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Allostery of 3-Deoxy-D-*arabino*-Heptulosonate 7-Phosphate Synthetase in *Clostridium*: Another Conserved Generic Characteristic

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Enzymological studies were done to characterize the allosteric control of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase in three species of *Clostridium*. Allosteric control was identified as feedback inhibition by phenylalanine and was qualitatively similar for the DAHP synthetases of *C. butyricum*, *C. acetobutylicum*, and *C. tetanomorphum*. Quantitative differences in the enzymology and kinetics of allosteric control distinguished *C. tetanomorphum* from *C. butyricum* and *C. acetobutylicum*. Crude extracts contained apparent proteolytic activity which could be fractionated from DAHP synthetase. The proteolytic activity was more labile than DAHP synthetase in extracts and was progressively inactivated by serial freeze-thaw treatments. Protease activity was at least partially inhibited by phenylmethylsulfonylfluoride. The method of comparative allostery of DAHP synthetase distinguishes the genera *Bacillus* and *Clostridium*, each having a strongly conserved pattern of regulation for DAHP synthetase. The data reinforce previous conclusions that allosteric control patterns governing the activity of DAHP synthetase are stable, reliable generic characteristics.

The pathway for aromatic amino acid synthesis is multibranched and diverging, a biochemical situation which poses certain complexities for regulation. The reaction steps of the biosynthetic pathway appear to be identical in most, if not all, microorganisms. This chemical uniformity implicates the ancient origin of the pathway as well as the probable limitation of feasible thermodynamic alternatives. In contrast to this uniformity, numerous and distinctly different patterns of allosteric control for the initial pathway enzyme, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase, have evolved in different groups of microorganisms. The allosteric control patterns that are found for microbial DAHP synthetases seem to be strongly conserved at the generic level (7, 10). The reliability of the allosteric pattern for DAHP synthetase as a group characteristic indicated its utility as a tool of microbial classification.

A detailed study of member species of the genus *Bacillus* (6, 11) showed that, with one exception, this rather diverse genus possessed a strongly conserved pattern of control for DAHP synthetase (sequential feedback inhibition [15]) which therefore constitutes a stable generic character. Since various taxa are not necessarily equivalent in hierarchical position, it was of interest to inquire whether this control pattern of *Bacillus* might extend to the taxonomic level of family, i.e., would the generic control pattern of *Clostridium* species also be that of sequential feedback inhibition? Unfortunately, *C. tetanomorphum*, the only species of *Clostridium* tested in the initial enzymological survey of microorganisms (7), did not display sufficient enzyme activity for inhibition analysis. *C. tetanomorphum* also possesses very low activities for enzymes of tryptophan synthesis in crude extracts (Twarog, *unpublished data*). Since crude extracts of another species, *C. butyricum*, possess much more activity for enzymes of tryptophan synthesis (Baskerville and Twarog, Bacteriol. Proc., p. 144, 1970), the analysis of the DAHP synthetase in *C. butyricum* seemed to be in order. An appreciation of some of the

enzymological characteristics of the *C. butyricum* enzyme then led to successful assay conditions for DAHP synthetase in several other species, including *C. tetanomorphum*.

MATERIALS AND METHODS

Microbial strains. *C. acetobutylicum* was obtained from the Northern Regional Research Laboratory of Peoria, Ill., as McCoy strain A-14, and probably is synonymous with ATCC 10132. *C. tetanomorphum* was obtained from the American Type Culture Collection as ATCC 3606. *C. butyricum* was obtained from the American Type Culture Collection as ATCC 6014.

Culture conditions. *C. butyricum* and *C. acetobutylicum* were grown in Hutner's mineral salts medium (1) containing glucose and acid-hydrolyzed casein at final concentrations of 1% and 0.025%, respectively. Based on an amino acid analysis of the casein supplement, the final concentrations of phenylalanine and tyrosine were 5.0 and 1.25 μg/ml, respectively. The medium was adjusted to *p*H 7.2 before autoclaving. Sterile glucose was added separately to the sterile medium.

