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Comparative Control of a Branch-Point Enzyme in Microorganisms

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Thirty-two genera of microorganisms were identified with one of six distinctive control patterns for the enzyme 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthetase. These patterns included sequential feedback inhibition, isoenzyme feedback inhibition, cumulative feedback inhibition, and three (apparent) simple one-effector patterns. Documentation is provided of an overwhelming tendency for control patterns to be strongly conserved among the member species of the various genera that were examined.

3-Deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthetase is an enzymatic link between multifunctional metabolites and the first intermediate specific to the pathway of aromatic amino acid biosynthesis. The activity of DAHP synthetase in *Bacillus subtilis* was found to be controlled by branch-point metabolites (13), and the overall pathway-wide interaction of regulatory enzymes was termed sequential feedback inhibition (21). The latter system of regulation differs from the amino acid-controlled isoenzymes of DAHP synthetase which had previously been described in *Escherichia coli* (25). Since the multi-branched pathway is complex, still other patterns of control for DAHP synthetase could conceivably exist in other microorganisms (28). Indeed, a number of other control patterns were observed. Therefore, it was of interest to inquire further into the extent and nature of this diversity through the comparative examination of a wide spectrum of microorganisms.

MATERIALS AND METHODS

Growth of cultures. A priori it appeared advisable to grow strains in simple defined media whenever possible. Theoretically, in the absence of exogenous amino acids, the metabolic stress on the output of biosynthetic pathways is relatively great, and at least partial derepression of enzyme synthesis in the aromatic pathway might be expected. Nevertheless, growth of cultures in complex media was often more satisfactory because (i) the nonrepressible enzyme activity was usually substantial, and (ii), in practice, the DAHP synthetase of many microorganisms grown in a minimal medium was already relatively repressed. The methodology of growing cells in a rich medium to support good growth followed by resuspension in a minimal medium to derepress biosynthetic enzymes (25) did not prove to be a useful technique.

Growth-assay code. The letter designations which appear under this heading in the tables correspond to the media listed below. The two numbers indicate the temperature of growth and the temperature of enzyme assay, respectively. In the tables, references describing some of the strains are cited in parentheses.

The media are as follows. A = (in grams per liter): trypticase, 17.0; phytone, 3.0; K_2HPO_4, 2.5; NaCl, 5.0; yeast extract, 10.0; and dextrose, 2.5. B = 0.25% lactate, Davis minimal salts (18). C = 0.5% glucose, Spizizen's minimal salts (26). D = medium C + 0.05% casein acid hydrolysate. E = medium C + 0.025% yeast extract. F = medium D + 0.25% yeast extract. G = 0.5% glucose, Davis minimal salts (18). H = Sauton's medium (in grams per liter): asparagine, 4.0; citric acid, 2.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7\,H_2O$, 0.5; iron ammonium citrate, 0.05; and glycerol, 60. I = McCormick's medium (N. C. McComick, Ph.D. Thesis, Univ. of Washington, Seattle, 1960). J = (in grams per liter) KNO_3, 0.5; Na-glycerophosphate, 0.1; tris(hydroxymthyl) aminomethane (Tris) buffer, 1.0; tryptone, 2.0; yeast extract, 2.0; in sea water at *p*H 7.0; Cu, Zn, Co, and Mo elements present at 0.01 ppm; B, Mn, and Fe elements present at 0.5 ppm. K = phytoflagellate medium as modified by Cirillo (4). L = Vogel's medium (30).

Organization of taxa. All data cited in the tables of this communication have been organized according to the taxonomy used in *Bergey's Manual of Determinative Bacteriology*, 7th ed., unless otherwise specified.

Identification of cultures. To eliminate aberrant results due to mislabelled or contaminated cultures, every attempt was made to secure authentic representatives of the various taxa. When appropriate, the purity and identity of a culture were determined on plates, by microscopic examination, staining proce-

dures, or diagnostic tests (such as the formation of indole). Many cultures were grown for us or identified by individuals working with them routinely. Many strains bear ATCC numbers, and, although this does not guarantee their authenticity, it was expected that any questionable cultures would always be available for subsequent examination. Species in our basic ATCC collection (obtained through a collaborative agreement with ATCC) have also been used by other investigators for taxonomic studies (8, 16, 20). Proper synonymy designations for cultures which were received with archaic labels are noted as such in the legends. This was not always done with cultures bearing ATCC numbers, since existing information on these strains appears in the ATCC culture catalogue.

Preparation of extracts. Bacterial cultures (whole cells) were often stored frozen at −20 C, occasionally as long as several months, before extracts were prepared for enzyme assay. Most cultures were disrupted by sonic oscillation in 0.04 M potassium phosphate buffer (*p*H 7.0). *Neurospora crassa* was grown at 37 C in Fernbach flasks and was harvested by pressing the mycelium on cheesecloth. The mycelial harvest was lyophilized and ground to a powder. Extracts were made by dissolving the powder in 0.04 M potassium phosphate buffer, (*p*H 7.0), followed by disruption with a Branson sonicator. Cells of *Polytoma obtusum* were broken in a Waring Blendor in 0.1 M Tris buffer (*p*H 7.5).

Dialysis. Extracts were dialyzed at 4 C against 1,000 volumes of 0.04 M potassium phosphate buffer for either 8 hr or overnight. It proved informative to assay crude extracts both before and after dialysis. Many of the results reported were obtained with undialyzed preparations. Sometimes enzymes were labile to dialysis, and occasionally it promoted a tendency of the enzyme to desensitize to inhibitor effects (*in preparation*). On the other hand, dialysis increased the activity of DAHP synthetase about 35 to 40% in most of the *Bacillus* species; it was absolutely necessary for the detection of any enzyme activity in *Micrococcus luteus*, *M. lysodeikticus*, *Streptococcus faecalis*, *S. bovis*, *Alcaligenes faecalis*, and *Achromobacter viscosus*.

