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Biochemical Diversity for Biosynthesis of Aromatic Amino Acids Among the Cyanobacteria

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We examined the enzymology and regulatory patterns of the aromatic amino acid pathway in 48 strains of cyanobacteria including representatives from each of the five major groupings. Extensive diversity was found in allosteric inhibition patterns of 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase, not only between the major groupings but also within several of the generic groupings. Unimetabolite inhibition by phenylalanine occurred in approximately half of the strains examined; in the other strains unimetabolite inhibition by tyrosine and cumulative, concerted, and additive patterns were found. The additive patterns suggest the presence of regulatory isozymes. Even though both arogenate and prephenate dehydrogenase activities were found in some strains, it seems clear that the arogenate pathway to tyrosine is a common trait that has been highly conserved among cyanobacteria. No arogenate dehydratase activities were found. In general, prephenate dehydratase activities were activated by tyrosine and inhibited by phenylalanine. Chorismate mutase, arogenate dehydrogenase, and shikimate dehydrogenase were nearly always unregulated. Most strains preferred NADP as the cofactor for the dehydrogenase activities. The diversity in the allosteric inhibition patterns for 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase, cofactor specificities, and the presence or absence of prephenate dehydrogenase activity allowed the separation of subgroupings within several of the form genera, namely, *Synechococcus*, *Synechocystis*, *Anabaena*, *Nostoc*, and *Calothrix*.

The cyanobacteria (blue-green algae) comprise a very large, diverse group of procaryotic organisms which are of tremendous importance because of their photosynthetic and nitrogen-fixing capabilities. Despite this importance, however, the cyanobacteria are poorly understood in terms of their physiology, development, and genetics. Moreover, it is not known to what extent enzymological diversity exists among the biosynthetic pathways of these photoautotrophic organisms.

The term "enzymological patterning" includes differences in allosteric control patterns for biochemical pathways, differences in cofactor specificities, and pathway multiplicity, i.e., different biochemical routes to the same end product. Pathway multiplicity among microorganisms has been recently reviewed and occurs in the biosynthesis of arginine, isoleucine, lysine, methionine, and serine (21). Different metabolic pathways for the biosynthesis of phenylalanine and tyrosine using the newly discovered intermediate L-arogenate (38) have been demonstrated in a number of microorganisms (10, 28), plants (4, 31), and fungi (1), and in *Euglena gracilis* (6). One study revealed that tyrosine was synthesized via arogenate in six diverse species of cyanobacteria (33), and the evidence suggested that all cyanobacteria might utilize the arogenate pathway as the exclusive, or primary, means of tyrosine biosynthesis. Differences in allosteric control patterns in the eubacteria have been reported in the biosynthetic pathways for the aromatic amino acids (5, 22, 24, 35) and the amino acids of the aspartate family (8), and in the oxidative dissimilation of aromatic compounds via the β-ketoadipate pathway (7).

Increasing evidence points to the conclusion that such enzymological diversity in biosynthetic pathways may reflect ancient events of evolutionary divergence and that any particular pattern of enzymological steps to the end product(s) of a pathway is a conserved trait within a taxonomic group (5, 7, 8, 21, 22, 24, 25, 35). Recent enzymological patterning characterizations in our laboratory with a large number of pseudomonad species have led to the separation of five subgroups which parallel exactly the five subgroups defined by rRNA/DNA hybridization (5, 35). Although it has been generally conceded

for years that only pragmatic microbial classifications will ever be possible, a growing conviction exists that phylogeny-based microbial classifications are possible after all. In addition to nucleic acid hybridization techniques and enzymological patterning, a third molecular technique termed oligonucleotide cataloging (11) has received considerable attention in recent years. To the extent that these independent techniques have overlapped in species coverage, agreement about phylogenetic relationships has been impressively good.

It seemed likely, therefore, that a group as large and as ancient as the cyanobacteria would also possess diversity in biochemical pathways or regulatory mechanisms, or both. To gain a deeper insight into the possibilities of enzymological patterning within the cyanobacteria, we examined the enzymology and allosteric inhibition patterns of the aromatic amino acid pathway in 48 strains of cyanobacteria, including representatives from each of the five major groupings (sections) proposed by Rippka et al. (30).

MATERIALS AND METHODS

Organisms. Forty-five strains belonging to 13 form genera and representative of the five major sections of cyanobacteria proposed by Rippka et al. (30) were grown at the American Type Culture Collection (ATCC) under the conditions recommended in *The American Type Culture Collection Catalogue of Strains I* (14th ed., 1980), i.e., in Stanier medium BG11 (30) (or medium MN [30] if a marine strain) at 26°C in shake flasks illuminated with fluorescent lights at an intensity of 2,000 lx. *Synechocystis* sp. strain ATCC 29108, *Anabaena* sp. strain ATCC 29151, and *Synechococcus* sp. strain ATCC 29404 (*Agmenellum quadruplicatum* BG1) were grown at the State University of New York at Binghamton under conditions previously described (15, 16, 19).

Cells were harvested at late log phase, pelleted, and then frozen and shipped to New York packaged in dry ice. Upon arrival, the frozen pellets were stored at −40°C before extract preparation. All strains examined are listed in Table 3, together with Pasteur Culture Collection (PCC) numbers. In a survey of this type, it is not practical to optimize growth conditions for all the strains. In our experience, however, severe growth conditions result in (i) cells with decreased phycocyanin pigments and resultant yellow-green cell pellets, (ii) cells with a granular appearance that lyse easily, (iii) both of the preceding effects, or (iv) no growth at all. The cell pellets from the ATCC were dark green, and microscopic examination of thawed cells revealed intact, healthy-looking cells and trichomes. Moreover, we have found that variable culture conditions do not cause changes in specific activities of enzymes of the aromatic pathway. For example, changes in the specific activity of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, the only aromatic pathway enzyme so affected in the cyanobacteria to date, was limited to a threefold difference, and that difference occurred only in mutant strains, regardless of nutritional conditions that might be expected to cause changes in the specific activity (15, 16). Culture conditions, unless extreme, are unlikely to effect qualitative changes in the allosteric behavior or cofactor specificities of enzymes because these are intrinsic properties of the enzyme protein.

