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Comparative Allostery of 3-Deoxy-D-*Arabino*-Heptulosonate-7-Phosphate Synthetase as a Molecular Basis for Classification

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The allosteric pattern of control for 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase has previously been shown to be strongly conserved among the member species of a given genus in bacteria. The implications of this finding as a procedural tool of bacterial phylogeny were pursued by a study of two organisms, *Sporosarcina ureae* and *Aeromonas formicans*, the taxonomic positions of which have been historically controversial. *S. ureae* has characteristics of both *Bacillaceae* and *Micrococcaceae*, and *A. formicans* has characteristics of both *Enterobacteriaceae* and pseudomonads. Since the patterns of control for DAHP synthetase in all four of these microbial groups are different from one another but internally homogeneous within each group, the results obtained from the two test organisms were unambiguous. It was concluded that *S. ureae* is properly classified within *Bacillaceae*, probably deserving generic rank, and that *A. formicans* belongs with the family *Enterobacteriaceae*.

The multibranched pathway that is used for the biosynthesis of aromatic amino acids in microorganisms is sufficiently complex to have allowed the evolutionary development of six or more alternative patterns of allosteric regulation. The enzyme, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase, catalyzes the initial biochemical reaction and is negatively controlled by feedback inhibition. Previous data documented the microbial control patterns for DAHP synthetase in 32 genera represented by more than 90 species (15). A striking conservation of the pattern of control for DAHP synthetase among the member species of all genera examined was found. These data have been recently updated (R. A. Jensen and J. L. Rebello, Dev. Ind. Microbiol., *in press*).

To investigate the validity of comparative allostery for assessing taxonomic relationships, the majority of microorganisms previously selected were those for which there appeared to be a consensus about taxonomic position. The resultant agreement of our analysis of control for DAHP synthetase with generally accepted taxonomic schemes stimulated a program of examination of microorganisms of uncertain, controversial taxonomic position. This communication deals with two microorganisms of long-standing interest: *Sporosarcina ureae* and *Aeromonas formicans*. Although *S. ureae* is a packet-forming coccus like species of *Micrococcus* and *Sarcinia*, its motility and ability to form true endospores are suggestive of *Bacillus* (21). The other genus, *Aeromonas*, displays morphological characteristics that are suggestive of pseudomonads but has the biochemical make-up of enteric bacteria. Species of *Aeromonas* have been suggested to be "intermediate" between pseudomonads and enteric bacteria (6). The taxonomic status of both of these has been examined recently by other molecular methodology (3, 9, 22, 23).

MATERIALS AND METHODS

Bacillus subtilis. NP 40, a prototroph obtained by spontaneous reversion of strain 168 (1; R. L. Armstrong, N. Harford, R. H. Kennett, H. L. St. Pierre, and N. Sueoka, Methods in Enzymology, *in press*) was used. Nutritional and enzymological methodology have been given in detail elsewhere (12, 15-17).

Sporosarcina ureae. A stock of *S. ureae* was obtained from J. Larkin. It was cultured in TSY liquid medium (15) with the *p*H adjusted to 8.4. Shake cultures at 30 C were harvested in the late-exponential logarithmic phase of growth and washed in 0.04 M potassium phosphate buffer (*p*H 6.8) after centrifugation. Precautions were taken to ensure that cultures of S. *ureae* were free from contamination. Cultures were verified to conform to their original description by phase-contrast microscopy examination. The authen-

ticity of the strain was confirmed with the visual observation of packet-formation, endospores, and motility.

Micrococcus (Sarcina) luteus. *M. luteus* ATCC 272 was obtained from the American Type Culture Collection under the name *Sarcina lutea*. It has been designated *M. luteus* by the recommendation of Rosypal et al. (26). It was cultured in TSY liquid medium at 25 C in shake cultures which were grown to the late-exponential phase of growth.