Preparation of inhibitors. The preparation of prephenate (PPA) and chorismate (CHA) has been described in detail (15). All amino acids (Calbiochem, Los Angeles, Calif.) were of the L configuration and were always appropriately mixed at 4 C with an equal volume of protein extract to yield the desired final concentration. The substrates were added to initiate the enzymatic reaction.

DAHP synthetase assay. The modified assay previously described (14) was used. It was impractical to determine optimal conditions for catalysis or feedback inhibition for enzymes from each of the microorganisms tested. The substrate concentrations used were high (2 mM). This tended to prevent the masking or underestimation of isoenzymes with the largest K_m values, a probable consequence of assay conditions for which concentrations of substrate(s) are nonsaturating. The final buffer concentration in the assay was 0.02 M potassium phosphate (*p*H 7.0).

Preliminary inhibition assay. At the time that initial assays were done to determine enzyme concentrations yielding accurate activity determinations in the colorimetric assay, it was convenient to obtain preliminary inhibition data. Inhibitions produced by 0.17 mM PPA, 0.35 mM ARO (mixture of tyrosine, phenylalanine, and tryptophan, each at the latter concentration, plus *p*-aminobenzoate and *p*-hydroxybenzoate at a 10-fold lower concentration), and 0.07 mM ARO were determined. This served to separate enzymes controlled by sequential feedback inhibition (inhibited by PPA, not by ARO) from those controlled by isoenzyme or cumulative feedback inhibitions (inhibited by ARO, but not by PPA). In the case of the latter, the two concentrations of ARO were used to allow an estimation of the most appropriate inhibitor concentrations for subsequent assays.

Follow-up assay. Enzyme preparations which were inhibited by ARO were tested for inhibition in the presence of the individual amino acids at a minimum of two concentrations. This protocol was designed to spotlight the pattern of control by relating inhibitions observed with single amino acids to inhibitions occurring with combinations of these inhibitors; i.e., the possible occurrence of isoenzyme, cumulative, and concerted feedback inhibitions (28), are distinguishable from one another with the analysis of these data. The use of several inhibitor concentrations was for the purpose of preventing misinterpreattions caused by possible nonspecific inhibitions occurring at high inhibitor concentrations, particularly since the data obtained from isoenzyme systems under these conditions tend to resemble those expected for cumulative feedback inhibition. The above protocol was designed to reveal possible instances of activation of DAHP synthetase by some effectors, antagonistic allosteric effects of end products, or appreciable contributions of the vitamin derivatives of the pathway to the control of DAHP synthetase. None of the latter possible variations were found. When PPA inhibited DAHP synthetase in the preliminary assay, 1.0 mM CHA was tested as an inhibitor in the follow-up assay.

Nonroutine protocols. When no enzyme activity was found, the preparation was dialyzed and a preliminary test was again made. Some preparations with low specific activities were successfully concentrated by $(NH_4)_2SO_4$ precipitation. When inhibition was not found or was weak (below 50%), the inhibition analysis was carried out by use of serial dilutions of the standard substrate mixture to detect possible competitive inhibitions. The assay temperature was also varied whenever weak inhibitions were encountered to detect temperature dependent effects similar to that of *B. subtilis* (15).

Nonspecific inhibition. Sometimes DAHP synthetase activity which was specifically inhibited by CHA and PPA was partially inhibited by 0.35 mM ARO, and this tended to be more pronounced at higher amino acid concentrations. Likewise, some of the isoenzymes which were specifically inhibited by one of the aromatic amino acids were partially inhibited by high concentrations of another aromatic amino acid or by CHA or PPA. For this reason, the most reliable results were obtained with low but still effective concentrations of inhibitors. We assume that the above effects of other inhibitors are nonspecific,

although it is conceivable that they could impose secondary, mutually reinforcing effects.

Comparative levels of DAHP synthetase. No attempt was made to assign significance to the specific activities of DAHP synthetase found in extracts of the various test microorganisms. Since the growth regimen varied substantially and no attempt was made to define optimal assay conditions for the enzyme of each microorganism, the quantitative meaning of these numbers on a comparative basis is dubious. Qualitatively, the enteric bacteria showed high specific activities in the range of several hundred. Species of *Aeromonas and Veillonella* approached these activity levels. The aerobic pseudomonads and species of *Hydrogenomonas*, *Staphylococcus*, and *Bacillus* showed moderately high enzyme levels (however, *Bacillus* species were rather heterogeneous in this respect). Other microorganisms yielded specific activities that were about two orders of magnitude lower than that of the enteric bacteria.