Extract preparation. Frozen cell pellets were thawed and suspended in 3 to 6 ml of cold 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol. Cell suspensions were sonicated on ice at 70 mV in an Ultratip Labsonic system (Lab-Line Instruments, Inc.). The number of 30-s pulses required for cell disruption varied with the organism. *Anabaena* sp. strains required only three pulses, whereas strains with well-developed sheaths required as many as eight pulses. Efficiency of cell disruption was determined microscopically. After sonication, the suspensions were centrifuged at 150,000 × *g* for 1 h at 5°C in a model L5-65 ultracentrifuge (Beckman Instruments, Inc.). Small molecules were removed by passing the resulting supernatants through a Sephadex G-25 column equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol.

Analytical procedures. The assay conditions chosen were those found to be optimal, or near optimal, for the aromatic pathway enzymes and to produce results consistent with in vivo patterns of regulation, as determined by mutant analyses, in *Synechocystis* sp. strain ATCC 29108 (15), *Synechococcus* sp. strains ATCC 27144 (unpublished data) and ATCC 29404 (20, 33), and *Anabaena* sp. strain ATCC 29151 (16; G. C. Hall and R. A. Jensen, Curr. Microbiol., in press). When the strain being examined was capable of growing under extreme environmental conditions, the assay procedure was adjusted to reflect the particular condition and is so noted in Results. Activities that were too low to provide reliable data (results not included in this report) were attributed to low protein concentrations, i.e., less than 2.0 mg of protein per ml of extract, coupled with low specific activity. As a result of the high sensitivity of the dehydrogenase assays, however, even extracts with low protein and low specific activity provided sufficiently high readings to be reliable. For example, at a final concentration of 250 μg of protein per ml in the reaction mixtures, *Synechocystis* sp. strain ATCC 29235 provided a fluorescence increment/minute (ΔFU/min) reading of 1.58 ± 0.08 for arogenate/NADP dehydrogenase (specific activity, 0.69).

Crude extracts were used because of the nature of this study and because partial purification results in the inactivation of some enzymes, e.g., prephenate dehydratase of *Synechocystis* sp. strain ATCC 29108 (unpublished data) and the tyrosine-sensitive DAHP synthase isozyme of *Anabaena* sp. strain ATCC 29151 (16). Protein concentrations were determined by the method of Bradford (3) as described in Bio-Rad Laboratories technical bulletin 1051. The method of Srinivasan and Sprinson (32) as modified by Jensen and Nester (23) was used to assay DAHP synthase. Reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM dithiothreitol, 1 mM each phosphoenolpyruvate and erythrose 4-phosphate, and 100 to 700 μg of extract protein.

Arogenate dehydrogenase, shikimate dehydrogenase, and prephenate dehydrogenase were assayed at 37°C by following the formation of NADH or NADPH

spectrophotofluorometrically in an Aminco-Bowman spectrophotofluorometer (excitation at 340 nm, emission at 460 nm). Reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.5), 1 mM NAD or NADP, 1 mM shikimate or prephenate, or 0.5 mM arogenate, 0.13 mM dithiothreitol, and 50 to 100 μg of extract protein. No interfering endogenous dehydrogenase activities were observed in any of the extracts. As an extra control extracts were also tested with acid-converted arogenate to eliminate any false activities due to trace contaminants in the arogenate preparation. The arogenate was treated with 0.1 N HCl for 10 min at 37°C. This treatment results in complete conversion of arogenate to phenylalanine (38). The preparation was neutralized with NaOH. No activity with acid-converted arogenate was found in any of the extracts. The tyrosine formed from arogenate via dehydrogenase activity was assayed fluorometrically (36) as previously described (15). The amount of tyrosine formed corresponded with the amount of NADPH produced.

Chorismate mutase and prephenate dehydratase were determined by measuring the formation of phenylpyruvate at 37°C as previously described (28). Reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.5), 0.25 mM dithiothreitol, 1 mM chorismate or prephenate, and 100 to 700 μg of extract protein. Arogenate dehydratase was measured as previously described (28). Phenylalanine hydroxylase was assayed as described by Guroff and Ito (14), with the fluorometric determination of tyrosine (36).

Biochemicals. Chorismate was isolated from culture supernatants of *Enterobacter aerogenes* 62-1 as described by Gibson (12). Prephenate was isolated from culture supernatants of *Salmonella typhimurium* (9). Arogenate (pretyrosine) was isolated from culture supernatants of a multiple aromatic mutant (ATCC 36373) of *Neurospora crassa* (26) and purified by a modified procedure (38). All other biochemicals were obtained from Sigma Chemical Co.

RESULTS

DAHP synthase. The first step unique to the aromatic amino acid pathway is the condensation of D-erythrose 4-phosphate and phosphoenolpyruvate to form DAHP as depicted in Fig. 1. This enzyme activity was assayed and tested for feedback inhibition by aromatic end products and pathway intermediates. Cobalt ions were found to stimulate activities in crude extracts by approximately twofold and so were routinely added to reaction mixtures at a concentration of 0.13 mM. Magnesium and manganous ions were either inhibitory or had no effect.

The inhibition patterns for DAHP synthase activities are presented in Tables 1 and 2. Those strains in which activities were too low to determine meaningful regulatory patterns are not listed in the tables. The predominant inhibition pattern in the cyanobacteria was unimetabolite inhibition by L-phenylalanine (retro-Phe), which occurred in over 55% of the strains examined. Only four examples of unimetabolite inhibition by L-tyrosine (retro-Tyr) were found, and inhibition in the presence of 0.1 mM tyrosine never exceeded 65%.

Additive, cumulative, and concerted patterns of inhibition were found in several strains, all of which belong to the section IV grouping of Rippka et al. (30). Several of the form genera in section IV (Table 2), including *Anabaena*, *Nostoc*, and *Calothrix*, contained strains with different inhibition patterns. In the form genus *Synechococcus* (Table 1), both retro-Phe and retro-Tyr patterns were found. The implications of these differing inhibition patterns with respect to taxonomic divisions are presented below.