Aeromonas formicans. *A. formicans* was obtained from I. P. Crawford who isolated it (4). It is also available through the American Type Culture Collection as ATCC 13137. It was grown at 32 C in modified Davis minimal medium (20) supplemented with 0.5% glucose and a mixture of nonaromatic amino acids (13) plus phenylalanine at a final concentration of 50 μg/ml. The presence of phenylalanine resulted in the physiological derepression of the tyrosine-sensitive isoenzyme (14) which was otherwise present at relatively low levels during growth in minimal medium.

Escherichia coli. *E. coli* strain C was obtained from Robert Harvey. It was cultured at 37 C in shake cultures. The liquid growth medium was modified Davis minimal salts containing 0.5% glucose.

Pseudomonas putida. *P. putida* ATCC 12633 was acquired from R. Y. Stanier (30). The carbon source and minimal salts composition were 0.25% lactate and modified Davis minimal salts, respectively. Shake cultures were incubated at 32 C.

Assay for DAHP synthetase. Trisodium phosphoenolpyruvate and dicyclohexylammonium D-erythrose 4-phosphate were products of Sigma Biochemical Co. and California Corp. for Biochemical Research (Calbiochem), respectively. Erythrose 4-phosphate was hydrolyzed as specified by Calbiochem and made up as a 9 mM stock solution. It was stored at 4 C to prevent dimer formation, which is induced by freezing (D. B. Sprinson, *personal communication*). In routine assays, the final concentration of erythrose 4-phosphate in reaction mixtures was 2 μmoles/ml for extracts of *Bacillus* species, 6 μmoles/ml for extracts of *S. ureae*, and 0.4 μmoles/ml for extracts of *M. luteus*. These concentration differences reflect the variation in K_m values for erythrose 4-phosphate exhibited by the various DAHP synthetases studied. Phosphoenolpyruvate was used at 2 μmoles/ml in every case. The routine assay temperature for both *A. formicans* and *P. putida* extracts was 32 C; the DAHP synthetases of other microorganisms were assayed at 37 C unless otherwise specified. Cells were disrupted at 4 C with a Biosonik Ultrasonic Disintegrator (Bronwill Scientific). Extracts were either dialyzed overnight against 1,000 volumes of buffer or passed through a Sephadex G-25 column at 4 C. The composition of buffer and other details of the assay were those previously reported for *B. subtilis* (17, 18) unless otherwise specified.

Because of its interaction with periodate, tryptophan interferes with the thiobarbituric assay for DAHP (29). The relative magnitude of this effect increases as lower enzyme activities are measured, a result which can lead to an apparent competitive inhibition of DAHP synthetase by tryptophan. This apparent inhibition was noted with the enzyme of *M. luteus*. Similar results had been obtained from *M. luteus* ATCC 398, *M. lysodeikticus* ATCC 4698, *Streptococcus faecalis* ATCC 4200 and *S. bovis* ATCC 9807 (Table 6 of reference 15). When we used the dephosphorylated derivative of DAHP isolated from a mutant of *B. subtilis* blocked in dehydroquinate synthetase as a control for this interference by tryptophan with the chemical assay for DAHP, the results were poorly reproducible for unknown reasons. A more reliable method for measuring the interference was to add tryptophan to the control reaction vessel after the reaction was terminated with acid just before the chemical assay for DAHP formed in the reaction was begun. Carefully controlled experiments showed that all of the apparent competitive inhibition of DAHP synthetase by tryptophan in *M. luteus* could be accounted for by its interference with the chemical assay system. Hence, *M. luteus* is a member of a group of microorganisms (which includes *M. lysodeikticus*, *S. faecalis*, and *S. bovis*) in which DAHP synthetase tentatively is classified as unregulated.

RESULTS

Sporosarcina ureae. *S. ureae*, a packet-forming, gram-positive coccus (like micrococci), but which produces true endospores (like *Bacillus*), has posed an intriguing dilemma for taxonomists seeking its proper taxonomic position. Since the pattern of control for DAHP synthetase for aerobic *Micrococcus* species is known to be distinct from that of *Bacillus* (15), it seemed likely that a comparison of the *S. ureae* enzyme with that of these two groups of microorganisms would lead to decisive conclusions.