RESULTS

It was not unexpected to find that species which are closely related to *B. subtilis* were endowed with the same control pattern for DAHP synthetase (e.g., *B. licheniformis* and *B. subtilis* var. *niger* and *atterimus*). The possibility that control patterns, indeed, tend to be strongly conserved in related species received increasing support when it was found that other species of this quite diverse genus (20) also possessed a DAHP synthetase subject to control by the pattern of sequential feedback inhibition. Data collected from a total of 35 strains representing 21 species of *Bacillus* are summarized in Table 1. With one exception, CHA or PPA inhibited the activity of DAHP synthetase, whereas amino acid end products did not. We assume that whenever DAHP synthetase is inhibited by the branch-point compounds, CHA and PPA, the levels of these inhibitory metabolites are in turn regulated by virtue of end-product control of the enzymes which utilize CHA and PPA as substrates. The latter pattern of four physiologically interacting circuits of feedback inhibitions has been called sequential feedback inhibition in *B. subtilis* (21). The *Bacillus* species studied include representatives of the three groups formulated on the basis of spore morphology by Smith et al. (24). These groups were quite similar in the details of the regulation of DAHP synthetase. An exception was the enzyme from *B. alvei* which was not sensitive to PPA, CHA, or the aromatic amino acids under the assay conditions cited in the legend of Table 1. Although it initially seemed likely that the enzyme merely possessed a labile inhibitor binding site, more careful studies revealed that tryptophan (trp), but not tyrosine (tyr) or phenylalanine (phe), was inhibitory at reduced substrate concentrations. To be certain that the culture of *B. alvei* had not been mislabeled or contaminated, the original parent strain ATCC 6348 and three other ATCC strains were tested (Table 2). DAHP synthetase activity measured in extracts from cultures of all four strains exhibited competitive inhibition by trp. The detailed kinetic studies required to define precisely the quantitative aspects of inhibitor and substrate interactions with the enzyme have not yet been carried out. *B. alvei* differs from the bulk of other *Bacillus* species in its maintenance of a constitutive tryptophanase (11, 12), its deoxyribonucleic acid base ratio (32.5% guanine + cytosine content; reference 20), and in the catalytic properties of several dehydrogenases (1). However, it is not clear that the observed difference in pattern of control can be specifically correlated with any of the latter, because *B. laterosporus* also produces indole (although the tryptophanase may not be constitutive) and *B. cereus* closely resembles *B. alvei* with respect to the other two characteristics. Nevertheless, the regulation of both *B. cereus* and *B. laterosporus* DAHP synthetases resembles that of other *Bacillus* species rather than that of *B. alvei* (Table 1). One species tested from the other genus of the family, *Bacillaceae*, yielded too little enzyme activity under the conditions used to make a determination (*Clostridium*, *see* Table 10).

Other generic groups displayed a similar conservative maintenance of a given control pattern. Since *E. coli* was already known to possess isoenzyme DAHP synthetases (25), we sampled representative genera in the family *Enterobacteriaceae* (Table 3). As in the case of *E. coli*, for which most DAHP synthetase activity exists as the phe-sensitive isoenzyme, the enzyme activity of other genera in this family was often inhibited predominantly by one of the amino acids. For convenience, we refer to these as "dominant" isoenzymes. Apparently, other isoenzymes are relatively repressed (or labile) under many growth conditions (*in preparation*). Possibly all genera in this family have isoenzyme DAHP synthetases, since there is usually detectable inhibition by at least two inhibitors, even though one or more of these may be quite minor quantitatively. The relative proportion of isoenzymes has been altered under certain nutritional and genetic conditions in *E. coli* (2, 19, 25; *in preparation*) and *S. typhimurium* and other microorganisms (*in preparation*). Unlike *E. coli* (2), a quantitatively minor trp-inhibited isoenzyme was found in species of *Aerobacter*, *Proteus*, *Shigella*, and *Serratia*. None was seen in extracts

TABLE 1. *Sequential feedback inhibition of DAHP synthetase in Bacillus species*

Bacillus species	Strain	Source	Growth-assay code[a]	Inhibition (%)	
				PPA 0.17 mM[b]	ARO 0.35 mM
B. subtilis	168	Univ. of Washington	C-37-37(3)	84 (74)	1
B. subtilis	23	Univ. of Washington	C-37-37(3)	89 (76)	4
B. licheniformis	9945A	C. Thorne	C-37-37(9)	85 (70)	0
B. licheniformis	NRS 243	H. Koffler	C-32-37	84 (83)	2
B. megaterium	K	Univ. of Washington	C-32-37	84 (83)	7
B. cereus	B-48	P. Bonventre	D-32-37	42 (61)	3
B. vulgatus[c]		R. Altenbern	C-37-37	82 (78)	0
B. pumilus		Univ. of Washington	E-37-37	89 (68)	0
B. pumilus	NRS 236	H. Koffler	E-32-37	82 (78)	0
B. brevis		R. Altenbern	D-32-37	79 (76)	2
B. coagulans		H. Koffler	A-37-37	74 (73)	9
B. globigii[c]		R. Altenbern	C-37-37	85 (88)	0
B. thuringiensis		R. Altenbern	D-32-37	82 (88)	19
B. firmus		H. Koffler	A-32-32	91 (76)	20
B. pulvifaciens	ATCC 13537	ATCC	A-37-37	64 (71)	34
B. laterosporus	ATCC 64	ATCC	A-32-32	67 (75)	20
B. pantothenticus	ATCC 14576	ATCC	A-37-37	82 (78)	22
B. sphaericus	ATCC 12300	J. Larkin/J. Stokes	F-32-32	93 (98)	0
B. sphaericus	ATCC 14577	ATCC	A-32-37	94 (98)	6
B. subtilis var. *niger*	ATCC 6455	ATCC	C-37-37	72 (71)	5
B. subtilis var. *atterimus*	ATCC 6460	ATCC	C-37-37	81 (83)	3
B. alvei	ATCC 6348-F	R. DeMoss	F-32-32(11)	0 (0)	7
B. circulans	ATCC 4513	J. Larkin/J. Stokes	A-37-37	84 (88)	25
B. anthracis[d]	Stern	P. Bonventre	A-37-37	70 (70)	1
Bacillus sp.[e]	W16A	J. Larkin/J. Stokes	F-20-32(17)	86 (85)	5
Bacillus sp.[e]	T38B	J. Larkin/J. Stokes	A-20-32(17)	59 (57)	1
Bacillus sp.[e]	T25B	J. Larkin/J. Stokes	A-20-32(17)	90 (94)	1
B. stearothermophilus	10	H. Koffler	A-65-37	59 (59)	0
B. stearothermophilus	2184	H. Koffler	A-65-37	79 (82)	0
B. stearothermophilus	CD	H. Koffler	A-65-37	80 (81)	4
B. stearothermophilus	FJW	H. Koffler	A-65-37	75 (85)	7
B. stearothermophilus	1130	H. Koffler	A-65-37	72 (77)	10
B. stearothermophilus	39	H. Koffler	A-65-37	75 (85)	0
B. stearothermophilus	CDS	H. Koffler	A-65-37	87 (88)	2

[a] Growth-assay code is explained in Materials and Methods.