C. tetanomorphum was grown in the following medium: L-glutamic acid, 15 g; yeast extract, 3 g; 1 M KH_2PO_4 (*p*H 7.4), 50 ml; $(NH_4)_2SO_4$, 1 g; sodium thioglycolate, 0.5 g; Hutner's mineral solution (1), 20 ml; plus water to a final volume of 1,000 ml. The *p*H of the medium was adjusted to 7.4 before autoclaving.

All three organisms were cultured at 37 C, and cells were harvested in the mid to late exponential phase of growth by centrifugation. The cells were washed once by resuspension in sterile growth medium (glucose was omitted in the medium used to wash *C. butyricum* and *C. tetanomorphum*), centrifuged, quick-frozen in liquid nitrogen, and then stored at −80 C.

Cells used as a source of deoxyribonucleic acid (DNA) were grown in a medium containing 0.5% peptone, 0.25% yeast extract, 0.2% K_2HPO_4, 0.05% sodium thioglycolate, and 1% glucose. The cells were harvested and washed in 1 mM dithiothreitol, 2 μg of pyridoxal-5-phosphate per ml buffered with 0.05 M tris(hydroxymethyl)aminomethane, *p*H 7.5.

Extract preparation. Whole cells (stored for up to 3 months at −80 C) were disrupted sonically with a Bronson sonic oscillator (Bronwill Instruments). Cells were suspended in 0.04 M potassium phosphate buffer, *p*H 7.0, containing 0.1 M KCl. Sonic treatment times were variable, depending upon cell concentration. One-minute bursts of sonic oscillation at 4 C were used to obtain extracts having 10 to 25 mg of protein per ml of extract. Nucleic acids were removed by protamine sulfate treatment. A fresh solution of 2% (w/v) protamine sulfate (Calbiochem) was made up in 0.1 M potassium phosphate, *p*H 7.2. The appropriate volume of this stock solution was added slowly to the crude extract with stirring at 4 C to give 0.15 mg of protamine sulfate per each mg of extract protein. Stirring at 4 C was continued for 20 min. The resulting precipitate obtained after centrifugation at 4 C was discarded. Small molecules were removed either by dialysis at 4 C against 1,000 volumes of 0.04 M potassium phosphate buffer (*p*H 7.0) containing 0.1 M KCl or by passage through a Sephadex G-25 column.

Storage of extracts. All extracts were stored at −80 C in a Revco refrigeration unit. Extracts were quick-frozen by using a dry ice-alcohol bath. Frozen extracts were thawed in a water bath thermostatted at 37 C before use.

Ammonium sulfate fractionation. Crude, undialyzed extracts of *C. tetanomorphum* which had been treated with protamine sulfate were fractionated by salting out with $(NH_4)_2SO_4$ (Mann, enzyme grade). A saturated (at 4 C) solution of $(NH_4)_2SO_4$ was adjusted to *p*H 7.0 with NH_4OH. A sufficient volume was mixed with extract to give a final saturation of 55%. After 30 min of slow stirring at 4 C, the precipitate was removed by centrifugation, and a fraction precipitating between 55 and 65% saturation was obtained. The latter fraction, resuspended in 0.04 M potassium phosphate buffer, *p*H 7.0, contained 41% of the original extract protein. The partial purification afforded by this step was probably two- to threefold.

Analytical assays. DAHP synthetase was assayed as previously described (9). Unless otherwise specified, erythrose-4-phosphate and phosphoenolpyruvate were present at 2 mM each. The specific activity of DAHP synthetase is expressed as units per milligram of extract protein where one unit equals an activity equivalent to the formation of 1 nmole of DAHP per min (9). Protein concentrations were estimated by the method of Lowry et al. (12).