Very little inhibition of DAHP synthase activities by the pathway intermediates, phenylpyruvate, prephenate, and chorismate, was observed. Exceptions included *Anabaena* sp. strain ATCC 29414, *Oscillatoria* sp. strain ATCC 29134, *Chroococcidiopsis* sp. strain ATCC 27900, and *Synechocystis* sp. strain ATCC 27171 with 64, 60, 39, and 37% inhibition, respectively. In each of these, however, the DAHP synthase activity was highly sensitive to inhibition by phenylalanine, which ruled out the possibility of a sequential mode of inhibition (24). No inhibition of DAHP synthase activity by the intermediate arogenate was observed.

Dehydrogenase activities. Shikimate, prephenate, and arogenate dehydrogenase activities were assayed for cofactor specificity as well as for possible inhibitory effects by aromatic end products.

Shikimate and arogenate dehydrogenase activities are listed in Table 3. Arogenate dehydrogenase activity was found in all but three of the cyanobacteria surveyed. No arogenate dehydrogenase or prephenate dehydrogenase activity was detected in *Anabaena* sp. strains ATCC 27899, 29211, and 29414. Different assay conditions may be necessary for detection of activity in these strains. For all arogenate dehydrogenase activities and the majority of the shikimate dehydrogenase activities, NADP was the preferred cofactor. Many species exhibited a high degree of specificity, and no shikimate dehydrogenase activity with NAD was detected in *Gloeothece* sp. strain ATCC 27152 and *Nostoc* sp. strain ATCC 29150. In contrast, NAD was the preferred cofactor for shikimate dehydrogenase activity in *Synechocystis* sp. strains ATCC 27171 and 27178 and *Synechococcus* sp. strains ATCC 29404 and 27180.

Significant prephenate dehydrogenase activity was found in 16 different strains (Table 4). In 12 of these strains, the activities had an absolute requirement for NADP as cofactor, whereas in the remaining four strains, the activity with NAD was less than 14% of the activity with NADP. Dehydrogenase activities with prephen-

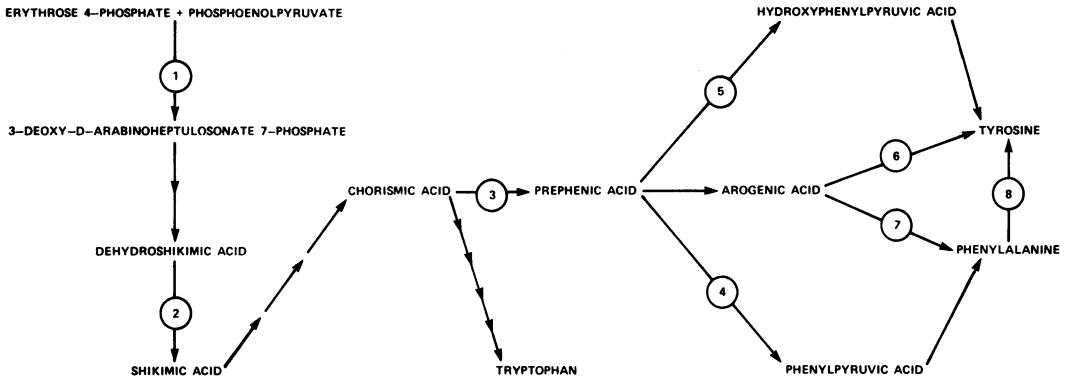

FIG. 1. Composite aromatic amino acid pathway including the various possible branchlets to phenylalanine and tyrosine that have been found among microorganisms. Enzymes: 1, DAHP synthase; 2, shikimate dehydrogenase; 3, chorismate mutase; 4, prephenate dehydratase; 5, prephenate dehydrogenase; 6, arogenate dehydrogenase; 7, arogenate dehydratase; 8, phenylalanine hydroxylase.

TABLE 1. Comparative allostery of DAHP synthase in sections I, II, III, and V cyanobacteria

Genus	PCC no.	ATCC no.	Sp act[a]	% Inhibition in presence of:				
				Phe[b]	Tyr[b]	Trp[b]	Aro[c]	Intmd[d]
Section I								
Synechococcus	6301	27144	2.90	8 (3)	25 (73)	0 (0)	0	4
	6908	27146	4.45	0 (0)	0 (59)	0 (1)	0	21
	6911	27192	0.76	0 (6)	9 (0)	0 (0)	0	23
	6910	27191	0.23	94	17	50	100	0
	6717	27180	0.98	97	7	0	95	17
	7202	29140	0.36	97	25	0	96	0
	73109	29404	1.02	98	17	0	98	0
	7502	29172	1.29	78	21	20	86	20
Synechocystis	6702	27171	2.30	97	11	20	96	37
	6308	27150	1.08	90	6	0	89	0
	6714	27178	1.08	88	14	0	83	0
	6902	29108	1.72	98	10	0	91	15
	6905	29109	2.50	92	11	0	93	24
	7008	29110	1.66	99	19	0	99	24
	7509	29235	0.89	32	0	9	37	12
Section II								
Chroococcidiopsis	7203	27900	0.28	57	0	0	0	39
Section III								
Oscillatoria	7112	29134	0.49	100	19	6	88	60
Lyngbya	6409	29119	0.31	42	0	0	30	0
	7114	29121	0.65	20	64	0	60	12
	7407	29126	1.10	93	11	0	87	0
Plectonema	6306	27894	2.74	92	15	11	87	17
Section V								
Chlorogleopsis	6912	27193	0.61	100	3	0	100	0
	6718	27181	1.98	97	7	0	98	0
Fischerella	7521	29538	3.07	50	0	6	52	16
	7414	29161[e]	1.14	42	6	2	47	25

[a] Expressed as nanomoles of product formed per minute per milligram of extract protein.
[b] Phenylalanine (Phe), tyrosine (Tyr), or tryptophan (Trp) present at a concentration of 0.1 mM. Values in parentheses were obtained at effector concentrations of 0.5 mM.
[c] Mixture of 0.1 mM each L-phenylalanine, L-tyrosine, and L-tryptophan.
[d] Mixture of 0.5 mM each phenylpyruvate, prephenate, and chorismate.
[e] Assayed at 50°C. At 37°C the specific activity for *Fischerella* sp. strain ATCC 29161 was 0.37.