[b] Numbers in parentheses denote per cent inhibition of DAHP synthetase in the presence of 1.0 mM chorismate.

[c] *B. vulgatus* and *B. globigii* are archaic designations in synonymy with *B. subtilis* and *B. licheniformis*, respectively (24).

[d] Unencapsulated variant of *B. anthracis*.

[e] These species are psychrophiles.

of *Escherichia*, *Salmonella*, or *Erwinia*, even under special genetic or nutritional conditions (*in preparation*).

The data summarized in Table 3, particularly those obtained at the lower inhibitor concentrations whose nonspecific effects are less troublesome (25), demonstrate considerable generic homogeneity in the sensitivity of the enzyme to inhibitory effects of one or more amino acids. Thus, *Escherichia* has a dominant phe-sensitive isoenzyme, *Aerobacter* and *Serratia* have dominant tyr-sensitive isoenzymes, and *Salmonella* and *Shigella* possess appreciable amounts of both the phe-sensitive and the tyr-sensitive DAHP synthetase. *Proteus* appears to have more variable levels of all three isoenzymes, although the tyr-sensitive DAHP synthetase is usually the most prominent. The two species of *Erwinia* were quite distinct; activity of one was only inhibited by phe, the other, only by tyr. In this connection, Kreig and Lockhart (16) found *E. amylovora* ATCC 7398 to depart significantly

TABLE 2. *Competitive inhibition in Bacillus alvei of DAHP synthetase by L-tryptophan as a function of concentration of substrates*

Amt of (μmoles/ml) substrate[a]	Per cent inhibition (0.35 mM trp)			
	ATCC 10871[b]	ATCC 6348	ATCC 6349	ATCC 6344
0.2	49	57	50	61
0.4	26	35	29	38
1.0	4	7	6	10
2.0	None	5	9	3

[a] Numbers represent the concentrations of both phosphoenolpyruvate and erythrose-4-P in the enzyme assay.

[b] All extracts were prepared from cultures grown in the minimal salts medium supplemented with 0.5% casein hydrolysate and 0.05% yeast extract.

from three other species including *E. carotovora* ATCC 495. The inhibitions obtained in the presence of ARO (Table 3) can be compared to the sum of the individual amino acid inhibitions. At inhibitor concentrations of 0.07 mM, the sum of the individual inhibitions was, within the range of experimental error, equal to the inhibition produced when all amino acids were simultaneously present (ARO). Such additive effects of inhibitors are expected when independent inhibitor sites are located on different molecules (i.e., isoenzymes).

The third major group of bacteria studied in detail were representative species of the aerobic pseudomonads (23). Only tyr-inhibitable activity was found in extracts obtained from most species in this group (Table 4). [One culture of *P. piscicida* 1 (10) yielded results identical with those from the latter pseudomonads. Curiously, three other cultures (strains 12, 14, and 15) produced extracts which were entirely without DAHP synthetase activity]. Growth of *P. fluorescens* strains in media supplemented with tyr repressed the level of DAHP synthetase several fold, but did not result in the derepression of any other isoenzymes. Our studies included representatives of all but one (*P. maltophilia*) of the species proposed by Stanier et al. (23). Our data did not differentiate any of these species from one another, except for Stanier's subgeneric category, the acidovorans group which consists of *P. acidovorans* and *P. testosteroni*. The observed differences in control pattern are consistent with the findings of Stanier et al. that the biochemical activities of the acidovorans group diverge sharply from those of other aerobic pseudomonads. These species possess significant phe-inhibitable enzyme in addition to the tyr-inhibitable enzyme, and the two inhibitors act together in a less than cumulative fashion. (Perhaps, the inhibitor binding sites mutually interfere with one another.) In addition, the inhibitor effects occur at substantially higher concentrations than usual, and the DAHP synthetases of these species are especially prone to "desensitize" to inhibitor effects spontaneously.

Strain 135, the representative tested of the group 1 pseudomonads of Delafield et al. (7) differs from both of the other pseudomonad groups, having a pattern of control similar to that of several hydrogen-oxidizing chemolithotrophs (Fig. 1 and Table 5). However, it differs from the latter in the sensitivity of its enzyme to trp in addition to phe and tyr. In their description of the aerobic pseudomonads, Stanier et al. (13) described distinctive "sub-generic" categories. It would be most consistent with our overall results to consider the acidovorans group (as well as the Delafield group) to separate from the other aerobic pseudomonads at the taxonomical level of genus, since a minimal requirement for similarity at the generic level in other taxa seems to be the conservation of the same control pattern.

DAHP synthetase in all of the hydrogen-oxidizing chemolithotrophs which were assayed was controlled by cumulative feedback inhibition (31). *H. facilis* (Table 5) exhibits this end-product pattern of control. Inhibition curves obtained in the presence of ARO for the enzyme of two other species (Fig. 1) conform quite well with the expected cumulative effect, calculated from the inhibitions that were measured in the presence of the individual inhibitors. The difference between expectations for cumulative effects (cumulative feedback inhibition) and the strictly additive effects (isoenzyme feedback inhibition) is maximal at high inhibitor concentrations, becoming insignificant at very low inhibitor concentrations. The phe binding site may tend to desensitize easily, since data similar to that shown for *H. eutropha* in Fig. 1 were also obtained in another experiment with another extract prepared from *H. pantotropha*. No measurable inhibition by trp was found in these strains. The good fit of experimental points with the theoretical curve throughout the inhibitor concentration ranges shown demonstrates excellent specificity. In like manner, inhibition curves that we obtained with a known system of isoenzymes (*in preparation*) closely follow theoretical expectations for strictly additive effects of inhibitor combinations.