It is clear from Table 1 that *S. ureae* possesses a DAHP synthetase that is regulated with the

TABLE 1. *Identification of feedback inhibitors of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase*

Compound tested[a]	Per cent inhibition of DAHP synthetase activity		
	Bacillus subtilis	*Sporosarcina ureae*	*Micrococcus (Sarcina) luteus*
Chorismic acid	71	64	≧5
Prephenic acid	84	73	≧5

[a] Final concentration of each compound tested as an inhibitor was 5×10^{-4} M at an assay temperature of 37 C. Phenylalanine, tyrosine, and tryptophan, tested individually at a final concentration of 5×10^{-4} M, in no case produced an inhibition greater than 5%. The combination of phenylalanine, tyrosine, and tryptophan, each at 5×10^{-4} M, plus *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and 3,4-dihydroxybenaldehyde, each at 5×10^{-5} M, also did not inhibit activity more than 5%.

same control pattern as that of *B. subtilis*. *B. subtilis* and other *Bacillus* species have DAHP synthetases that are feedback inhibited by the branch point metabolites, chorismate and prephenate, a physiological control designated Sequential Feedback Inhibition (16, 25). *M. luteus*, on the other hand, probably possesses an unregulated DAHP synthetase. It is, of course, possible that the enzyme of *M. luteus* is easily desensitized or that it is regulated in some novel fashion. However, it is sufficient for our purposes that the two generic control patterns are recognizably different, and that these patterns are reliable characteristics of most or all member species in each genus.

Other enzymological experiments were carried out to examine the quantitative details of the control properties of the DAHP synthetases of *B. subtilis*, *M. luteus*, and *S. ureae*. Substrate saturation curves (both substrates) conformed with Michaelis-Menten kinetics for the DAHP synthetase of all three microorganisms. The *M. luteus* enzyme (Fig. 1) was strongly inhibited by high erythrose 4-phosphate concentrations. Substrate inhibition was reversed by increasing the concentration of phosphoenolpyruvate. As shown in the inset of Fig. 1, a 10-fold increase in phosphoenolpyruvate concentration decreased substrate inhibition almost completely. The reaction mechanism involves an ordered chemical pathway with respect to the two reactants. The order of substrate addition is obligatory, since parallel lines were observed in double reciprocal plots by using several series of substrate saturation curves carried out with various concentrations of the fixed substrate (7). The presence of possible allosteric metabolites did not influence the shape or magnitude of the substrate saturation curve in *M. luteus*. Possible inhibitors that were tested also did not influence the substrate saturation curve when phosphoenolpyruvate was the variable substrate. Hence, the potent substrate inhibition by erythrose 4-phosphate in *M. luteus* did not implicate any subtle allosteric regulatory pattern that we could recognize [as, for example, is the case with the DAHP synthetase of *Rhodomicrobium vannielii* (R. A. Jensen and W. C. Trentini, J. Biol. Chem., *in press*)]. Substrate inhibition of DAHP synthetase activity was not found in extracts from *B. subtilis* or *S. ureae*.

The quantitative response of enzyme activity from *S. ureae* to variable concentrations of prephenate is indicated in Fig. 2. The inhibition curve obtained at an assay temperature of 15 C exhibits first-order kinetics with 50% inhibition of activity occurring at 3.7×10^{-6} M prephenate. At 40 C (data not given), the enzyme was 45 times less sensitive to prephenate (50% inhibition