TABLE 2. Comparative allostery of DAHP synthase in section IV cyanobacteria

Genus	PCC no.	ATCC no.	Sp act[a]	% Inhibition[b] in presence of:				Ratio[c]	Predicted inhibition in presence of aro if:		Inhibition pattern
				Phe	Tyr	Trp	Aro		Additive	Cumulative[d]	
Anabaena	7120	27893	0.93	58	12	7	76	1.0	77	66	Additive
	6411	27898	2.79	23 (21)	35 (77)	0 (10)	55 (95)	1.1 (1.1)	58 (100)	50 (84)	Additive
	7119	29151	2.60	25 (29)	30 (71)	0 (0)	48 (98)	1.1 (1.0)	55 (99)	48 (80)	Additive
	—	29413	1.34	33 (42)	30 (53)	12 (20)	56 (77)	1.3 (1.5)	75 (100)	59 (78)	Cumulative
	7118	27892	0.84	79	14	0	65	—	—	—	Retro-Phe
	7122	27899	0.22	45 (55)	14 (9)	0 (0)	50	—	—	—	Retro-Phe
	6309	29211	1.40	19	0	0	9	—	—	—	Retro-Phe
	—	29414	6.40	90	0	0	90	—	—	—	Retro-Phe
Nostoc	73102	29133	0.54	57	72	43	79	2.2	100	94	Cumulative
	6719	29105	0.90	16	3	0	44	0.4	—	—	Concerted
	7524	29411[e]	0.47	16 (33)	8 (18)	0	18 (56)	1.3 (0.9)	24 (51)	23 (45)	Additive
	7422	29132	1.27	72	17	4	81	1.1	90	66	Additive
Calothrix	7507	29112	1.01	24	34	28	62	1.4	86	64	Cumulative
	7103	27905	0.23	33	0	0	81	0.4	—	—	Concerted
	6303	29156	0.92	73	0	0	60	—	—	—	Retro-Phe
	7504	29158	0.25	84	24	0	76	—	—	—	Retro-Phe
	7102	27901	0.57	49	0	0	32	—	—	—	Retro-Phe
Scytonema	7110	29171	0.46	89	2	0	71	—	—	—	Retro-Phe

[a] Expressed as nanomoles of product formed per minute per milligram of extract protein.

[b] Inhibitors present at 0.1 mM concentration. Numbers in parentheses represent values obtained in the presence of 0.5 mM inhibitor. Aro represents an equimolar mixture of phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp).

[c] The ratio (R) is (the sum of the inhibitions found in the presence of each of the individual amino acids) ÷ (the inhibition observed in the simultaneous presence of the three inhibitors). If $R = 1$, the pattern is isoenzymic or single effector; if $R > 1$, the pattern is cumulative and if $R \ll 1$, the pattern is concerted (24).

[d] Predicted cumulative inhibition equals $100 - (\text{activity}_1 \times \text{activity}_2 \times \text{activity}_3)/\text{activity}_0$, where activity_0 represents activity in the absence of effectors, and activity_1, activity_2, etc., represent activity in the presence of the respective inhibitors (37).

[e] Assayed at 50°C. At 37°C the specific activity for *Nostoc* sp. strain ATCC 29411 was 0.29.

TABLE 3. Comparison of shikimate and arogenate dehydrogenase activities[a] with cofactors NAD and NADP

Section	Genus	PCC no.	ATCC no.	Shikimate		Arogenate	
				NAD	NADP	NAD	NADP
I	*Synechocystis*	6308	27150	0.78	6.40	0.58	2.26
		6902	29108	0.69	2.47	0	9.40
		7509	29235	0.36	2.15	0	0.69
		6714	27178	4.20	3.73	0.54	10.00
		6702	27171	1.63	1.28	0.18	3.46
		6905	29109	0.21	0.48	0.06	3.84
		7008	29110	0.06	0.20	0.08	3.58
	Synechococcus	7502	29172	0.34	3.10	0.73	2.87
		6717	27180	1.66	1.26	0.22	3.40
		6910	27191	0.15	0.82	0.20	3.58
		6911	27192	0.24	1.50	0.35	2.24
		6908	27146	1.47	8.25	3.11	17.33
		6301	27144[b]	2.64	10.56	3.95	22.25
		7009	29203	0.10	0.58	0.44	1.76
		73109	29404[c]	2.23	1.13	7.89	11.70
		7202	29140	0.45	1.05	0.93	2.05
	Gloeothece	6909	27152	0	1.28	7.20	20.20
II	*Chroococcidiopsis*	7203	27900	0.26	5.36	0.60	8.52
III	*Oscillatoria*	7112	29134	1.17	1.53	1.69	4.69
	Plectonema	6306	27894	1.07	2.53	4.02	15.10
		6402	27902	0.25	1.36	2.76	18.00
	Lyngbya	7407	29126	0.73	5.52	2.37	6.49
		7114	29121	1.10	1.64	1.26	5.64
		6409	29119	0.47	0.97	1.96	11.90
IV	*Anabaena*	7122	27899	1.18	1.33	0	0
		6309	29211	0.52	0.82	0	0
		—	29414	1.32	4.11	0	0
		7118	27892	0.32	1.22	3.30	14.40
		7120	27893	0.16	1.30	1.48	16.52
		6411	27898	0.04	2.50	0.54	8.74
		7119	29151	0.03	1.36	0.37	7.94
		—	29413	0.05	1.10	0.50	6.69
	Nostoc	7422	29132	0	0	0	9.62
		7107	29150	0	0.10	0	1.72
		6719	29105	0.24	2.40	0.65	6.65
		73102	29133	0.67	4.10	0.70	5.96
		7524	29411	0.16	1.30	1.60	19.46
	Cylindrospermum	7417	29204	0.27	0.42	0.05	1.29
	Scytonema	7110	29171	0.18	2.07	0.39	4.03
	Calothrix	7102	27901	1.14	5.00	1.16	30.40
		7103	27905	0.18	3.86	0.38	6.00
		7507	29112	0.34	2.06	0.43	10.28
		6303	29156	0.22	3.20	0.08	6.58
		7504	29158	0.15	0.90	0.25	3.30
V	*Fischerella*	7521	29538	0.24	1.42	0.39	3.66
		7414	29161[d]	0.14	0.34	0.24	1.16
	Chlorogleopsis	6912	27193	0.12	0.61	0.11	1.14
		6718	27181	0.04	1.26	0.25	1.17

[a] Activities expressed as nanomoles of product formed per minute per milligram of protein. A specific activity of "0" represents a flat line on the spectrophotofluorometer recording instrument (specific activity < 0.005).

