. A schematic of an efficient flow chart of steps for this analysis is shown in Fig. 2. Thus, the presence or absence of arogenate/NADP dehydrogenase activity separates groups I, IV, and V from groups II and III. Group II species possess a tyrosine-inhibited arogenate/NADP dehydrogenase, whereas the activity of group III species is insensitive to inhibition. Among groups I, IV, and V, only member species of group IV possess prephenate/NADP dehydrogenase. Finally, group I species can be distinguished from group V species through observation of whether prephenate/NAD dehydrogenase or arogenate/NAD dehydrogenase is most sensitive to feedback inhibition by tyrosine. Our overall enzymological patterning studies, especially this dehydrogenase analysis and the DAHP synthetase analysis (Whitaker et al., submitted for publication), suggest that, of the five groups, groups II and III are nearest each other. This is also suggested by DNA-rRNA hybridization experiments using *Janthinobacterium hiridum* as a test organism (10).

The comprehensive scope of this analysis introduces interesting possibilities for improved appreciation of gene-enzyme relationships and regulation in nature. Where dual routes to tyrosine exist in vitro, the fractional contribution of each sequence to total end product formed in vivo is of interest. In some cases the in vitro data allow a reasonable guess. For example, one might expect that member species of group V rely mainly on prephenate dehydrogenase for tyrosine biosynthesis based upon the excellent sensitivity of this enzyme to allosteric control and the sometimes-absence of arogenate dehydrogenase (e.g., *X. axonopodis*). The diversity of enzymological patterns with respect to presence

TABLE 6. *Activities and sensitivity to feedback inhibition of prephenate dehydrogenase and arogenate dehydrogenase in species of Alcaligenes*

ATCC no.	Species	Sp act[a]			
		Prephenate dehydrogenase		Arogenate dehydrogenase	
		NAD	NADP	NAD	NADP
17697	*A. eutrophus*	11.2 (0)	16.0 (0)	16.0 (75)	21.6 (62)
15554	*A. faecalis*	10.0 (0)	23.7 (0)	1.2 (0)	5.4 (0)
8750	*A. faecalis*	2.7 (0)	10.5 (0)	0.7 (0)	3.5 (0)
15173	*A. faecalis*	4.2 (0)	10.9 (0)	2.3 (0)	3.1 (0)
17713	*A. paradoxus*	11.7 (0)	11.7 (0)	6.6 (0)	7.0 (0)

[a] Specific activities are expressed as nanomoles of product per minute per milligram of protein. In parentheses are the calculated percentages of inhibition by 0.5 mM tyrosine.

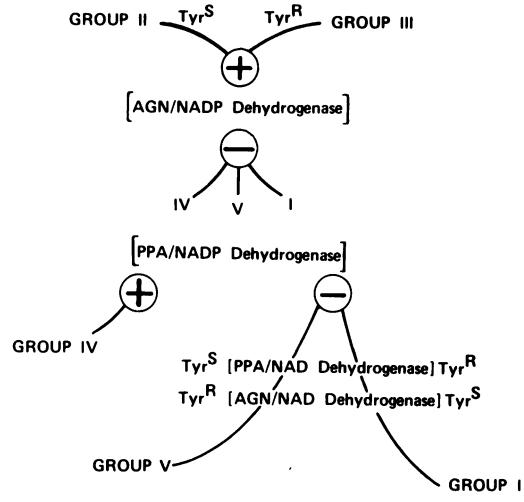

FIG. 2. *Use of dehydrogenase patterning to separate five taxa, denoted groups I through V. The analysis of greatest procedural efficiency is shown in the form of a flow chart (top to bottom). The assay of arogenate/NADP dehydrogenase is the first step, its presence (+) separating groups II and III from other groups where the enzyme activity is absent (−). Group II species possess a tyrosine-sensitive dehydrogenase (Tyr^S), in contrast to group III species having a dehydrogenase resistant to tyrosine feedback inhibition (Tyr^R). The second basic step is the assay of prephenate (PPA) dehydrogenase activity capable of reaction in the presence of NADP. Finally, groups I and V are separated by comparison of the relative allosteric sensitivities of NAD-dependent dehydrogenases.*

or absence of dual pathways to tyrosine (or phenylalanine), the specificity for pyridine nucleotide cofactor, and the allosteric regulation(s) in force should ultimately be interpretable in relationship to the equally diverse patterning of enzymes positioned elsewhere in the pathway, such as DAHP synthetase. The correlating patterns in nature of variation in one enzyme system with diverse patterns in another may assist the understanding of the subtleties of complex interaction exerted between segments of a highly branched biochemical pathway. We have equated the phenomenon of reluctant auxotrophy in *P. aeruginosa* to the presence of separate (dual) branchlets to both tyrosine and phenylalanine (30, 31). In *A. eutrophus*, phenylalanine auxotrophs, but not tyrosine auxotrophs, were successfully isolated (15). This is consistent with the presence of one pathway to phenylalanine (via prephenate dehydratase; unpublished data) in contrast to the presence of two pathways to tyrosine via prephenate dehydrogenase and arogenate dehydrogenase (this paper).

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