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Taxonomic Implications of Temperature Dependence of the Allosteric Inhibition of 3-Deoxy-D-*arabino*-Heptulosonate 7-Phosphate Synthetase in *Bacillus*

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The qualitative pattern of control for 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase is a stable and strongly conserved trait of related bacteria and constitutes a reliable generic character. In *Bacillus*, the generic control pattern for DAHP synthetase is sequential feedback inhibition, a regulatory pattern in which branch-point metabolites are feedback inhibitors. Member species of this genus have DAHP synthetases which vary quantitatively in the effect of temperature upon the sensitivity of the enzyme to feedback inhibition by prephenate. The magnitude of this temperature effect has been expressed quantitatively as the allosteric temperature ratio. The species clusters definable by allosteric temperature ratios correlate exceedingly well with subgroups previously distinguished on the basis of sporangial structure. Hence, two independently derived arrangements of *Bacillus* subgroups, depending upon very different methodologies, matched for all but 3 of the 24 species considered. The taxonomic position of these subgroups of the genus *Bacillus* is discussed.

Several different patterns of allosteric control for the regulation of the aromatic acid pathway of biosynthesis exist in microorganisms (6, 10). A given pattern of control of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase is in general shared by bacteria of the same genus. An attempt has been made to use this property as an aid in determining the relationships of certain bacteria whose taxonomic position is doubtful (11). The taxonomic and evolutionary implications of such studies were reviewed recently (10).

One of the control patterns for DAHP synthetase is sequential feedback inhibition (7, 16), which is characteristic of the genus *Bacillus*. In *B. subtilis*, the effectiveness of the inhibition of DAHP synthetase by chorismate and prephenate varies as an inverse function of temperature (9). The exact magnitude of the influence of temperature upon the sensitivity of DAHP synthetase to feedback inhibition varies considerably in different species of *Bacillus*. In this paper, it is shown that taxonomically significant subgroups of *Bacillus* can be recognized in terms of the allosteric temperature ratio, a quantitative expression of the modulation by temperature of the allostery of DAHP synthetase.

MATERIALS AND METHODS

Microbiological technique. Cultures of the various strains were examined to confirm the presence of appropriate nutritional, physiological, and microscopic characteristics. (Significantly, occasional contaminated or improperly labeled cultures were usually recognized as suspect cultures from enzymological data before verification by other procedures.)

Microorganisms. The *Bacillus* species in which stable activity for DAHP synthetase was measured are identified in Table 1. No DAHP synthetase activity was found in extracts of *B. larvae* and *B. popilliae*. Enzyme activities in *B. finitimus*, *B. bombycis*, and *B. pulvifaciens* were too low or unstable to permit the determination of an accurate allosteric temperature ratio. The conditions of growth and enzyme assay for *Sporosarcina ureae* were previously given (11).

Conditions of cultivation. Cultures were grown in volumes of 200 to 500 ml in flasks of 5 to 10 times that volume. Vigorous aeration was achieved on a New Brunswick reciprocal shaker. Most strains were grown in a minimal salts medium (4) containing 0.5% glucose, a mixture of nonaromatic amino acids (5), and 0.01% yeast extract. The presence of nonaromatic amino acids in the absence of the aromatic amino acids usually derepressed the synthesis of DAHP synthetase. Iron was often omitted from the trace elements mixture since this tends to increase the