Inhibitor assays. Unless indicated otherwise, compounds under test as inhibitors were incubated with the extract protein for 20 or more min over ice at 4 C. The DAHP synthetase reaction was then started by the addition of the substrates.

DNA base composition. The molar percentage of guanine plus cytosine (%GC) was calculated from the buoyant density in cesium chloride as previously described (13).

Chemicals. Chorismate and prephenate preparations were made by the method of Gibson as previously described (8), and were estimated to be 89 and 93% pure, respectively. The trisodium salt of phosphoenolpyruvate and the sodium salt of erythrose-4-phosphate were obtained from Sigma Chemical Co. Small batches of stock substrate solutions containing phosphoenolpyruvate and erythrose-4-phosphate at concentrations of 20 mM in 0.04 M potassium phosphate buffer, *p*H 7.0, and containing 0.1 M KCl were stored at 4 C. The concentrations of erythrose-4-phosphate given are not exact due to its lability in solution. In some cases the concentration cited may be incorrect by as much as 30 to 40%. It was not deemed necessary to carry out the tedious methods (9) for estimating exact concentrations of erythrose-4-phosphate in these experiments. However, experiments such as those for the determination of substrate saturation curves were, of course, done by using serial dilutions of a common stock substrate mixture to ensure that the kinetic data re-

flected correct relative numbers. Phenylpyruvate was obtained from Sigma, *p*-hydroxyphenylpyruvate from Mann, and anthranilate from Calbiochem. L-Amino acids were purchased from Calbiochem, and all other biochemicals were from Sigma. All chemicals were of the best reagent quality commercially available. Stock solutions of phenylmethylsulfonylfluoride (PMSF) (Sigma) were prepared at 50 mg per ml of isopropyl alcohol and diluted 1,000-fold into crude extracts immediately after sonic treatment.

RESULTS

In previous comparative analyses (7, 10) which included large groups of microorganisms, it was not feasible to optimize assay conditions for each of the DAHP synthetases under study. As long as qualitative patterns of control could be determined for a given DAHP synthetase, quantitative accuracy was not deemed necessary. In an early survey (7) the activity of DAHP synthetase in *C. tetanomorphum* was too low to permit inhibition assays. In retrospect it appears that the explanation for these results involves the presence of active proteolytic enzymes in this representative of the amino acid fermenting clostridia.

In contrast to the results obtained from crude extracts of *C. tetanomorphum*, relatively high levels of DAHP synthetase activity were found in crude extracts of *C. butyricum*. This activity showed an apparent temperature optimum of about 26 C. Surprisingly, stored extracts increased in specific activity with use to a value which leveled off at about three to four times the original activity. Assay at 37 C showed the factor of increase in specific activity to be even greater (about 20-fold) as shown in Fig. 1. The results given in Fig. 1 are readily interpreted after examination of the data in Fig. 2, which show a striking shift in the temperature optimum for catalysis from 26 C to about 40 C following aging and freeze-thaw treatment of the sample. Presumably, DAHP synthetase is susceptible to one or more protease activities which are relatively active at higher temperatures and which are labile to freeze-thaw treatment. Although some decrease in proteolytic activity may be the result of time-dependent storage inactivation, the major fraction of inactivation probably results from freezing or thawing, or both, of extracts. Qualitatively similar results were obtained with extracts of *C. acetobutylicum* and *C. tetanomorphum*. One culture of *C. butyricum*, grown in the presence of glutamic acid (1 mg/ml) plus 0.1% $(NH_4)_2SO_4$ yielded a crude extract originally having about three times the specific activity observed in other crude extracts. This probably does not reflect a nutritional influence on the repression control of DAHP synthetase but rather a nutritional effect upon the level of proteolytic activity. This conclusion is supported by the finding that inactivation was less than usual, producing a final specific activity of DAHP synthetase that was similar to that of other crude extracts.