[b] *Anacystis nidulans* strain Tx 20.

[c] *Agmenellum quadruplicatum* BG-1.

[d] At 50°C no shikimate dehydrogenase activity was detected, and the specific activity of arogenate/NADP dehydrogenase was 1.28.

TABLE 4. Dehydrogenase activities with prephenate as substrate

Genus	PCC no.	ATCC no.	Prephenate/NADP dehydrogenase activity[a]	As % of activity with arogenate[b]
Synechococcus	6301	27144	0.68	3
	6908	27146	1.60	9
	6911	27192	0.44	20
Synechocystis	6905	29109	0.04	1
	6714	27178	0.09	1
	7509	29235	0.33	48
Lyngbya	6409	29119	0.59	5
	7114	29121	0.08	1
	7407	29126	0.16	2
Anabaena	7120	27893	0.38	2
	6411	27898	0.56	6
	7119	29151	1.14	14
	—	29413	0.26	4
Nostoc	6719	29105	1.37	20
	73102	29133	2.60	44
Fischerella	7512	29538	0.06	2

[a] Activity expressed as nanomoles of product formed per minute per milligram of extract protein.
[b] Arogenate/NADP dehydrogenase specific activities are listed in Table 3.

ate as substrate were generally very low when compared with activities with arogenate as substrate. In *Synechococcus* sp. strain ATCC 27192, *Synechocystis* sp. strain ATCC 29235, and *Nostoc* sp. strains ATCC 29105 and 29133, however, the prephenate/NADP dehydrogenase activities were at least 20% or more of the arogenate/NADP activities. Whether the prephenate dehydrogenase activities were the result of arogenate dehydrogenase substrate ambiguity or of the presence of a separate gene product could not be determined from the available data.

No consistent patterns of inhibition were observed for any of the dehydrogenase activities. Several species possessed shikimate/NADP dehydrogenase activity that was inhibited by a mixture containing 0.5 mM each phenylalanine, tyrosine, and tryptophan (Table 5). In extracts of *Gloeothece* sp. strain ATCC 27152, *Anabaena* sp. strain ATCC 27893, and *Nostoc* sp. strain ATCC 29150, the inhibition was greater than 50%.

Inhibition of arogenate dehydrogenase by tyrosine was infrequently observed (Table 6), and in only one case, *Nostoc* sp. strain ATCC 29133, was inhibition greater than 40%. In all species, inhibition occurred only when NAD was present as cofactor. Because high concentrations (0.5 mM) of L-tyrosine were used, a trivial decrease in activity would not be unexpected as a result of mass action effects. No L-phenylalanine activation of arogenate dehydrogenase or prephenate dehydrogenase was found.

Chorismate mutase. Chorismate mutase catalyzes the conversion of the branch-point compound, chorismate, to prephenate (Fig. 1). Very little regulation of chorismate mutase by L-phenylalanine and L-tyrosine, singly or in combination, was observed, even at concentrations of 0.5 mM. Only in an extract of *Cylindrospermum* sp. strain ATCC 29204 was chorismate mutase significantly inhibited by L-tyrosine (94%). L-Phenylalanine inhibition was observed in three strains: *Gloeothece* sp. strain ATCC 27152 (100%), *Anabaena* sp. strain ATCC 27893 (58%), and *Calothrix* sp. strain ATCC 27901 (72%).

Chorismate mutase activities in two thermophilic strains, *Fischerella* sp. strain ATCC 29538 and *Synechococcus* sp. strain ATCC 27180, were assayed at both 37°C and 50°C. In *Fischerella* sp. strain ATCC 29538 extracts the activity of chorismate mutase at 37°C was 91% inhibited by 0.5 mM L-tryptophan, whereas at 50°C the activity was only 9% inhibited. In *Synechococcus* sp. strain ATCC 27180, at 37°C the activity of chorismate mutase was 100% inhibited by L-phenylalanine, but at 50°C it was only 10% inhibited.

Prephenate dehydratase. Prephenate dehydratase converts prephenate to the aromatic intermediate phenylpyruvate, which is subsequently transaminated to form L-phenylalanine (Fig. 1). In several extracts the presence of aminotransferase activity interfered with the determination of regulation by L-tyrosine and L-phenylalanine.

TABLE 5. Inhibition of shikimate/NADP dehydrogenase activities by aromatic amino acids

Genus	PCC no.	ATCC no.	ΔFU per min		% Inhibition
			Control	Aro[a]	
Gloeothece	6909	27152	0.54	0.10	81
Synechocystis	6905	29109	1.50	1.00	33
Plectonema	6402	27902	1.36	0.76	44
Anabaena	7120	27893	15.00	6.26	58
Nostoc	7107	29150	0.24	0.10	60
Calothrix	7507	29112	3.60	2.58	29

[a] Aromatic amino acids, L-phenylalanine, L-tyrosine, and L-tryptophan, present at a concentration of 0.5 mM each.

Deamination of L-tyrosine produces 4-hydroxyphenylpyruvate which absorbs at 330 nm, and deamination of L-phenylalanine produces phenylpyruvate. Attempts to specifically inhibit the aminotransferase activity were unsuccessful. However, corrections for the presence of 4-hydroxyphenylpyruvate could be made by reading the absorbance at wavelengths from 320 to 330 nm. The λ_{max} was indicative of the relative amounts of phenylpyruvate and 4-hydroxyphenylpyruvate present in the assay mixture. The formation of phenylpyruvate via phenylalanine deamination often masked L-phenylalanine inhibition of prephenate dehydratase in the absence of L-tyrosine, but the level produced was insufficient to mask the inhibition of L-tyrosine activation.