Other bacteria that are classified within the suborder *Pseudomonadineae* exhibit a variety of control patterns (Table 5). Both phe- and

TABLE 3. *Distribution of DAHP synthetase isoenzymes in the family Enterobacteriaceae*

Tribe	Genus-species	Strain	Source	Growth-assay code	Per cent inhibition[a]			
					Phe	Tyr	Trp	ARO
Escherichieae	*Escherichia coli*	M1 30	R. Harvey	G-32-37	80 (76)	18 (0)	0 (0)	91 (75)
	E. coli	C	K. Paigen	G-32-37	70 (66)	31 (15)	0 (0)	96 (81)
	E. coli	K-10	F. Neidhardt	G-37-37	89 (84)	8 (3)	0 (0)	95 (87)
	E. coli	K-12	Reference 19	Reference 19	91 (68)	6 (0)	0 (0)	93 (69)
	E. coli	W	Reference 2	Reference 2	89	7	0	
	E. coli	B	Univ. of Washington	G-37-37	84 (79)	14 (0)	0 (0)	96 (79)
	E. coli	ATCC 11775	ATCC	G-37-37	68 (60)	51 (38)	0 (0)	97 (81)
	E. anindolica	ATCC 6879	ATCC	G-37-32	76 (68)	12 (8)	3 (3)	95 (80)
	Aerobacter aerogenes		Univ. of Washington	G-37-37	24 (18)	77 (41)	5 (1)	89 (64)
	Enterobacter aerogenes[b]	ATCC 13048	ATCC	G-37-32	36 (31)	77 (60)	16 (0)	100 (88)
	E. cloacae[b]	ATCC 13047	ATCC	G-37-37	32 (23)	57 (39)	0 (0)	95 (69)
Erwinieae	*Erwinia amylovora*	ATCC 7398	ATCC	G-32-32	2 (0)	91 (55)	0 (0)	97 (63)
	E. carotovora	ATCC 13047	ATCC	F-32-32	59 (46)	0 (0)	0 (0)	84 (57)
Proteae	*Proteus americanus*	ATCC 4675	ATCC	G-37-37	9	28	28	73
	P. ammoniae	ATCC 7002	ATCC	G-37-37	2 (0)	61 (40)	20 (18)	90 (61)
	P. mirabilis	ATCC 14273	ATCC	G-37-37	0 (0)	85 (41)	15 (14)	97 (68)
	P. morganii	ATCC 9917	ATCC	G-37-37	13	33	0	44
	P. rettgeri	ATCC 9250	ATCC	G-37-37	45 (42)	72 (37)	7 (0)	98 (86)
	P. rettgeri	ATCC 9919	ATCC	G-37-37	21 (10)	83 (53)	0 (0)	95 (69)
	P. vulgaris	ATCC 13315	ATCC	G-37-37	4 (3)	89 (75)	17 (0)	100 (80)
Salmonelleae	*Salmonella choleraesuis*	ATCC 13312	ATCC	G-37-37	26 (25)	68 (40)	0 (0)	95 (70)
	S. typhimurium	ATCC 13311	ATCC	G-37-37	35 (32)	60 (45)	0 (0)	95 (74)
	S. typhimurium	UC-3187	Upjohn	G-37-37	29 (27)	66 (41)	0 (0)	95 (74)
	Shigella dysenteriae	ATCC 13313	ATCC	F-37-37	74 (66)	15 (9)	13 (7)	91 (81)
	S. boydii	ATCC 9207	ATCC	F-37-32	45 (43)	65 (51)	19 (8)	97 (87)
	S. flexneri	UC-3187	Upjohn	F-37-37	88 (80)	10 (2)	7 (2)	92 (82)
Serratieae	*Serratia marcescens*	ATCC 13880	ATCC	G-32-32	7 (3)	67 (33)	5 (0)	89 (37)
	S. marcescens subsp. *kiliensis*	ATCC 8101	ATCC	G-32-32	9 (2)	72 (44)	15 (0)	81 (47)

[a] Inhibitors used at concentration of 0.35 mM. Figures in parentheses refer to results with inhibitors at a concentration of 0.07 mM.
[b] ATCC generic designation is *Enterobacter* rather than *Aerobacter* (Intern. Bull. Bacteriol. Nomen. Taxon. 13:23, 1963).

TABLE 4. *Distribution of isoenzymes in the genus Pseudomonas*[a]

Genus-species	Strain	Per cent inhibition							
		0.35 mM				0.07 mM			
		Phe	Tyr	Trp	ARO	Phe	Tyr	Trp	ARO
P. aeruginosa	ATCC 17503	0	96	0	97	0	77	0	73
P. aeruginosa	ATCC 10145	0	96	0	90	0	80	0	70
P. fluorescens	ATCC 13525	0	96	3	98	0	67	4	65
P. fluorescens	ATCC 17815	0	93	0	97	1	58	0	66
P. fluorescens	ATCC 17559	0	84	0	88	0	60	0	64
P. fluorescens	ATCC 9446	3	81	0	82	0	70	0	64
P. fluorescens	ATCC 13985	0	92	0	92	0	58	2	55
P. putida	ATCC 12633	0	89	0	94	3	55	0	72
P. putida	ATCC 17472	13	70	0	86	14	71	0	79
P. alcaligenes	ATCC 14909	0	91	13	95	0	67	7	74
P. pseudoalcaligenes	ATCC 17440	0	94	14	95	0	67	3	82
P. multivorans	ATCC 17759	0	75	0	79	0	60	0	52
P. stutzeri	ATCC 17587	15	100	0	100	0	93	0	89
P. stutzeri	ATCC 17588	4	98	0	96	0	84	0	85
P. piscicida[b]	1	0	95	0	97	0	67	0	71
P. acidovorans	ATCC 15668	62	38	0	76	57	0	0	52
P. testosteroni	ATCC 11996	33	67	0	63	30	30	B	61
Pseudomonas sp.[c]	#135	13	39	43	67	14	2	15	28

[a] Strains designated by ATCC numbers are from the collection of the aerobic pseudomonads studied by Stanier et al. (23). Growth-assay code: B-32-32.