Compared with the other two species, the proteolytic activity of *C. tetanomorphum* was relatively stable. Storage and freeze-thaw treatment of the *C. tetanomorphum* extracts only produced an increase of DAHP synthetase activity of about 150%, and a similar modest increase in activity was obtained in the presence of PMSF. However, as shown in Table 1, the protein fraction salting out between 55 and 65% of saturation with $(NH_4)_2SO_4$ contained more than 14 times the original DAHP synthetase activity. Since $(NH_4)_2SO_4$ addition to crude extracts did not stimulate activity, an activating effect of NH_4^+ or SO_4^{2-} ions can be eliminated. Apparently proteolytic activity

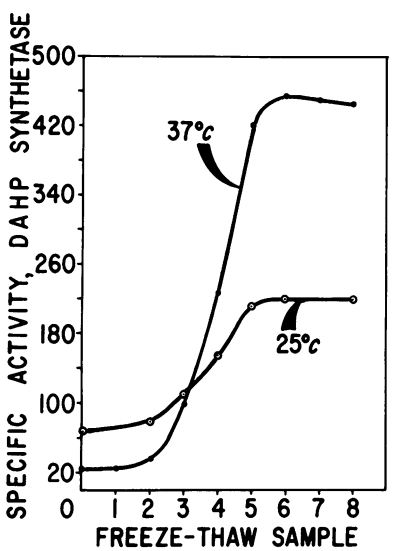

FIG. 1. *Storage-dependent increase of DAHP synthetase activity in C. butyricum. Sample 0 is a crude, dialyzed extract with no previous history of freezing. Sample 1 is sample 0 after storage at −80 C in a Revco cabinet and subsequent thawing at 37 C. In like manner each sample number indicates the number of freeze-thaw treatments in the history of the extract. The time intervals, in days, between sample 1 and 2, 2 and 3, ..., 7 and 8 were 1, 2, 6, 7, 15, 27, and 41, respectively. Enzyme activities were determined at assay temperatures of 25 and 37 C. Specific activities of DAHP synthetase are given in nanomoles of DAHP per minute per milligram of extract protein.*

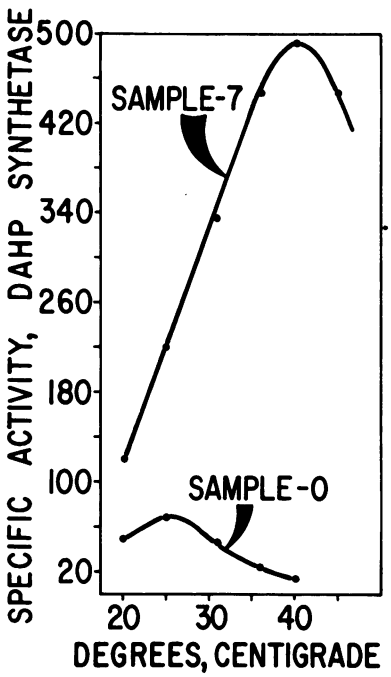

FIG. 2. *Profile of DAHP synthetase activity of C. butyricum as a function of assay temperature. Designations of extracts as sample 0 or sample 7 are as defined under Fig. 1. The crude extract of C. butyricum contained a protein concentration of 19.7 mg/ml. Specific activities of DAHP synthetase are given in nanomoles of DAHP per minute per milligram of crude extract.*

TABLE 1. *Desensitization of DAHP synthetase in C. tetanomorphum*

Extract condition	Relative specific activity[a]	Inhibition by 0.3 mM phenylalanine (%)
Crude	1.0	6
Crude + PMSF[b]	1.9	9
55–65% $(NH_4)_2SO_4$ fraction	14.3	91

[a] A relative specific activity of 1.0 corresponds to a specific activity of 12.1 nmoles of DAHP per min per mg of protein. The crude extract was frozen and thawed four times over a period of 3 weeks, resulting in a final increase in activity of about 2.5-fold over that of the original preparation shown above.