TABLE 1. *Strain information*

Bacillus sp.	Strain	Source	Growth temp
			C
1. *B. alvei*	ATCC 6344	ATCC	32
2. *B. alvei*	ATCC 6348	ATCC	32
3. *B. alvei*	ATCC 6348-F	Ralph DeMoss (3)	32
4. *B. alvei*	ATCC 6349	ATCC	32
5. *B. alvei*	ATCC 10871	ATCC	32
6. *B. anthracis*[a]	M-36R	Robert Williams	32
7. *B. anthracis*[a]	Stern	Peter Bonventre	32
8. *B. brevis*	8185 NA	Robert Altenbern	32
9. *B. cereus*	ATCC 6464	ATCC	32
10. *B. cereus*	BA 25	Robert Williams	32
11. *B. cereus*	B-48	Peter Bonventre	32
12. *B. cereus* var. *mycoides*[b]	ATCC 6462	ATCC	32
13. *B. cereus* var. *thuringiensis*[c]		Robert Altenbern	32
14. *B. circulans*	ATCC 11043	ATCC	32
15. *B. circulans*	ATCC 4513	John Larkin	32
16. *B. coagulans*	ATCC 7050	ATCC	55
17. *B. firmus*		Henry Koffler	32
18. *B. laterosporus*	ATCC 64	ATCC	32
19. *B. licheniformis*	9945 A	Curtis Thorne	37
20. *B. licheniformis*	NRS 243	Henry Koffler	37
21. *B. licheniformis*[d]		Robert Altenbern	37
22. *B. macerans*		Henry Koffler	32
23. *B. megaterium*	ATCC 13632	ATCC	32
24. *B. pantothenticus*	ATCC 14576	ATCC	32
25. *B. pasteurii*	ATCC 11859	ATCC	32
26. *B. psychrophilus*	ATCC 23304	John Larkin (13)	18
27. *B. psychrosaccharolyticus*	ATCC 23296	John Larkin (13)	18
28. *B. pumilus*	NRS 236	Henry Koffler	32
29. *Bacillus* sp.[e]	T 38 B	John Larkin	18
30. *B. sphaericus*	ATCC 12300	ATCC	32
31. *B. sphaericus*	ATCC 14577	ATCC	32
32. *B. stearothermophilus*	#10 (Nebraska)	Henry Koffler	65
33. *B. stearothermophilus*	FJW (Formosa)	Henry Koffler	65
34. *B. subtilis*	168 (1)		37
35. *B. subtilis*	23 (1)		37
36. *B. subtilis*	ATCC 6051	ATCC	37
37. *B. subtilis*[f]		Robert Altenbern	37
38. *B. subtilis* var. *aterrimus*	ATCC 6460	ATCC	37
39. *B. subtilis* var. *niger*	ATCC 6455	ATCC	37
40. *Sporosarcina ureae*	#860 (14)	John Larkin	32

[a] Both strains of *B. anthracis* are unencapsulated, avirulent laboratory strains.
[b] Received under the name of *B. mycoides;* renamed according to reference 19.
[c] Received under the name *B. thuringiensis;* renamed according to reference 19.
[d] Received under the name *B. globigii;* renamed according to reference 19.
[e] T 38 B is a psychrophilic *Bacillus* which belongs in the subtilis group (J. Stokes, *personal communication*).
[f] Received under the name *B. vulgatus;* renamed according to reference 19.

production of various phenolic derivatives of chorismic acid (18), a phenomenon which correlates with the derepression of DAHP synthetase. The *Bacillus* species *bombycis, circulans, coagulans, finitimus, laterosporus, macerans, pantothenticus, pasteurii, psychrosaccharolyticus, pulvifaciens, sphaericus, stearothermophilus,* and T 38 B were grown in a trypticase-yeast extract medium (6). The medium for *B. anthracis* was that of Thorne et al. (22). The culture of *S. ureae* has been described (11). *B. pasteurii* medium contained 3% urea with the *p*H adjusted to 8.4.

Assay for DAHP synthetase. Dicyclohexylammonium D-erythrose 4-phosphate and trisodium phosphoenolpyruvate were products of Calbiochem, Los Angeles, Calif., and Sigma Biochemical Co., St. Louis, Mo., respectively. The assay was performed as previously described (8). In the case of some *Bacillus* species which possessed an extremely high K_m for erythrose 4-phosphate, enzyme activity was ele-

vated by increasing the final concentration of erythrose 4-phosphate in the reaction mixture from 2 to 6 μmoles/ml. Phosphoenolpyruvate was always used at a final concentration of 2 μmoles/ml.

Control on enzyme desensitization. Although allosteric proteins have frequently been reported to "desensitize" at temperatures which leave catalytic activity unimpaired, none of the *Bacillus* DAHP synthetases became insensitive to inhibition by prephenate at 37 C. This control was done by incubating the extract at 37 C for 30 min, followed by another incubation at 15 C, this time in the presence of the enzyme substrates. The sensitivity of this enzyme preparation to feedback inhibition by prephenate was compared to that determined at 15 C in the absence of preincubation at 37 C. In *B. subtilis*, both the catalytic activity and allosteric sensitivity of DAHP synthetase are stable at 60 C for at least 30 min (8).