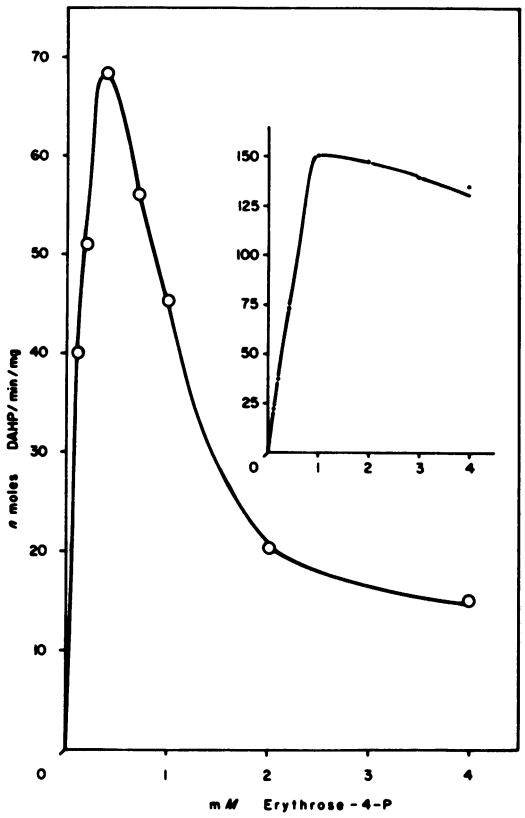

FIG. 1. *Substrate saturation curve of DAHP synthetase in a crude extract of M. (Sarcina) luteus. The specific activity in nanomoles of DAHP formed per minute per milligram of protein is plotted as a function of erythrose 4-phosphate concentration. Phosphoenolpyruvate was fixed at a concentration of 1 mM. The inset at the upper right shows the result of increasing the phosphoenolpyruvate concentration to 10 mM in the same experiment. The reaction mixtures contained 0.40 mg of protein.*

at 1.7×10^{-4} M) than was the case at 15 C. The experiment shown in Table 2 shows that the decreased sensitivity of the enzyme to inhibition by prephenate at the higher temperature was not due to a permanent desensitizing effect of the heat treatment. The enzyme was stable at 40 C, and the rate of the reaction was increased 49% (*see* specific activity data). A comparison of columns 1 and 3 shows that preincubation of the enzyme at 40 C for 30 min did not alter the activity of DAHP synthetase or its sensitivity to inhibition by prephenate when it was subsequently assayed for activity at 15 C. Such a temperature-mediated modulation of inhibitor sensitivity of DAHP synthetase is a common characteristic of *Bacillus* species (R. A. Jensen, Bacteriol. Proc., p. 114, 1967). The allosteric

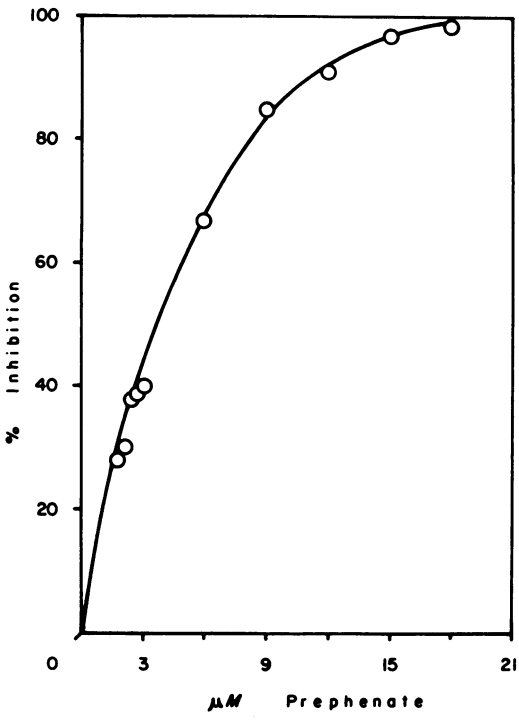

FIG. 2. *Inhibition curve of DAHP synthetase measured at 15 C in S. ureae. Ordinate, per cent inhibition of control enzyme activity* = $(v_0 - v_i)/v_0$; *abscissa, concentration of prephenate prepared as the barium salt. The reaction mixture contained 0.33 mg of protein.*

TABLE 2. *Effect of temperature on the sensitivity of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase to feedback inhibition in Sporosarcina ureae*

Assay conditions	Per cent inhibition[a]	Specific activity[b]
15 C, no preincubation	51	18.6
40 C, no preincubation	None	27.7
15 C, preincubation at[c] 40 C	54	18.6

[a] Inhibition assays were done at a final prephenate concentration of 3.6×10^{-6} M.

[b] Expressed as nanomoles of DAHP per minute per milligram of protein. All reaction mixtures contained 0.27 mg of protein.