[b] Phenylmethylsulfonylfluoride.

was fractionated away or inactivated, or both, by $(NH_4)_2SO_4$ treatment. Possible indirect effects of proteolysis such as the release of free phenylalanine from degraded protein cannot be ruled out. In the case of *C. tetanomorphum*, inhibition of DAHP synthetase activity by L-phenylalanine exhibited a striking increase following $(NH_4)_2SO_4$ fractionation. This result probably indicates that the allosteric site is more susceptible to proteolytic disruption than is the catalytic site. If one mixes extracts from *C. butyricum* (which has relatively high levels of tryptophan biosynthetic enzymes [Baskerville and Twarog, Bacteriol. Proc., p. 144, 1970]) and *C. tetanomorphum*, the activities of anthranilate synthetase and tryptophan synthetase decay at a perceptible rate (Twarog, *unpublished data*). These results are further consistent with the presence of proteolytic activity in *C. tetanomorphum*.

Additional evidence consistent with the degradation of DAHP synthetase by proteolysis in crude extracts is shown in Table 2. An equal-volume mixture of sample 0 (active protease) and sample 7 (inactive protease) resulted in a low specific activity approaching that of sample 0. The addition of PMSF, an inhibitor of proteolytic enzymes, to sample 0 increased the activity nine to tenfold, about 50% of the sample 7 preparation. As expected, PMSF did not influence the sample 7 activities. In *C. tetanomorphum* (Table 1) PMSF was relatively ineffective (twofold increase in activity) compared to its effect in the other two species.

The qualitative pattern of allosteric control for DAHP synthetase is the same for all three species. As shown in Table 3, L-phenylalanine is the only allosteric effector. Other aromatic end products and intermediates produced no significant enzymatic effects. Inhibition curves are shown in Fig. 3. Kinetic data plotted for

TABLE 2. *Effect of freeze-thaw treatment and PMSF on DAHP synthetase activity in Clostridium butyricum and Clostridium acetobutylicum*

Extract sample[a]	Relative specific activity[b]	
	C. butyricum	*C. acetobutylicum*
0	1.0	1.0
7	21.4	22.7
0 + 7	2.6	2.4
7 + PMSF	20.5	22.7
0 + PMSF	9.4	9.9

[a] A crude extract prepared as described in the experimental section is designated sample 0. Sample 7 was frozen and thawed seven times. Phenylmethylsulfonylfluoride (PMSF) was added as described in Materials and Methods after the cells were disrupted sonically.

[b] Specific activities of DAHP synthetase were normalized by arbitrarily assigning to sample 0 a value of 1.0. Values of 1.0 in *C. butyricum* and *C. acetobutylicum* correspond to specific activities of 23.2 and 21.5 nmoles of DAHP per min per mg of protein, respectively.

TABLE 3. *Specificity of DAHP synthetase for inhibitors*[a]

Concn (mM)	Inhibitor(s)	Percent inhibition		
		C. butyricum	*C. acetobutylicum*	*C. tetanomorphum*[b]
0.05	L-Phenylalanine	96	97	89
1.0	L-Tyrosine	3	1	1
0.1	L-Tryptophan	1	0	0
0.1	Aromatic amino acids[c]	97	98	90
0.5	Phenylpyruvate	2	0	2
0.5	*p*-Hydroxyphenylpyruvate	0	0	0
0.1	Anthranilate	0	1	0
1.0	Chorismate	1	0	0
1.0	Prephenate	4	1	5
0.5	Shikimate	0	0	0

[a] Control reactions lacking test compounds as inhibitors were run in duplicate. The results are the average of two experiments. Error in these assays is less than 5%. The specific activity of the extracts prepared from *C. butyricum*, *C. acetobutylicum*, and *C. tetanomorphum*, respectively, were 299, 319, and 169.

[b] The preparation from *C. tetanomorphum* was the 55 to 65% ammonium sulfate fraction (see Materials and Methods).