Extract preparation. Extracts for enzyme assay were prepared from cells in late logarithmic or early stationary phase of growth. The cells were disrupted by a 15- to 30-min treatment at 34 C with lysozyme (200 μg/ml) and deoxyribonuclease (5 μg/ml), followed by sonic treatment for 1 min in a Biosonik Ultrasonic Disintegrator (Bronwill Scientific Co.). Additional sonic treatments of 1 min, for a total period of as much as 5 min, were necessary to lyse some of the strains examined. The lysed extract was clarified by centrifugation, and small molecules were removed by gel filtration with Sephadex G-25. Occasionally, DAHP synthetase in crude extracts became unstable after Sephadex treatment (i.e., in *B. laterosporus*, *B. macerans*, *B. pantothenticus*, and *B. thuringiensis*), and these enzymes were therefore assayed without removing small molecules.

Chemicals. Barium prephenate was prepared as previously described (7). The purities of the several batches used ranged from 80 to 86% (uncorrected for solvation). Lysozyme (3× crystallized) was purchased from Sigma Chemical Co. and deoxyribonuclease and amino acids were obtained from Calbiochem. All other chemicals were of the highest purity available commercially.

Allosteric temperature ratios. The allosteric temperature ratios (defined under Table 2) were determined by obtaining complete inhibition curves of DAHP synthetase activity as a function of prephenate concentration at 15 and at 37 C. This was essential to determine accurately the 50% inhibition point. In *B. subtilis*, chorismate inhibits DAHP synthetase about one-eighth as well as prephenate on a molar basis. Chorismate and prephenate exhibit identical kinetic effects upon enzyme activity in *B. subtilis* (7). Inhibitor studies with chorismate were not performed because of the complications posed by the potential conversion of chorismate to prephenate, both nonenzymatically (especially at higher temperatures) and enzymatically via chorismate mutase.

RESULTS

Inhibition of DAHP synthetase by prephenate in Bacillus. Sequential feedback inhibition (7, 16) is the term describing the physiological pattern of feedback inhibition of DAHP synthetase by prephenate and chorismate, the branch-point metabolites of the aromatic amino acid pathway. Plots of the inhibition of enzyme velocity as a function of inhibitor concentration typically reveal sigmoid inhibition curves in *Bacillus*. Figure 1 illustrates an inhibition curve having a pronounced sigmoid shape, obtained with the DAHP synthetase from *B. brevis*. This implicates cooperative interactions exerted between inhibitor-binding sites on the enzyme. As also shown in Fig. 1, the *B. brevis* enzyme is equally susceptible to inhibition by prephenate at 15 and 37 C since the inhibition curves obtained at the two temperatures superimpose. Therefore, the allosteric temperature ratio is unity.

Modulation of feedback inhibition by temperature. In contrast to results with the *B. brevis* enzyme, the degree of inhibition of DAHP synthetase activity by prephenate is strongly dependent upon temperature in the majority of *Bacillus* species studied. This is illustrated by the inhibition curves obtained at 37 and at 15 C from *B. sphaericus* DAHP synthetase (Fig. 2). The amount of prephenate required to produce 50% inhibition is greater by a factor of nine at the higher temperature; therefore, the allosteric temperature ratio is 9. The enzymological details of this temperature phenomenon have been studied in the greatest detail in *B. subtilis* (9) in which the allosteric temperature ratio is about 3. The interaction coefficients between inhibitor binding sites (determined by Hill plots) at different temperatures were identical. Hence, in *B. subtilis*, temperature does not influence cooperative interactions between the inhibitor-binding sites of DAHP synthetase, but probably alters the over-all dissociation constant (9).

The DAHP synthetase of *S. ureae* displays by far the most extreme influence of temperature upon inhibitor sensitivity (11); the allosteric temperature ratio is 43. The shape of the inhibition curve changes from sigmoid at 37 C to that of a rectangular hyperbola at 15 C (Fig. 3). Hence, a decrease in interaction between inhibitor binding sites at lower temperatures probably does play an important role in this microorganism to accomplish the tremendous variation of inhibitor sensitivity observed, in contrast to the response described above for the enzyme of *B. subtilis*, which is probably characteristic for most *Bacillus* species.