[b] *P. piscicida* was obtained from O. B. Weeks (10). Growth-assay code: J-32-32.

[c] The group I species of Delafield et al. (7) was grown and assayed according to the protocol: B-32-32.

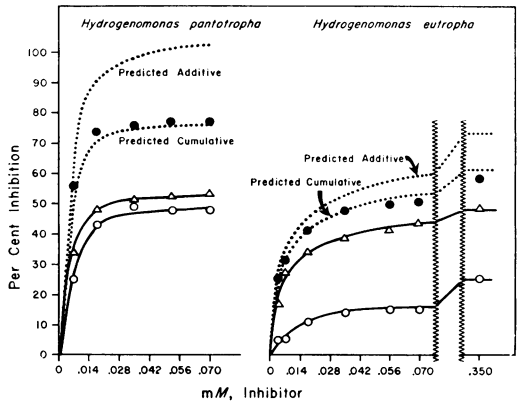

FIG. 1. *Cumulative feedback inhibition in Hydrogenomonas species. H. pantotropha 350 and H. eutropha 337 were obtained from R. Stanier. Growth-assay code: B-32-32. Symbols: ○, phe; △, tyr; ●, ARO. Upper dotted line is the theoretical additive sum of the individual inhibitions shown, and lower dotted line is the theoretical cumulative effect of inhibitions calculated from the observed individual inhibitions. Predicted cumulative inhibition equals (per cent inhibition by phe) + [(per cent inhibition by tyr) × (100% − per cent inhibition by phe)].*

tyr-sensitive isoenzymes were found in species of *Aeromonas* quite similar to the results obtained with *Aerobacter* (Table 3); inhibitions were additive at inhibitor concentrations of 0.07 mM. Both isoenzymes were rather labile to storage under our handling conditions. The DAHP synthetase of *Acetobacter suboxydans* was only inhibited by trp. As is true for most trp-sensitive DAHP synthetases (see Tables 2 and 6) which we have examined, preliminary evidence suggests that the inhibitor effect is competitive with respect to substrate(s). This species is not polarly flagellated and therefore does not fit the criteria of Stanier et al. (23) for its inclusion with the aerobic pseudomonads. It will be instructive to determine whether the control pattern of other acetic acid bacteria which do fit these criteria will resemble the pseudomonads or *A. suboxydans*. The control pattern for DAHP synthetase of *Xanthomonas* species has tentatively been identified as sequential feedback inhibition. However, this merits further study because several differences set it apart from other DAHP synthetases with this control pattern. Although CHA is a potent inhibitor, PPA has little inhibitory effect. Trp also produces a degree of

TABLE 5. *Inhibition patterns observed in various microorganisms in the suborder Pseudomonadineae*

Genus-species	Strain	Growth-assay code	Per cent inhibition									
			0.35 mM				0.07 mM					
			Phe	Tyr	Trp	ARO	Phe	Tyr	Trp	ARO	CHA[a]	PPA[b]
Aeromonas liquifaciens	ATCC 14715	F-37-32	34	88	14	100	29	64	0	95	2	6
A. hydrophilea	ATCC 9071	C-37-32	61	69	26	95	46	35	0	84	12	3
Acetobacter suboxydans	UC-625	F-32-32	0	0	23	25	0	0	18	15	11	9
Xanthomonas hyacinthi[c]	ICPB-XH110	C-30-32	0	13	68	66	0	11	33	41	88	10
X. campestris[c]	ICPB-XC135	C-30-32	21	0	6	24	0	0	16	24	86	0
Hydrogenomonas facilis[d]	332	B-32-32	45	72	0	91	47	60	0	87	5	5

[a] Final concentration chorismate (CHA), 1.0 mM.
[b] Final concentration prephenate (PPA), 0.17 mM.
[c] The two species of *Xanthomonas* were obtained from M. Starr.
[d] *H. facilis* is a hydrogen-oxidizing chemolithotroph received with the name *Pseudomonas facilis* from the collection of R. Stanier.

TABLE 6. *Competitive inhibition of DAHP synthetase by trp in species of Micrococcus and Streptococcus*

Genus-species	Strain	Growth-assay code	Per cent inhibition by 0.35 mM		
			.40[a]	.08	.04
Micrococcus luteus	ATCC 398	A-25-32	28	36	61
M. lysodeikticus	ATCC 4698	A-25-32	14	45	65
Streptococcus faecalis	ATCC 4200	A-37-37	33	49	77
S. bovis	ATCC 9807	A-37-37	45	54	71

[a] Refers to micromoles of substrate present in a final reaction volume of 0.2 ml.

inhibition that appears too substantial to be a nonspecific effect.

Four genera in the order *Actinomycetales* were found to be represented by two patterns of control. In species of *Mycobacterium* and *Nocardia*, tyr appeared to be the only effector, and only trp inhibited the enzyme of *Streptomyces* and *Micromonospora* species (Table 7). Relatively high inhibitor concentrations were required to achieve significant inhibitions in these microorganisms. Again, trp inhibition depends upon substrate concentration. Generic conservation of the control pattern was found in four species of *Mycobacterium* and in five species of *Streptomyces*. Inhibition of *S. virginiae* resembles that of other species of *Streptomyces* when lower substrate concentrations are used (see Fig. 1; reference 13). The typical competitive interaction of inhibitor and the enzyme substrates usually found when trp is the only detectable effector is illustrated in Table 6 with data obtained from species of *Micrococcus* and *Streptococcus*.