[c] Aromatic amino acids refer to the combination of tryptophan, phenylalanine, and tyrosine, each at the indicated concentration.

the DAHP synthetases of *C. acetobutylicum* and *C. butyricum* were superimposable. These enzymes are very sensitive to phenylalanine inhibition (50% inhibition at less than 0.01 mM), and inhibition approached 100% at higher inhibitor concentrations. Similar first-order kinetics of inhibition were found for the *C. tetanomorphum* enzyme except that it was about an order of magnitude less sensitive to phenylalanine inhibition. In all three systems, prior incubation of extract protein with either substrate or inhibitor did not significantly influence the magnitude of inhibition.

The DAHP synthetases of *C. butyricum* and *C. acetobutylicum* yielded similar results with respect to the kinetic analysis of the phenylalanine inhibition (Fig. 4). Inhibition was strictly competitive with respect to erythrose-4-phosphate and noncompetitive with respect to phosphoenolpyruvate. Although phenylalanine inhibition of the DAHP synthetase of *C. tetanomorphum* was also noncompetitive with phosphoenolpyruvate, it appeared to be noncompetitive with erythrose-4-phosphate. With all three species, substrate saturation curves for DAHP synthetase displayed Michaelis-Menten kinetics for both substrates, and no substrate inhibition was noted.

It is perhaps significant that one of the relatively few microorganisms previously shown (7) to possess single-effector control of DAHP synthetase by phenylalanine was *Veillonella alcalescens* (4), another anaerobe. Possibly, for unknown reasons, this particular pattern of control is appropriate for strictly anaerobic metabolism. In view of the difficulty of understanding the physiological efficiency of single-effector control (10) in a branched pathway, the following possibility was considered. Perhaps a low, partially inhibitory concentration of phenylalanine might act synergistically with high concentrations of the other aromatic amino acids. This control relationship might make physiological sense if intracellular concentrations of tyrosine and tryptophan were high compared to that of phenylalanine. Table 4 shows data obtained with concentrations of phenylalanine adjusted to give about 10% inhibition. Under these conditions the additional presence of high concentrations of both tryptophan and tyrosine did not produce concerted inhibitory effects, and therefore the hypothesis was negated. Hence, the efficiency of the control pattern in the physiological sense remains unclear, but the characteristic nevertheless

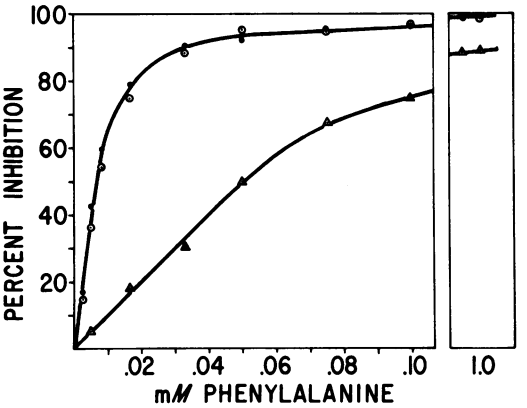

FIG. 3. *Inhibition of DAHP synthetase by phenylalanine in species of Clostridium. Percent inhibition (ordinate) was calculated by relating velocities observed in the presence of the indicated concentration of phenylalanine to the average of duplicate determinations of reaction velocity in assay mixtures lacking phenylalanine. Sample 6 (⊙) and sample 8 (●) as described under Fig. 1 were used as extract preparations for C. butyricum and C. acetobutylicum, respectively. The 55 to 65% fraction of $(NH_4)_2SO_4$ from C. tetanomorphum was used (▲). The assay temperature was 37 C. The extracts of C. butyricum, C. acetobutylicum, and C. tetanomorphum contained 19.7, 14.9, and 6.8 mg of protein per ml, respectively.*