Analysis of allosteric temperature ratios in Bacillus. Enzymological studies of DAHP synthetase were carried out in 40 strains of *Bacillus* representing 24 species. The results are listed in the order of increasing values for the allosteric

TABLE 2. *Analysis of temperature modulation of feedback inhibitor sensitivity of DAHP synthetase in Bacillus*

Bacillus sp.[a]	Prephenate concn (mM) for 50% inhibition at		Allosteric[b] temp ratio	Sporangial[c] type
	15 C	37 C		
8. *B. brevis*	.085	.082	1.0	2
13. *B. circulans*	.005	.005	1.0	2
15. *B. circulans*	.005	.005	1.0	2
32. *B. stearothermophilus*	.112	.115	1.0	2
33. *B. stearothermophilus*	.099	.103	1.0	2
7. *B. anthracis*	.010	.016	1.6	1
6. *B. anthracis*	.011	.019	1.7	1
10. *B. cereus*	.0025	.0045	1.8	1
16. *B. coagulans*	.011	.020	1.8	1; 2(?)
11. *B. cereus*	.0020	.0038	1.9	1
22. *B. macerans*	.035	.067	1.9	2
9. *B. cereus*	.0020	.0041	2.0	1
13. *B. thuringiensis*	.001	.002	2.0	1
29. *Bacillus* sp.	.002	.004	2.0	1
21. *B. licheniformis*	.030	.062	2.1	1
23. *B. megaterium*	.0013	.0029	2.2	1
28. *B. pumilus*	.017	.038	2.2	1
12. *B. cereus* var. *mycoides*	.009	.021	2.3	1
17. *B. firmus*	.037	.088	2.4	1
37. *B. subtilis*	.009	.023	2.6	1
39. *B. subtilis* var. *niger*	.010	.026	2.6	1
35. *B. subtilis*	.007	.019	2.7	1
36. *B. subtilis*	.007	.019	2.7	1
27. *B. psychrosaccharolyticus*	.007	.019	2.7	2
19. *B. licheniformis*	.025	.069	2.8	1
20. *B. licheniformis*	.024	.067	2.8	1
18. *B. laterosporus*	.032	.089	2.9	2
38. *B. subtilis* var. *aterrimus*	.009	.027	3.0	1
34. *B. subtilis*	.009	.028	3.1	1
25. *B. pasteurii*	.008	.043	5.4	3
30. *B. sphaericus*	.004	.035	8.8	3
31. *B. sphaericus*	.004	.036	9.0	3
26. *B. psychrophilus*	.012	.129	10.8	3
22. *B. pantothenticus*	.005	.060	12.0	3
40. *Sporosarcina ureae*	.003	.130	43.3	

[a] Numbers on the left in this column relate these strains to those identified with corresponding numbers in Table 1. All five strains of *B. alvei* had DAHP synthetases which were insensitive to inhibition by prephenate.

[b] Allosteric temperature ratio is defined as the ratio of the concentration of prephenate required to inhibit 50% at 37 C to the concentration required to inhibit 50% at 15 C.

[c] Sporangial type, as defined by Smith, Gordon, and Clark (19).

temperature ratio in Table 2. The numbers fall into several clusters: 1.0; 2–3; 5–12; and 43. Each value denoting the concentration of prephenate required to inhibit enzyme activity 50% at 15 or at 37 C was estimated from inhibition curves of the type shown in Fig. 1–3.

The absolute concentration of prephenate that inhibits DAHP synthetase activity by 50% at any given temperature in species of *Bacillus* varies as much as 100-fold. It does not appear to be of taxonomic significance, and may vary in strains of a single species. One species of *Bacillus*, *B. alvei*, is an isolated exception to the *Bacillus* generic control pattern of sequential feedback inhibition (6). The exceptional status of this species has been verified in the five strains listed at the top of Table 1. In every case, the DAHP synthetase of this species appears to be unregulated (10).

Bacillus subgroup 1. Smith, Gordon, and Clark (19) distributed 24 species of *Bacillus* into three subgroups on the basis of cytological observations of sporangial structure. Table 3 lists the species of their group 1 together with the allosteric

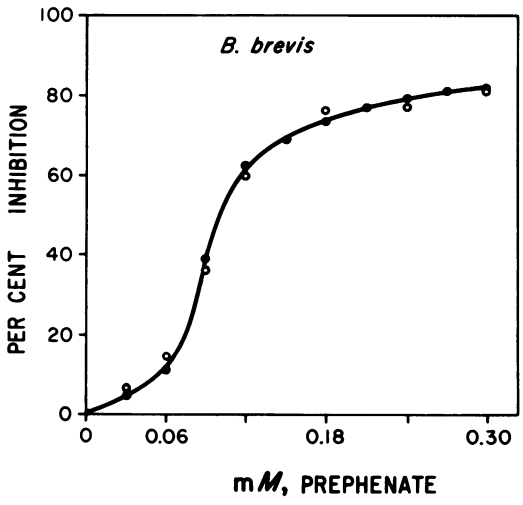

FIG. 1. *Inhibition curve of DAHP synthetase activity as a function of prephenate concentration in B. brevis. Percentage of inhibition on the ordinate was calculated by relating the enzyme activity measured in the presence of prephenate to the activity in the control tube lacking prephenate. The open symbols denote data obtained at 15 C; closed symbols refer to data obtained at 37 C. Prephenate was added to the substrate-enzyme reaction mixture at 4 C in ice to give the final concentrations indicated. The reaction mixture contained 420 μg of protein.*