[c] Extract was incubated 30 min at 40 C, placed in an ice bath for 5 min, and then assayed at 15 C.

temperature ratio [concentration of prephenate required to inhibit 50% at 40 C/concentration of prephenate required to inhibit 50% at 15 C] of 45 for *S. ureae* is the largest ratio that has been observed to date. Allosteric temperature ratios found in other *Bacillus* species range from 1 to 12. *M. luteus* DAHP synthetase was totally insensitive to any of the inhibitors tested in assays within the temperature range of 15 C to 40 C.

Aeromonas formicans. Pseudomonads possess DAHP synthetases subject to unimetabolite control by L-tyrosine (15). *E. coli*, in contrast, possesses several isoenzymic DAHP synthetases which are separately regulated (28). It should be noted here that proper methodology (14) is necessary to distinguish the two. For example, phenylalanine is an analogue of tyrosine and sufficiently high concentrations of phenylalanine will usually produce a nonspecific inhibition of a tyrosine-sensitive isoenzyme. Also, one isoenzyme is apt to be "dominant" so that other isoenzymes may be revealed only by repressing the synthesis of the dominant isoenzyme. The effect of aromatic amino acids upon the activities of the DAHP synthetases of *P. putida*, *A. formicans*, and *E. coli* are compared in Table 3. DAHP synthetase in *P. putida* was inhibited strongly by tyrosine in extracts from cells grown in minimal medium. Although the presence of 50 μg of tyrosine per ml in the minimal growth medium repressed the specific activity of DAHP synthetase about fivefold, the sensitivities of the enzyme preparations to inhibition by L-tyrosine were identical. This constitutes a "uni-metabolite" pattern of control of a single DAHP synthetase by tyrosine. In contrast, a fraction of the *E. coli*

TABLE 3. *Comparison of the Aeromonas inhibition pattern with that of other microorganisms*

Compound tested[a]	Per cent inhibition of DAHP synthetase activity[b]					
	Pseudomonas putida		*Aeromonas formicans*		*Escherichia coli* C	
	− Tyrosine[c]	+ Tyrosine	− Phenylalanine	+ Phenylalanine	− Phenylalanine	+ Phenylalanine
Tryptophan	0	0	7	40	0	2
Tyrosine	89	91	18	77	31	63
Phenylalanine	1	2	79	55	70	33
Aromatic end products	94	90	98	93	96	94

[a] The metabolites listed were tested as inhibitors at a final concentration of 0.2 mM. See Table 1 for the composition of aromatic end products.

[b] The reaction mixtures for *P. putida*, *A. formicans*, and *E. coli* contained 0.41, 0.32; 0.07, 0.09; and 0.06, 0.05 mg of protein per 0.3-ml volume, respectively, from left to right above.

[c] For each microorganism, the data in the left-hand column were obtained from cultures grown in minimal medium, and data in the right-hand column were obtained from cultures grown in minimal medium supplemented with either tyrosine or phenylalanine at 50 μg/ml.

enzyme activity is inhibited by phenylalanine, and another fraction is inhibited by tyrosine. The phenylalanine-sensitive isoenzyme predominates in extracts from cultures grown in minimal medium. Growth in phenylalanine-supplemented medium results in the repression of the phenylalanine-sensitive isoenzyme and derepression of the tyrosine-sensitive enzyme. It is qualitatively clear that enzymatic activity of *A. formicans* resembles that of *E. coli*, differing substantially from that of *P. putida*. The isoenzymes of *A. formicans* displayed greater overlapping specificities for inhibitors than those of *E. coli;* good additivity of inhibitor combinations was not found above inhibitor concentrations of 5.0×10^{-5} M. Figure 3 indicates the result of a Sephadex G-100 gel fractionation of the isoenzymic DAHP synthetases from *A. formicans*. The leading peak of activity eluted contained the phenylalanine-sensitive isoenzyme (inhibited 61% by 0.05 mM phenylalanine). The second peak of enzyme activity eluted was a tyrosine-sensitive isoenzyme (inhibited 81% by 0.05 mM tyrosine). The lowest molecular weight fraction was mainly inhibited by tryptophan (76%, 0.05 mM). The isoenzymes in crude extracts were inhibited by combinations of inhibitors at 0.03 mM in accordance with expectations for strictly additive inhibitions produced by individual inhibitors acting independently upon different enzyme molecules. Kinetic studies have not been done to determine whether the isoenzymes are subject to noncompetitive inhibition with respect to both substrates, as in the case of *E. coli* (28). The inset in the upper left corner of Fig. 3 shows the ability of 0.1 mM concentrations of each amino acid to inhibit the activity of DAHP synthetase in the unfractionated extract for comparison with the inhibition histograms obtained from the eluate fractions. Eluate tubes 30, 35, and 42, slightly displaced from the apparent activity peaks, were used for inhibition analyses to avoid possible inaccuracies resulting from the slight overlapping of the peaks. The dotted lines represent an estimate of the true peaks. The results with *A. formicans* resemble previous results obtained with crude extracts of *A. liquifaciens* ATCC 14715 and *A. hydrophila* ATCC 9071 (15).