In addition to *Bacillus* (and possibly *Xanthomonas*) species, a number of other microorganisms proved to use the pattern of sequential feedback inhibition for the control of DAHP synthetase (Table 8). These included species of *Staphylococcus* and *Gaffkya*. Most of the microorganisms used in this study are representatives of taxa in which the taxonomy has been the most rigorously defined. Results obtained with species of *Flavobacterium*, *Achromobacter*, and *Alcaligenes*, representatives of three highly questionable taxa, having sequential feedback-inhibited DAHP synthetases are also shown in Table 8. Other species that are also presently classified in the two latter genera were found to have phe-sensitive DAHP synthetases that were not inhibited by PPA or CHA (See Table 9). In other words, *Alcaligenes faecalis* is more like *Achromobacter viscosus* than like *Alcaligenes visolactis*, and the latter is more like *Achromobacter parvulus* than like *Alcaligenes faecalis*. It seems quite possible that further amplification of the differences found in *Alcaligenes* and *Achromobacter* might have predictive value suitable for a contribution to the eventual revision of these taxa. Interestingly, Focht and Lockhart came to a similar conclusion using the same four ATCC cultures (8). *A. faecalis* and *A. viscosus* also resembled one another in the necessity to dialyze extracts prepared from these cultures before enzyme activity could be detected.

Data obtained with three other bacterial

TABLE 7. *Amino acid inhibition of DAHP synthetase in the order Actinomycetales*

Genus-species	Strain designation[a]	Growth-assay code	Per cent inhibition[b]			
			Tyr	Phe	Trp	ARO
Mycobacterium avium	ATCC 7992	H-37-32	50	None	17	70
M. phlei	ATCC 354	H-37-32	29	None	3	33
M. rhodochrous	ATCC 271	H-25-32	47	3	14	57
M. smegmatis		H-37-32	88	None	10	92
Nocardia corallina	UC-2161	D-25-32	45	8	None	49
Streptomyces acidomyceticus	UC-2327	D-32-32	None	None	39	37
S. endus	UC-2020	D-32-32	None	None	23	20
S. griseus	UC-2137	C-37-37	None	None	49	49
S. virginiae	UC-2002	C-30-32	None	None	2	1
S. albidoflavus		D-32-32	None	None	58	50
Micromonospora sp.	UC-2259	D-37-32	None	None	24	26

[a] UC strains were received from Upjohn Pharmaceutical, Kalamazoo, Mich. *M. smegmatis* and *S. albidoflavus* were obtained from the University of Washington.
[b] Inhibitor concentration, 0.35 mM.

TABLE 8. *Distribution in genera other than Bacillus of DAHP synthetase activity regulated by sequential feedback inhibition*

Genus-species	Strain	Source	Growth-assay code	Per cent inhibition	
				PPA[a]	ARO[b]
Staphylococcus roseus	ATCC 418	ATCC	A-32-37	83 (79)	30
S. aureus	ATCC 12600	ATCC	F-37-37	100 (74)	2
Staphylococcus albus		Univ. of Washington	F-37-37	75 (92)	0
Gaffkya tetragena	ATCC 10875	ATCC	F-37-37	71 (70)	0
Flavobacterium sp.		Univ. of Washington	A-25-32	38 (29)	13
F. devorans	ATCC 10892	ATCC	A-25-32	69 (56)	4
Achromobacter parvulus	ATCC 4335	ATCC	A-25-32	73 (55)	15
Alcaligenes viscolactis	ATCC 9036	ATCC	A-30-32	79 (83)	9

[a] prephenate (PPA) final reaction concentration, 0.17 mM. In parentheses, per cent inhibition by 1.0 mM chorismate (CHA).
[b] ARO final reaction concentration, 0.35 mM.

strains are tabulated in Table 9. Species of *Neisseria*, *Veillonella*, and *Myxococcus* yielded extracts in which the enzyme was inhibited strongly by a single amino acid end product: tyr, phe, and trp, respectively. Also included in Table 9 are results obtained with a few nonbacterial microorganisms. Among the yeasts, *Saccharomyces cerevisiae*, an ascomycete, proved to have isoenzymic DAHP synthetases, whereas the basidiomycete, *Sporobolomyces odorus*, exhibited cumulative feedback inhibition. The mold *N. crassa* possessed DAHP synthetase isoenzymes as did a protozoan, *Polytoma obtusum*. A list of the microorganisms in which DAHP synthetase activity was absent or too low to permit an accurate determination appears in Table 10. No attempt was made to recover enzyme activity in these strains by altering growth or assay conditions, or preparative procedures for making extracts.

In general, DAHP synthetase was well suited to these studies because the enzyme assay is sensitive, because enzyme activity was reasonably stable in most microorganisms tested, and because the residual nonrepressible activity was usually sufficient, such that easily measurable enzyme activities could be found even under conditions of maximal repression in a rich medium. A total of 127 strains proved to have sufficient levels of DAHP synthetase to permit a satisfactory analysis of the control pattern.