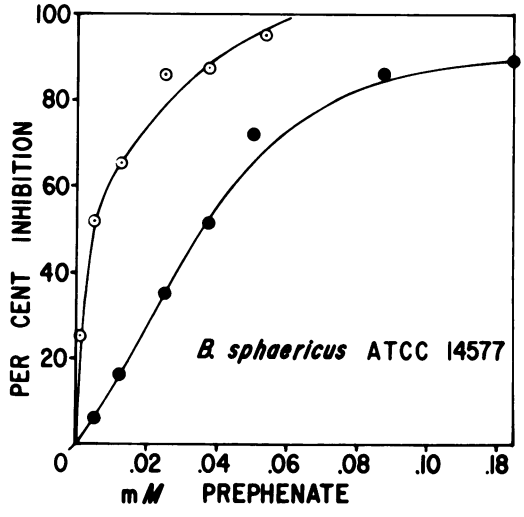

FIG. 2. *Inhibition curve of DAHP synthetase activity as a function of prephenate concentration in B. sphaericus. Symbols: ⊙, assay at 15 C; ●, assay at 37 C. The strain is described in Table 1.*

temperature ratios that were measured. This subgroup is homogeneous with respect to the ratios which range between 2 and 3. The ratios for *B. subtilis* and *B. licheniformis* approach 3, whereas

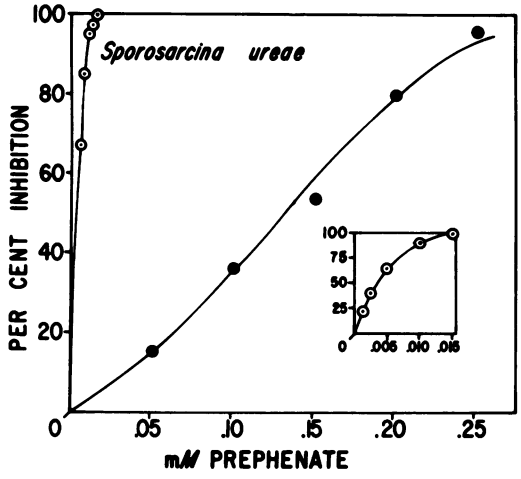

FIG. 3. *Inhibition curve of DAHP synthetase activity as a function of prephenate concentration in S. ureae. Symbols: ⊙, assay at 15 C; ●, assay at 37 C. The strain is described in Table 1. Inset shows the kinetic shape of the inhibition curve obtained at 15 C on an expanded scale of prephenate concentration.*

TABLE 3. *Group 1 subgroup of Bacillus*[a]

Bacillus sp.[b]	Allosteric temp ratio[c]	Avg ratio
B. coagulans[d]	1.8	1.8
B. cereus	1.8, 2.0	
B. cereus var. *anthracis*	1.6, 1.7	
B. cereus var. *mycoides*	2.3	2.0
B. cereus var. *thuringiensis*	2.0	
B. megaterium	2.2	2.2
B. pumilus	2.2	2.2
B. firmus	2.4	2.4
B. licheniformis	2.8	2.8
Bacillus sp.[e]	2.7	
B. subtilis	2.7, 2.7, 3.1	2.8
B. subtilis var. *niger*	2.6	
B. subtilis var. *aterrimus*	3.0	

[a] Sporangial structure; spores not swollen (19).

[b] Strain designations and histories are detailed in full under Table 1. All species included in the studies of Smith, Gordon, and Clark (19) are represented except *B. lentus*. The designations of these authors are used even though a few of them are now not generally accepted.

[c] Defined in Table 2.

[d] *B. coagulans* overlapped both groups 1 and 2 of Smith, Gordon, and Clark (19).

[e] *Bacillus* sp. T 38 B was placed in this sporangial morphology group by Larkin and Stokes (13).