L-Tyrosine activated, and L-phenylalanine inhibited, prephenate dehydratase activity in 56% of the strains examined. In many strains activity in the absence of L-tyrosine was below detectable levels. In no instance was L-tyrosine activation present without accompanying L-phenylalanine inhibition. In *Synechocystis* sp. strain ATCC 29108 inhibition by L-phenylalanine was competitive with respect to both activator (L-tyrosine) and substrate (unpublished data). In eight strains, no prephenate dehydratase activity could be detected even in the presence of L-tyrosine, or when an extract of one of these, *Anabaena* sp. strain ATCC 29151, was prepared in the presence of L-tyrosine. The absence of activity was attributed to a high degree of enzyme lability since, even in those extracts in which activity was present, the activity rapidly diminished with time unless L-tyrosine was present. In three strains (*Gloeothece* sp. strain ATCC 27152, *Oscillatoria* sp. strain ATCC 29134, and *Nostoc* sp. strain ATCC 29411) prephenate dehydratase activity was observed, but L-tyrosine was ineffective as an activator and L-phenylalanine was an effective inhibitor. In *Scytonema* sp. strain ATCC 29171 the activity was unchanged by the presence of either effector.

Arogenate dehydratase and phenylalanine hydroxylase. Arogenate dehydratase catalyzes the conversion of arogenate to phenylalanine. Of the 48 strains of cyanobacteria included in this survey, 23 were assayed for arogenate dehydratase, but no activity was detected in any of the strains. Similarly, no phenylalanine hydroxylase activity was found in any of these strains.

Implications of diversity. The cyanobacteria in sections I and II are unicellular (single cells or colonial aggregates), and sections III, IV, and V contain filamentous organisms. Grouping of the cyanobacteria into five sections by Rippka et al. (30) was determined solely by reproductive and divisional characters, and further classification into generic groups was based on additional morphological characters. Although some cellular characters may truly indicate taxonomic relatedness, they are often hard to determine, being subject to variable cultural conditions and

TABLE 6. Inhibition of arogenate/NAD dehydrogenase activities by L-tyrosine

Genus	PCC no.	ATCC no.	ΔFU per min		% Inhibition
			Control	Tyr[a]	
Synechococcus	6717	27180	1.92	1.56	18
	6910	27191	2.10	1.27	40
	6911	27192	4.22	3.20	23
Synechocystis	7509	29235[b]	1.58	1.03	35
Nostoc	73102	29133	1.78	0.42	77

[a] L-Tyrosine present at 0.5 mM.
[b] Cofactor was NADP. No activity was detected with NAD as cofactor.

interpretations. In the form genus *Synechococcus*, for example, are included 28 strains representing seven different genera as defined by previous classification schemes. This suggests that such a form genus could possibly be broken down into subgenera by using more reliable taxonomic characters such as nucleic acid sequence homologies and enzymological patterning, as has been done with the pseudomonads (5, 35). Our data on aromatic amino acid biosynthesis served to delineate generic groupings in the cyanobacteria.

The correlation of DAHP synthase regulation with dehydrogenase data and DNA base composition for strains of *Synechococcus* and *Synechocystis* is presented in Table 7. The most striking feature was the separation that occurred with regard to patterns of DAHP synthase regulation.

As a group, the various *Synechocystis* sp. strains were characterized by potent inhibition of DAHP synthase by L-phenylalanine (90% or better inhibition in the presence of 0.1 mM L-phenylalanine). In *Synechocystis* sp. strain ATCC 29108 this sensitivity is accompanied by growth inhibition in the presence of exogenous phenylalanine (15). All the *Synechocystis* sp. strains exhibited an overwhelming preference of arogenate dehydrogenase activity for the cofactor NADP. The low level of prephenate dehydrogenase activity detected in strains ATCC 27150 and 27178 could possibly be due to a small degree of arogenate dehydrogenase substrate ambiguity. The low prephenate dehydrogenase specific activities as compared with arogenate dehydrogenase activities (Table 4) support this conclusion. Within the genus *Synechocystis*, Rippka et al. (30) recognized two subgroups which were distinguished by mean DNA base compositions of low (35 to 37) and high (42 to 48) mol% guanine plus cytosine (G+C). Our examination of enzyme activities in the aromatic pathway revealed the possible existence of a third subgrouping listed as group 3 in Table 7. This group was distinguished by a complete lack of arogenate dehydrogenase activity with the cofactor NAD. *Synechocystis* sp. strain ATCC 29235, however, is distinguished from strain ATCC 29108 by the presence of prephenate dehydrogenase activity and is further distinguished from other *Synechocystis* sp. strains by its larger genome size (17) and its ability to grow photoheterotrophically at the expense of sucrose (30). In group 2 strains ATCC 27171 and 27178 there was a slight preference for NAD over NADP as the cofactor for shikimate dehydrogenase. This preference, plus the difference in arogenate NADP/NAD activity ratios, would seem to preclude identity of strain ATCC 27171 with strain ATCC 29109, as has been suggested (17, 18, 30), but provides additional evidence for the identity of strains ATCC 27171 and 27178.

The strains designated as members of the genus *Synechococcus* by Rippka et al. (30) fell into two quite distinct groupings (Table 7). Group 1 was characterized by (i) a DAHP synthase mildly sensitive to tyrosine or not regulated at all, (ii) significant prephenate dehydrogenase activities with NADP as cofactor, and (iii) very high mol% G+C. These strains are considered by us to be representative of the genus *Synechococcus*.

Synechococcus sp. strains in group 2 were characterized by (i) sensitivity of DAHP synthase to inhibition by L-phenylalanine and (ii) the absence of any prephenate dehydrogenase activity in the presence of NAD or NADP. Sequence analyses of 16S rRNA by Bonen et al. (2) indicate that *Synechococcus* sp. strains ATCC 29404 and 29172 are less closely related to *Synechococcus* sp. strain ATCC 27144 than to *Synechocystis* sp. strain ATCC 27178, both of which are considered reference strains for their respective genera by Rippka et al. (30). Indeed, according to the S_{AB} values, a measure of relatedness, *Synechococcus* sp. strain ATCC 29404 is more closely related to *Synechocystis* sp. strain ATCC 27178 than is *Synechocystis* sp. strain ATCC 27179 (2). *Synechococcus* group 2 was further subdivided into two groups, A and B, on the basis of DNA composition as reported by Herdman et al (18).

The genera of section III include the nonheterocystous, filamentous strains. Our survey included only a few representative strains of this group, but the enzymological data provided a basis for the subgrouping presented in Table 8. The *Oscillatoria* and *Plectonema* sp. strains were characterized by retro-Phe inhibition of DAHP synthase and the absence of any detectable prephenate dehydrogenase activity. The *Lyngbya* sp. strains were characterized by the presence of prephenate/NADP dehydrogenase activity, but, although two strains were characterized by the retro-Phe pattern of inhibition for DAHP synthase, one (ATCC 29121) possessed a retro-Tyr pattern of inhibition and most likely represents an additional grouping within the LPP-B group as defined by Rippka et al. (30).