DISCUSSION

Distribution of control patterns. A multiplicity of examples illustrating the diversity of metabolic signals that can serve to mediate control functions have emerged from studies of various multibranched biosynthetic systems of enzymes in different organisms (28). These include isoenzyme feedback inhibition (27), cumulative feedback

TABLE 9. *Inhibition patterns in various microorganisms*

Genus-species	Strain	Source	Growth-assay code	Per cent inhibition[a]				
				Tyr	Phe	Trp	ARO	PPA
Alcaligenes faecalis	ATCC 8750	ATCC	A-32-32	0	100	0	100	1
Achromobacter viscosus	ATCC 12448	ATCC	A-32-32	0	91	0	93	5
Veillonella alcalescens	ATCC 14894	Univ. of Washington	1-37-37[b]	0	86	0	85	0
Neisseria sp.		Univ. of Washington	F-37-37	97	0	0	99	0
Myxococcus xanthus		Univ. of Washington		0	5	83	76	0
Saccharomyces cerevisiae	XT-300	J. DeMoss	C-32-32	44	46	5	98	0
S. cerevisiae		Univ. of Washington	C-32-32	59	34	0	94	0
Sporobolomyces odorus		Univ. of Washington	C-32-32	66	86	14	93	0
Polytoma obtusum	P10	L. Provosoli	K-32-32 (4)	8	15	31	54	0
Neurospora crassa[c]		Univ. of Washington	L-37-37	72	42	12	99	0

[a] Amino acid inhibitors were used at a final concentration of 0.35 mM in the enzyme assay; PPA was used at a final concentration of 0.17 mM.

[b] Further description of this strain is found in N. C. McCormick, Ph.D. Thesis, Univ. of Washington, Seattle, 1960.

[c] Detailed inhibition data contained in another report (*in preparation*).

TABLE 10. *Microorganisms in which DAHP synthetase activity was low or absent*

Microorganism	Source
Bacillus macerans	H. Koeffler
Caulobacter crescentus	R. Stanier
Chondrococcus sp.	Univ. of Washington
Clostridium tetanomorphum	Univ. of Washington
Corynebacterium sp.	Univ. of Washington
Cytophaga hutchinsonii	Univ. of Washington
Lactobacillus casei	ATCC 334
Leuconostoc citrovorum	ATCC 8082
L. mesenteroides	ATCC 12291
Micrococcus aerogenes	Univ. of Washington
Sarcina lutea	ATCC 272
Sporocytophaga myxococcoides	Univ. of Washington

inhibition (31), concerted feedback inhibition (6), and sequential feedback inhibition (13, 21). Our assessment of the control patterns of DAHP synthetase in 91 species of 32 genera turned up examples of at least six distinctive patterns: (i) sequential feedback inhibition, (ii) cumulative feedback inhibition, (iii) isoenzyme feedback inhibition, (iv) inhibition by tyr, (v) inhibition by phe, and (vi) inhibition by try. No synergistic patterns of control were found, i.e., concerted feedback inhibition or cooperative feedback inhibition (28). Isoenzyme and sequential feedback inhibition patterns were observed most frequently. Variations found in the Delafield and acidovorans groups of pseudomonads and in *Xanthomonas* may represent still other distinctive patterns of control for DAHP synthetase.

Nature of single-effector enzymes. DAHP synthetases which were inhibited by only a single amino acid require further study to understand the physiological interactions underlying the control function. Microorganisms in this category which were capable of growth on minimal media (e.g., species of *Pseudomonas*) were not inhibited by exogenous supplements of the amino acid capable of inhibiting DAHP synthetase in vitro. Therefore, the enzyme in vivo was still active in the normal synthesis of precursors for the remaining end products of the pathway under these conditions. These cells were obviously permeable to the exogenously supplied amino acid since repression of enzyme synthesis was observed. The existence of "single-effector" enzymes can be interpreted in at least three ways. (i) The possibility that a single DAHP synthetase controlled by one amino acid could operate effectively in the complex branched pathway will be discussed separately (R. Jensen, *in preparation*). (ii) The pathway itself might differ so that amino acids which do not behave as effectors might arise from distinctly different pathways. Such a situation would reduce the control situation to the status of a simple unbranched pathway. Separate routes of biosynthesis for tyr and phe seem especially unlikely in view of their closely analogous structures. (iii) Some or all of these could be isoenzyme systems. The undetected isoenzymes could be labile to the preparative procedures for making extracts, or their synthesis could be maximally repressed under many or most nutritional conditions. The latter possibility must be seriously considered, because the known isoenzyme systems tend to exhibit "dominant isoenzymes" under most growth conditions (3), and isoenzymes frequently differ in physical properties such as stability. It is important to note that, regardless of whether these classes eventually prove to consist of single enzymes or of stable or dere-

pressed isoenzymes, they are nevertheless easily distinguished operationally and appear to represent empirically useful categories.

Generic conservation of control patterns. Almost without exception, all of the species in a given genus possessed a DAHP synthetase controlled by the same regulatory pattern. One notable and well-substantiated exception was *B. alvei* (four strains) which differed from the control pattern found for 35 strains of 21 species in *Bacillus*. The application of this approach to the definition of evolutionary relationships between microorganisms and, accordingly, its utility for taxonomical studies will be discussed elsewhere (R. Jensen, *in preparation*). Particularly in the case of isoenzymes, the literature contains numerous examples in which the genetic control of the enzymes is virtually identical throughout a species (1, 5). An exception such as *B. alvei* represents could be attributed to (i) the inadequacy of its present classification, (ii) a case of exaggerated evolutionary divergence from the rest of the genus [this is not necessarily separate from (i) above], or (iii) an example of an occasional lack of correlation due to limitation of the method.

It would be important to discover whether similar conservation of control characteristics in related taxa would be found for other allosteric enzymes that are complexly related to multiple end products. It is interesting in this connection that a study of the control of arginine biosynthesis led to the conclusion that species-specific control mechanisms exist in bacteria (29), although the number of strains examined in the latter study was quite limited.

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ADDENDUM IN PROOF

M. Mandel has kindly pointed out to us that the anomalous results obtained with strain 1 relative to those obtained with the other strains of *P. piscicida* are consistent with the finding that this strain is not pure (Mandel, Weeks, and Colwell, J. Bacteriol. **90**:1492, 1965). Our results with this strain are also consistent with the probable identity of the contaminant as a pseudomonad species.

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