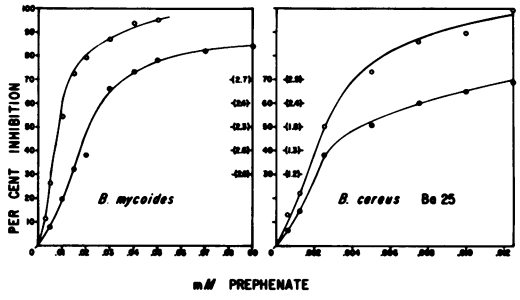

FIG. 4. *Inhibition curves of DAHP synthetase activity as a function of prephenate concentration in B. mycoides (left) and B. cereus (right). Percentage of inhibition on the ordinate was calculated by relating the enzyme activity measured in the presence of prephenate to the activity in the control tube lacking prephenate. Numbers given in the central portion of the chart denote the allosteric temperature ratios (see legend of Table 2) calculated at the following levels of inhibition: 30, 40, 50, 60, and 70% (from bottom to top). Strains are described in Table 1. Symbols: ○, assay at 15 C; ●, assay at 37 C.*

the ratios for other members of the group cluster around a value of 2. Figure 4 shows the inhibition curves obtained from *B. cereus* var. *mycoides* (on the left) and from *B. cereus* (on the right). The inhibition curves obtained with *B. cereus* var. *mycoides* are symmetrical and quite typical. The allosteric temperature ratio calculated in the usual manner comparing the concentration of prephenate required to inhibit activity 50% at 37 C with that required at 15 C does not differ a great deal from analogous ratios calculated using the 30 or 70% inhibition point in place of 50% inhibition. All strains of all groups yielding enzymes with allosteric temperature ratios of 2.2 or better displayed such symmetrical inhibition curves. On the other hand, all strains having allosteric temperature ratios of 2 or less characteristically had a DAHP synthetase in which the inhibition curve at the lower temperature deviated in shape from the inhibition curve obtained at 37 C. Thus, in *B. cereus* (Fig. 4) the ratios differ substantially, depending on the inhibition level from which they are calculated. The possible significance of this observation (e.g., to discriminate between the *subtilis* and *cereus* groups) has not been further pursued.

Bacillus subgroup 3. Sporangial structure group 3 (19) also formed a homogeneous subgroup in terms of allosteric temperature ratios (Table 4). The values vary from 5.4 for *B. pasteurii* to 12.0 for *B. pantothenticus*; these represent a cluster of ratios since no ratios were calculated in any *Bacillus* species between 3.0 and 5.4 or between 12.0 and 43.3. Inhibition data for *B. sphaericus*, a representative member of this group, are given in Fig. 2.

Bacillus subgroup 2. Table 5 lists the member species of sporangial structure subgroup 2 (19). Unlike the other two groups, subgroup 2 was heterogeneous with respect to allosteric temperature ratios. *B. alvei*, a member of this cytological subgroup, does not even share with other *Bacillus* species the same qualitative pattern of control (6). In three other species, the inhibition of DAHP synthetase by prephenate was not influenced by temperature; the allosteric temperature ratio was unity. In four other species, the ratios were between 2 and 3, similar to those characteristic of subgroup 1. Of these, *B. coagulans* was reported by Smith et al. to be intermediate in terms of sporangial structure between subgroups 1 and 2. The placement of *B. psychrosaccharolyticus* is also questionable: although the spores are ellipsoidal and swell the sporangia (subgroup 2), it has other cytological characteristics (13) of the *B. megaterium-B. cereus* group (subgroup 1). Accordingly, only the species *B. macerans*, *B. laterosporus*, and *B. alvei* provide exceptions to the correlation between sporangial structure and allosteric temperature ratio groupings.

Possible physiological relationship of allosteric temperature ratio and temperature range for growth. The only thermophilic organisms examined were strains of *B. stearothermophilus*. The sensitivity of DAHP synthetase to prephenate inhibition in these strains is not affected by temperature. Figure 5 shows that 0.17 mM prephenate inhibits about as well at 55 as at 25 C. In contrast, the DAHP synthetase of one of the first psychrophilic strains examined by us, *B. psychrophilus*, exhibited a large allosteric temperature ratio: 0.03 mM prephenate inhibited the activity of DAHP synthetase 94% at 15 C, but only 12% at 37 C. This suggested the possibility that the effect of temperature upon the allosteric properties of DAHP synthetase might be correlated with the temperature range for growth.