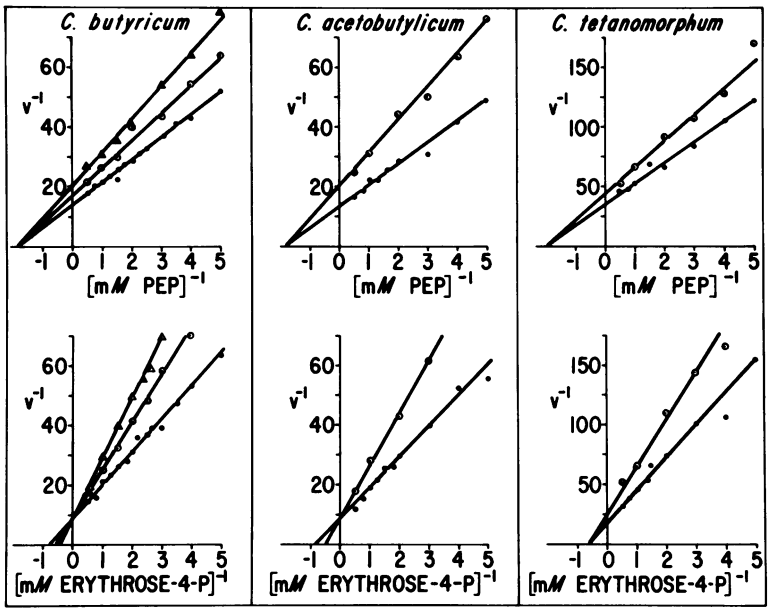

FIG. 4. *Kinetics of inhibition of DAHP synthetase by phenylalanine in species of Clostridium. Upper horizontal row of graphs: phosphoenolpyruvate (PEP) was the fixed substrate at a concentration of 2 mM. Lower horizontal row: Erythrose-4-phosphate was the fixed substrate at a concentration of 2 mM. The reciprocal of velocity (v^{-1}) is expressed on the ordinate scale as micromoles of DAHP per minute per milliliter of reaction mixture. Phenylalanine was present at a final concentration of 15 nM (⊙) or 40 nM (▲). Control reactions lacking phenylalanine are designated ●. The crude extracts of C. butyricum (sample 8) and C. acetobutylicum (sample 6) were assayed for 10 min at 37 C and contained 23 and 22 μg of protein, respectively (per 0.2-ml reaction volume). A 41-μg amount of the 55 to 65% $(NH_4)_2SO_4$ fraction of C. tetanomorphum was used in a reaction of 30 min duration at 37 C.*

appears to be a stable and reliable taxonomic feature.

DNA was extracted from cells of each representative of *Clostridium*. The buoyant densities of the DNA preparations from species of *Clostridium* in cesium chloride all clustered at values corresponding to 32% guanine plus cytosine (GC) (Table 5). *V. alcalescens*, an anaerobe representing another taxon, possesses DNA having significantly higher GC content (41%).

DISCUSSION

The rod-shaped, endospore-producing, gram-positive bacteria are classified as the family *Bacillaceae*, a taxon which contains two genera. The genus *Bacillus* contains aerobic species with DNA having GC contents which vary over a broad range of about 33 to 53% (5). The genus *Clostridium* contains anaerobic species having GC contents in DNA ranging between 26 to 35% (2, 3). The distinct difference between DNA base compositions of *Bacillus* and *Clostridium* implies a substantial evolutionary divergence of these groups. The generic control pattern of sequential feedback inhibition (15) is well documented for DAHP synthetase in *Bacillus* (6, 11). It now appears that a conservative allosteric control pattern also is characteristic of *Clostridium* species, the generic control pattern being single-effector control by phenylalanine. The nature and frequency of single-effector control patterns for DAHP synthetase in microorganisms has been reviewed (10). Although only three species have been examined with respect to the enzymology of DAHP synthetase, the qualitative pattern of control will probably prove to characterize the other species of *Clostridium* for the following reasons. (i) The GC base compositions are fairly narrow in this group. (ii) Not a single convincing exception to the thesis that member species of a genus share a common control mechanism for DAHP synthetase has yet been found (6, 7), all exceptions being organisms whose classification is suspect. (iii) The species of *Clostridium* tested represent the two subgroups which were defined by amino acid-fermenting capabilities (14).