Of the six generic groups in section IV (heterocystous, uniseriate, filamentous forms), four were included in our survey. Inhibition patterns for DAHP synthase were quite varied both within and between form genera. Table 9 correlates the DAHP synthase inhibition patterns with other enzymological characters and defines proposed groupings. Rippka et al. (30) separated the *Anabaena* sp. strains into two distinct groups primarily on the basis of cultural and morphological characteristics. Our enzymological data sep-

TABLE 7. Enzymological comparison of *Synechocystis* sp. and *Synechococcus* sp. strains

Genus	PCC no.	ATCC no.	DAHP synthase inhibition	Dehydrogenase activities			Mol% G+C[c]	Group
				Shikimate NADP/NAD[a]	Prephenate[b] as % of arogenate activity	Arogenate NADP/NAD[a]		
Synechocystis	6308	27150	Retro-Phe	8.2	0	3.9	35.2	1
	6902	29108	Retro-Phe	3.6	0	—	42.1	3
	7509	29235	Retro-Phe	6.1	48	—	42.5	
	6714	27178	Retro-Phe	0.9	1	19	47.7	
	6702	27171	Retro-Phe	0.8	0	19	47.4	2
	7008	29110	Retro-Phe	3.3	0	45	44.9	
	6905	29109	Retro-Phe	2.3	1	63	47.1	
Synechococcus	6911	27192	None	6.3	20	6.0	66.3	
	6908	27146	Retro-Tyr	5.5	9	5.6	55.8	1
	6301	27144	Retro-Tyr[d]	4.1	3	5.6	55.1	
	6717	27180	Retro-Phe	0.76	0	15	52.0	
	6910	27191	Retro-Phe	5.9	0	18	47.8	2A
	73109	29404	Retro-Phe	0.76	0	1.5	48.7	
	7502	29172	Retro-Phe	9.0	0	3.9	40.5	2B
	7202	29140	Retro-Phe	2.3	0	2.2	39.0	

[a] Ratio of activity with NADP to activity with NAD as calculated from the data in Table 3.
[b] Activity determined with NADP as cofactor, and calculated from the data in Tables 3 and 4.
[c] From reference 18.
[d] References 19 and 34.

TABLE 8. Enzymological comparison of section III genera

Genus	PCC no.	ATCC no.	DAHP synthase inhibition	Prephenate dehydrogenase[a]
Oscillatoria	7112	29134	Retro-Phe	0
Plectonema	6306	27894	Retro-Phe	0
	6402	27902	Retro-Phe	0
Lyngbya	7407	29126	Retro-Phe	2
	6409	29119	Retro-Phe	5
Lyngbya	7114	29121	Retro-Tyr	1

[a] As percentage of activity with arogenate as substrate, and calculated from the data in Tables 3 and 4.

arated the *Anabaena* sp. strains into the same groupings but with the possible addition of a third grouping. Group 1 was characterized by an additive pattern of DAHP synthase inhibition, the presence of prephenate dehydrogenase, and a relatively high specificity of shikimate dehydrogenase for NADP as cofactor. *Anabaena* sp. strain ATCC 29413 fit the above criteria except for the apparent cumulative pattern of DAHP synthase inhibition and thus may represent an additional grouping (group 3). Strains assigned to group 2 were characterized by the retro-Phe pattern of DAHP synthase inhibition, the absence of prephenate dehydrogenase activities, and increased relative activity of shikimate dehydrogenase with NAD as cofactor. In three of the group 2 strains, no arogenate or prephenate dehydrogenase activity could be measured. This may be a result of some as yet undetermined reaction requirement common to these strains.

Anabaena sp. strains ATCC 27899 and 29211 were considered by Rippka et al. (30) to repre-

TABLE 9. Enzymological comparison of section IV genera

Genus	PCC no.	ATCC no.	DAHP synthase inhibition	Dehydrogenase activities			Group
				Shikimate[a] NADP/NAD	Prephenate[b]	Arogenate[a] NADP/NAD	
Anabaena	7120	27893	Additive	8.1	2	11.2	
	6411	27898	Additive	62.5	6	16.2	1
	7119	29151	Additive	45.3	14	21.5	
Anabaena	7118	27892	Retro-Phe	3.8	0	4.4	
	7122	27899	Retro-Phe	1.1	0	NA[c]	2
	6309	29211	Retro-Phe	1.6	0	NA[c]	
	—	29414	Retro-Phe	3.1	0	NA[c]	
Anabaena	—	29413	Cumulative	22.0	4	13.9	3
Nostoc	7524	29411	Additive	8.1	0	12.2	1
	7422	29132	Additive	NA[c]	0	—	
Nostoc	6719	29105	Concerted	10.1	20	10.2	2
	73102	29133	Cumulative	6.1	44	8.5	3
Calothrix	6303	29156	Retro-Phe	14.5	0	82.3	
	7504	29158	Retro-Phe	6.0	0	13.8	1
	7102	27901	Retro-Phe	4.4	0	26.2	
Calothrix	7507	29112	Cumulative	6.1	0	23.9	2
Calothrix	7103	27905	Concerted	21.4	0	15.8	3
Scytonema	7110	29171	Retro-Phe	11.5	0	10.3	

[a] Ratio of activity with NADP as cofactor to activity with NAD as cofactor, and calculated from the data in Table 3.
[b] As percentage of activity with arogenate as substrate, and calculated from the data in Tables 3 and 4.
[c] No activity detected.

sent the same species. DNA-DNA reassociation values also indicated a high degree of similarity between these strains (27). The retro-Phe pattern of DAHP synthase inhibition and the similarity of shikimate/NADP to shikimate/NAD dehydrogenase ratios support this conclusion. However, strain ATCC 27892 was quite clearly similar to the strains in group 2 and was not identical with the strains in group 1 as previously suggested (30). Strains ATCC 29151 and 27898 appeared to be identical because of the similarities in dehydrogenase specific activities (Table 3), DAHP synthase inhibition data (Table 2), and the presence of prephenate dehydrogenase (Table 4). On the basis of these comparisons, however, strain ATCC 27893 appeared to represent a species or strain different from strains ATCC 27898 and 29151 because of the large difference in shikimate dehydrogenase NADP/NAD activity ratios.