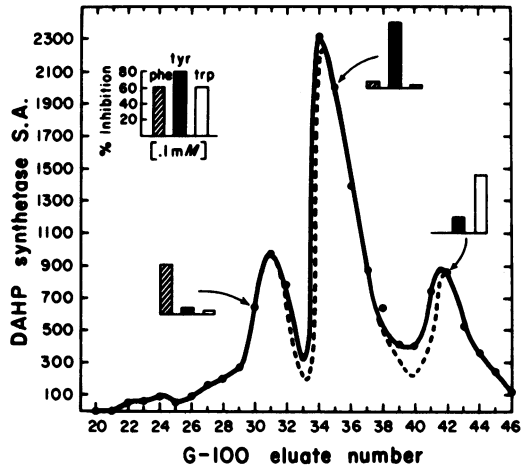

FIG. 3. *Gel filtration profile of DAHP synthetase isoenzymes of A. formicans. Cells from the middle-exponential phase of growth were disrupted by sonic treatment. Extract (1 ml) containing a total of 9.1 mg of protein was passed through a Sephadex G-100 gel column (75 cm by 3.2 cm) equilibrated with 0.04 M potassium phosphate buffer containing 0.1 M KCl (pH 7.2). Seven-drop fractions were collected at a flow rate of 14 ml/hr. Specific activity, on the ordinate, is nanomoles of DAHP formed per minute per milligram of protein at 37 C. The bar graphs indicate the result of inhibition analyses carried out with eluate fractions (indicated by an arrow) corresponding to the three isoenzymes that were separated. The inset at the upper left indicates the results of an inhibition analysis on the crude, unfractionated extract.*

DISCUSSION

The classical methodology for defining taxonomic relationships in microorganisms has recently been supplemented with new molecular techniques (22, 23). Procedures, such as DNA (deoxyribonucleic acid)-DNA and DNA-RNA (ribonucleic acid) homology are inspired by the hypothesis that remnants of evolutionary history are conserved in the informational content of genes. In general, the conclusions about taxonomic relationships which derive from the use of newer techniques correspond well with existing taxonomies. It now seems certain that the newer techniques will assist in the making of more difficult discriminations and will contribute additional information concerning the most appropriate taxonomy of microorganisms which are now difficult to classify.

We have undertaken a comparative analysis of the metabolic control patterns governing the activity of DAHP synthetase. This allosteric protein is subject to alternative, complex patterns of multi-metabolite regulation. A given pattern of control is consistently found among the member species of various genera which have been examined (15). We suggested that the basis for the strong conservation of control patterns which regulate DAHP synthetase relates to the existence of regulatory interactions that are exerted between various metabolic pathways, a phenomenon termed "metabolic interlock" (13). Thus, a change in a regulatory specificity in one pathway

which is coupled with regulatory processes in one or more other pathways may perturb metabolism sufficiently to override the potential benefits of the particular change. It now appears that the allosteric control of other enzymes positioned at metabolic branch points may also reflect evolutionary relationships as is the case with DAHP synthetase (3). If the foregoing speculations relating to the significance of metabolic interlock are correct, then one would expect a comparable degree of conservation in control patterns for other complex pathways as well.