TABLE 4. *Lack of synergistic interaction of aromatic amino acids as inhibitors of DAHP synthetase*

Concn (nM)	Inhibitor(s)	Percent inhibition[a]		
		C. butyricum	*C. acetobutylicum*	*C. tetanomorphum*[b]
100 each	Tryptophan + tyrosine	0	0	0
2	Phenylalanine	10	12	
8	Phenylalanine			13
100 each	Tryptophan + tyrosine +	9	14	
2	phenylalanine			
100 each	Tryptophan + tyrosine +			15
8	phenylalanine			

[a] Percent inhibition was determined by relating the activities found in the presence of inhibitor to the activities of the average of duplicate control reactions lacking inhibitors. The specific activities of the extracts of *C. butyricum*, *C. acetobutylicum*, and *C. tetanomorphum* were 318, 300, and 173, respectively.

[b] The partially purified $(NH_4)_2SO_4$ fraction, 55 to 65% of saturation, was used. Extracts of *C. butyricum* and *C. acetobutylicum* were sample 9 and sample 6, denoted as described under Table 2 and Fig. 1.

TABLE 5. *Buoyant density in CsCl and guanine plus cytosine molar composition (GC) in DNA of some anaerobes*

Culture designation	ρ_{CsCl}(g/cm^3)[a]	GC (moles %)
Clostridium acetobutylicum	1.692 ± .0016	32.6 ± 1.6
C. butyricum	1.691 ± .0016	31.6 ± 1.6
C. pasteurianum	1.692 ± .0015	32.9 ± 1.5
C. tetanomorphum	1.691 ± .0015	31.3 ± 1.5
Veillonella alcalescens[b]	1.700	40.8

[a] Mean ± standard deviation. Three analyses were performed for each species of *Clostridium;* two were performed for *V. alcalescens.*

[b] Frozen cells were obtained from H. R. Whiteley. The growth and origin of this strain, available from American Type Culture Collection as ATCC 14894 (originally designated *Micrococcus lactilyticus*), has been described (7).

C. tetanomorphum deviated quantitatively from *C. butyricum* and *C. acetobutylicum* in every enzymological detail examined, e.g., specific activities, K_i for phenylalanine inhibition, kinetic mechanism of inhibition with erythrose-4-phosphate, and various characteristics of the presumed protease activity. Further studies with additional species might establish the usefulness of some of these characteristics for the possible definition of subgroups. A precedent for this possibility was the finding that the allosteric temperature ratio in *Bacillus*, defined in terms of differential effect of temperature upon the sensitivity of DAHP synthetase to feedback inhibition, correlates fairly well with subgroups arranged according to sporangial structure (6).

One of the difficulties in comparing taxonomic data compiled by different research groups (particularly when they are done at different times) is the question of whether the cultures examined are, in fact, the same. An independent check for culture authenticity that would ordinarily reveal contaminated or misnamed cultures is the characterization of the GC content of the DNA. Hence, one can be certain, for example, that *V. alcalescens* (Table 5) is not actually a misnamed species of *Clostridium*. Furthermore, the GC base content would be expected to remain relatively stable over a period of years during which mutations affecting various single characteristics could accumulate.

Richmond (16) recently pointed out that the new molecular data have tended to be accompanied by an atomistic philosophy which ignores earlier insights provided by Dobzhansky and Simpson. He states the case that "selection does not act on separate genes or traits but on whole genotypes and phenotypes." It seems likely to us that the observed general reliability of the method of comparative allostery of DAHP synthetase reflects the strongly conserved maintenance of a highly integrated group of genes.

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