Three groups were discerned in the form genus *Nostoc*. Group 1 organisms were characterized by an additive DAHP synthase inhibition pattern, group 2 by a concerted pattern, and group 3 by cumulative inhibition. It should be noted that in *Nostoc* sp. strain ATCC 29133 the arogenate/NAD dehydrogenase activity was inhibited by L-tyrosine and there was a very high level of prephenate dehydrogenase activity. DNA-DNA reassociation values also show only a moderate degree of interrelatedness between strain ATCC 29133 and other *Nostoc* sp. strains (27). The absence of arogenate dehydrogenase activities with the cofactor NAD (Table 2) in two strains in group 1 may characterize a fourth grouping.

Strains in the form genus *Calothrix* separated into three groups on the basis of DAHP synthase regulation: group 1 characterized by the retro-Phe pattern, group 2 by the cumulative pattern, and group 3 by the concerted pattern. No prephenate dehydrogenase activity was found in any of the *Calothrix* strains.

The base compositions of all strains in section IV are in a very narrow range (38 to 44 mol% G+C) and provide no basis for subdivisions (18).

DISCUSSION

The results presented in this report reveal that the cyanobacteria possess a diverse array of regulatory patterns for aromatic biosynthesis at the first committed enzymatic step, accompanied by a more modest degree of diversity in the enzymology of subsequent reactions in the common portion of the pathway (shikimate dehydrogenase) and in the diverging branchlets to tyrosine and phenylalanine. It is perhaps not surprising that the more sophisticated inhibition patterns for DAHP synthase (additive, cumulative, and concerted) occur in those strains that are considered to be more complex in that they exhibit filamentous growth with the differentiation of heterocysts and akinetes. The additive patterns of DAHP synthase inhibition in several of the section IV strains suggest the presence of separate regulatory isozymes. Indeed, mutant analyses and physical separation of DAHP synthase activities have confirmed the existence of DAHP synthase regulatory isozymes in *Anabaena* sp. strain ATCC 29151 (16).

The diversity in subsequent reactions resides primarily in the specificities of the shikimate and arogenate dehydrogenase enzymes for the cofactors NAD and NADP, and in the presence of prephenate dehydrogenase activity in several strains. The NADP/NAD dehydrogenase activity ratios should be useful in determining the identity, or nonidentity, of separately isolated strains. It has not yet been determined whether the prephenate dehydrogenase activity is due to a separate enzyme or to a modest degree of substrate ambiguity on the part of arogenate dehydrogenase. The results clearly indicate, however, that the arogenate pathway to tyrosine is a common conserved trait among the cyanobacteria.

In an effort to correct and define cyanobacterial genera which had previously been classified with the eucaryotic algae and so were characterized on the basis of field observations and herbarium specimens, Rippka et al. (30) reclassified the cyanobacteria on the basis of nutritional, physiological, and morphological characteristics. The cyanobacteria were grouped into five major sections, and these sections were further subdivided into generic groups or "form genera." Many of these form genera very likely consist of more than one generic group, and biochemical characterizations are needed to differentiate these groups. The enzymological patterns found in the aromatic pathway of the cyanobacteria, as reported here, did define subgroups within the form genera *Synechococcus*, *Synechocystis*, *Anabaena*, *Nostoc*, and *Calothrix*. Consideration should be given to the possibility of grouping group 2 *Synechococcus* sp. strains with the enzymologically similar group 2 *Synechocystis* sp. strains. These two groups differ in cell shape and mode of cell division, but such characteristics are deemed to have little taxonomic significance (11). Comparative analyses of 16S rRNA sequence homologies have been used to determine phylogenetic relationships among the procaryotes (11, 13), and Bonen et al. (2) have extended these studies to include several strains of cyanobacteria. It was found that *Synechococcus* sp. strain ATCC 29404 and *Synechocystis* sp. strain ATCC 27178 are more closely related to each other than each is to

other strains in its own form genus. Consideration should also be given to reclassifying the strains in section IV.

It should be noted that *Synechococcus* sp. strain ATCC 27144 (*Anacystis nidulans*) has been the subject of many studies on cyanobacterial physiology. Yet, according to its DAHP synthase inhibition pattern and mol% G + C (18), this strain appears to represent a minority group of cyanobacteria.

On the basis of their studies, Bonen et al. (2) postulated that filamentous forms of the genera *Nostoc* and *Fischerella* arose from within the *Synechocystis* group. Our data revealed significant differences in DAHP synthase inhibition patterns between strains in the *Nostoc* and *Synechocystis* form genera. However, the enzymology of strains in group-2 *Anabaena* sp. is very similar to that of the *Synechocystis* sp. strains, so it remains possible that these strains arose from within the *Synechocystis* group. The two *Fischerella* sp. strains examined did exhibit retro-Phe inhibition of DAHP synthase, which is characteristic of the *Synechocystis* sp. strains.

Comparative 16S rRNA homology studies have also indicated that the chloroplasts of the red alga *Porphyridium cruentum* are of definite cyanobacterial origin whereas *Euglena* chloroplasts are possibly of non-cyanobacterial origin (2). Studies (6) have shown that in *E. gracilis* arogenate is an obligatory intermediate in the synthesis of tyrosine, that arogenate dehydrogenase and DAHP synthase are highly sensitive to inhibition by low levels of tyrosine, and that arogenate can also serve as an intermediate in phenylalanine synthesis. The latter two characteristics differentiate *Euglena* from presently existing cyanobacteria. In the study cited, however, whole cell extracts rather than isolated chloroplast extracts were used. In this context it is also interesting that mung bean seedlings contain an unregulated arogenate dehydrogenase activity in addition to a regulated prephenate dehydrogenase activity, and it was postulated that the arogenate dehydrogenase might be localized within the chloroplasts (31). Further analyses of the aromatic amino acid pathway in red algae such as *P. cruentum* and plant chloroplasts would contribute a great deal to our understanding of the origins of chloroplasts and to the determination of the validity of the proposed polyphyletic origin of chloroplasts (29).

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