S. ureae, a packet-forming coccus, is morphologically reminiscent of species of the family *Micrococcaceae*. Yet its ability to form true endospores (11, 24, 31), its guanine plus cytosine (GC) ratio (11), and its motility under certain nutritional conditions (8, 21) are all suggestive of its proper placement in the genus *Bacillus*. The *Bacillus* group and the aerobic *Micrococcus* group are internally homogeneous with respect to the control pattern for DAHP synthetase. The two groups are easily distinguished by a qualitatively different pattern of control for DAHP synthetase. The control of DAHP synthetases of numerous *Bacillus* species has been studied and found to be similar; most of the data cited in this paper were obtained from *B. subtilis*, taken to be a representative organism. *M. luteus* was selected as the representative of the *Micrococcaceae*. We have not considered the microaerophilic-anaerobic *Sarcina* species, *S. ventriculi* and *S. maxima*. The aerobic sarcinae are clearly different from the anaerobic, sugar-fermenting species in GC base ratios in the DNA, and the aerobic sarcinae are properly designated as *Micrococcus* (2). It is the aerobic sarcinae which seem to be appropriate for taxonomic comparison with *S. ureae*. *B. subtilis* and other *Bacillus* species display a pattern of regulation termed Sequential Feedback Inhibition (25). *M. luteus* typifies a group of microorganisms which appear to have unregulated DAHP synthetases. *S. ureae*, like species of *Bacillus*, exhibited the qualitative pattern of control for DAHP synthetase, Sequential Feedback Inhibition. DAHP synthetases of *Bacillus* species and *S. ureae* are feedback inhibited by chorismate and prephenate; the DAHP synthetases of *Micrococcus* probably are not feedback inhibited at all.

S. ureae was compared with the various subgroups of *Bacillus* which have been formulated on the basis of the allosteric temperature ratio (R. A. Jensen, Bacteriol. Proc., p. 114, 1967; Jensen, *unpublished data*). The temperature analysis resulted in the recognition of four discrete groups of allosteric temperature ratios. *S. ureae* constituted one of the subgroups by itself. The other three subgroups correlate fairly well with the three cytological groups of Smith, Gordon, and Clark (27). Hence, the qualitative pattern of control observed for DAHP synthetase agrees with the placement of *S. ureae* in the family *Bacillaceae*, whereas the quantitative measurement of allosteric temperature ratios agrees with previous suggestions (21) that it be given rank as another genus in that family, i.e., genus *Sporosarcina*. It appears probable to us that other subgroups will eventually be sufficiently well-defined to acquire generic designation within *Bacillaceae*.

Our second example again involves a dilemma in classification arising from the conflicting conclusion made when morphological and biochemical characteristics are compared. Species of *Aeromonas* have features that are suggestive of pseudomonads (morphological) as well as of enteric bacteria (biochemical references 5 and 6). *A. formicans* was selected as our test strain because it has been the subject of other recent studies using molecular biological techniques. *A. formicans* resembles *E. coli* rather than pseudomonads with respect to tryptophan enzymes, isoenzymic aspartokinases, and properties of β-galactosidase (3; R. Y. Stanier, *personal communication*). In our studies, three isoenzymic DAHP synthetases were separated from *A. formicans* by gel filtration. Each fractionated isoenzyme proved to be specifically inhibited by a single aromatic amino acid (Fig. 3). Thus, both *Aeromonas* and *Escherichia* species elaborate isoenzymic DAHP synthetases possessing individual allosteric specificities. Pseudomonads, on the other hand, have a single DAHP synthetase inhibited by tyrosine.

It seems clear that whenever a microorganism appears to be taxonomically related to several other groups, the analysis of allosteric control patterns for DAHP synthetase would be instructive. Provided that the groups with which the controversial microorganism is to be compared display distinct control patterns, it is likely that comparative allostery will have meaningful implications for defining taxonomic relationships.

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