TABLE 4. *Group 3 subgroup of Bacillus*[a]

Bacillus sp.[b]	Allosteric temp ratio[c]
B. pasteurii	5.4
B. sphaericus	9.0
B. psychrophilus[d]	10.8
B. pantothenticus	12.0

[a] Sporangial structure; spores swollen and round (19).

[b] Strain designations and histories are detailed in full under Table 1. All of the species studied by Smith, Gordon, and Clark (19) are represented.

[c] Defined in Table 2.

[d] *B. psychrophilus* was placed in this sporangial morphology group by Larkin and Stokes (13).

TABLE 5. *Group 2 subgroup of Bacillus*[a]

Bacillus sp.[b]	Allosteric temp ratio[c]
B. circulans	1.0, 1.0
B. brevis	1.0
B. stearothermophilus	1.0, 1.0
B. coagulans[d]	1.8
B. macerans	1.9
B. psychrosaccharolyticus[e]	2.7
B. laterosporus	2.8
B. alvei[f]	Prephenate insensitive

[a] Sporangial structure; spores swollen and oval (19).

[b] Strain designations and histories are detailed in full under Table 1. All of the species studied by Smith, Gordon, and Clark (19) are represented except *B. polymyxa* and *B. pulvifaciens*.

[c] Defined in Table 2.

[d] *B. coagulans* overlapped both groups 1 and 2 of Smith, Gordon, and Clark (19).

[e] *B. psychrosaccharolyticus* was placed in this spore morphology group by Larkin and Stokes (13).

[f] *B. alvei* is the only *Bacillus* species found which has a DAHP synthetase that is not sensitive to inhibition by prephenate (6, 10).

However, results obtained with two other psychrophiles did not support this supposition. The allosteric temperature ratio from each of the three psychrophiles examined resembled that of its mesophilic counterpart, rather than that of the other psychrophiles. The enzymes of *B. psychrosaccharolyticus* and *Bacillus* species T 38 B have allosteric temperature ratios of 2.7 and 2.0, respectively. *B. psychrophilus* has been identified as a psychrophilic form of *B. sphaericus* (13), and its allosteric temperature ratio resembles that of mesophilic strains of *B. sphaericus*. Each of the three psychrophiles, representing all three cytological groupings (13), resembles a related mesophilic strain in allosteric temperature ratio.

Since strains of *B. stearothermophilus* cluster with *B. brevis* and *B. circulans* in group 2 (*see* Table 5), it seems clear that insensitivity to the temperature effect is not obligately correlated with thermophily. Hence, the results obtained with both psychrophiles and thermophiles indicate that the temperature range supporting growth has no obvious physiological relationship to the allosteric temperature ratio of DAHP synthetase.

In general, DAHP synthetase is a thermostable enzyme in *Bacillus* species. One of the steps of purification of *B. subtilis* DAHP synthetase is heat treatment at 60 C (8). Even the enzymes from the psychrophilic *Bacillus* species were stable at 37 C for at least 30 min, although temperature lability has been reported for many other enzymes in psychrophiles (20). The physiological significance of the temperature effect, expressed quantitatively as the allosteric temperature ratio in in vitro studies, remains unclear. For example, a good portion of the inhibition curve shown for the enzyme from *B. psychrophilus* in Fig. 5 was obtained at temperatures which will not sustain growth. In any event, our results indicate that psychrophily is not an important taxonomic characteristic, a conclusion which seems to follow a priori from the probability that psychrophiles are represented in most taxa (20).

DISCUSSION

The comparative analysis of control patterns for enzymes is a newly recognized method of determining taxonomic relationships (10, 15). We have found that the qualitative pattern of control for DAHP synthetase is a reliable generic character. We further suggested (10) that the existing control pattern resists evolutionary changes due to metabolic interlock (5). A multigenic basis probably accounts for the conservation of the observed pattern of control for DAHP synthetase as a stable generic characteristic. We suggest that multiple genetic changes would be necessary in order to replace one control pattern with another for the following reasons: (i) it is doubtful that the gene specifying an enzyme could mutate from one complex control pattern to another (e.g., from sequential feedback inhibition to isoenzymic feedback inhibition) by a single mutation or even by several mutations; and (ii) once a given control pattern is integrated with those governing other metabolic pathways (metabolic interlock), a mutated control pattern which could successfully compete with that of

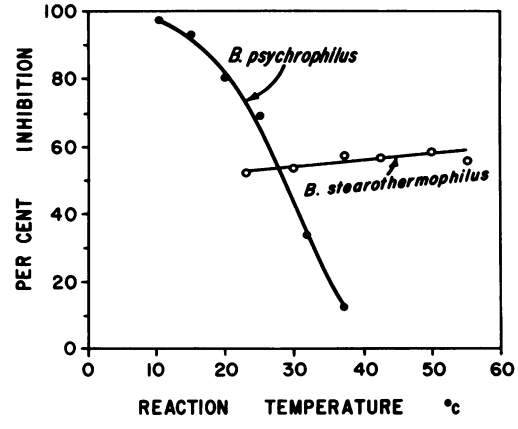

FIG. 5. *Inhibition of DAHP synthetase as a function of assay temperature in B. psychrophilus and B. stearothermophilus. The concentrations of prephenate were fixed at 0.03 and 0.17 mM in B. psychrophilus and B. stearothermophilus, respectively. Strains are described fully in Table 1.*

the parental organism would undoubtedly require additional mutational adjustments in other pathways.

It was of interest to determine whether any generic subgroupings could be distinguished by a comparative study of the effect of temperature on allosteric control. We selected the genus *Bacillus* partly because of its internal diversity (as indicated by the wide span of deoxyribonucleic acid base ratios) and partly because Smith et al. (19) previously proposed three subgroups on the basis of sporangial structure. Additionally, *Bacillus* DAHP synthetase was known to vary in susceptibility to feedback inhibition as a function of temperature (9), similar to several other enzymes (17, 21). Hence, a quantitative parameter of the control pattern, conveniently expressed as the allosteric temperature ratio, was available as a variable characteristic in *Bacillus*.

Allosteric temperature ratios varied from values of 1.0 in three *Bacillus* species to 43.3 in *S. ureae*. It was thought appropriate to include *S. ureae* in these studies as a consequence of previous results (11). Ratios which were calculated from data obtained from 35 strains fell into four ranges: 1.0, 2–3, 5–12, and 43. Species which were placed into the first three groups by this analysis correlated very well with sporangial morphology groups 2, 1, and 3, respectively (19). Only 3 species of the 24 examined were clearly ambiguous when the two methods of subgrouping were compared. All three are members of group 2; they are *B. alvei*, *B. macerans*, and *B. laterosporus*. Hence, all of the discrepancies were confined to the group-2 species, constituting a rather large fraction of the eight group-2 species studied.

Since the alignment of subgroups by the use of allosteric temperature ratios in *Bacillus* correlates well with subgroups defined by the morphological analysis of sporangia, these two methodologies overlap with respect to the hierarchical level of classification at which they discriminate. It was of interest to compare our results with those obtained from other studies involving other taxonomic approaches. Some of our data may reveal smaller taxonomic clusters. For example, the *B. cereus* cluster (average ratio, 2.0) shown in Table 3 appears to be distinctly separate from the *B. subtilis* cluster (average ratio, 2.8). Hence, some of our data may provide confirmation of various intrageneric relationships inferred from gross phenotypic analyses (12, 19). More strains must be examined to pursue this possibility more decisively. The genus *Bacillus* is quite heterogeneous with respect to mean deoxyribonucleate base composition (2). The analysis of deoxyribonucleate base ratios is useful in discriminating very closely related microorganisms. No meaningful correlations could be drawn from a comparison of our results with deoxyribonucleate base composition data. Hence, deoxyribonucleate base ratio analysis and the method of allosteric temperature ratio analysis seem to be useful at entirely different hierarchical levels of taxonomic analysis.

We concluded from the results of a broader, more general study (6) that the comparative control of the branched aromatic amino acid pathway provides useful and reliable clues to taxonomic relationships in microorganisms. It now appears possible that a more detailed comparison of certain properties of an appropriate regulatory enzyme in genera in which member species have qualitatively similar patterns of control may serve to distinguish related subgroups at a lower taxonomic level. A related group of microorganisms undoubtedly tends to acquire more complex hierarchial structure in classification schemes in direct proportion to the attention that the group has received from taxonomists. For example, the genus *Bacillus* undoubtedly does not occupy the same hierarchical level as the member genera of *Enterobacteriaceae*. The separation of three subgroups of *Bacillus* by two different methodologies, one a classic method of cytological observation and the other a molecular approach, seems to us to be a striking observation. It may suggest a future elevation of each subgroup to the generic level with *S. ureae* given rank as a fourth genus. The few species which remain ambiguous after examination by the two methods could reflect the limitations of one or both methodologies. On the other hand, some or all of these could represent still other subgroups.

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