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Regulation of the Biosynthesis of Amino Acids of the Aspartate Family in Coliform Bacteria and Pseudomonads

G. N. COHEN,[1] R. Y. STANIER,[2] AND GISELE LE BRAS
Laboratoire d'Enzymologie du Centre National de la Recherche Scientifique, Gif-sur-Yvette, 91, France

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The control of aspartokinase and homoserine dehydrogenase activities was compared in aerobic and fermentative pseudomonads (genera *Pseudomonas* and *Aeromonas*), and in coliform bacteria representative of the principal genera of the *Enterobacteriaceae*. Isofunctional aspartokinases subject to independent end-product control occur in the *Enterobacteriaceae* and in *Aeromonas*. In *Pseudomonas*, there appears to be a single aspartokinase, subject to concerted feedback inhibition by lysine and threonine. Within this genus, the sensitivity of aspartokinase to the single allosteric inhibitors varies considerably: the aspartokinase of the acidovorans group is little affected by the single inhibitors, whereas that of the fluorescent group is severely inhibited by either amino acid at high concentration. In all bacteria examined, homoserine dehydrogenase activity is inhibited by threonine; inhibition is more severe in aerobic pseudomonads than in the other groups. In most of the bacteria examined, either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate can serve as a cofactor for this enzyme, though the relative activity with the two pyridine nucleotides varies widely. Aerobic pseudomonads of the acidovorans group contain a homoserine dehydrogenase that is absolutely specific for NAD. The taxonomic implications of these findings are discussed.

The branching pathway for the biosynthesis of the amino acids of the aspartate family (Fig. 1) is subject in bacteria to primary regulation at two points. One is the phosphorylation of L-aspartate, mediated by aspartokinase (EC 2.7.2.4), which controls the primary flow of carbon to all the amino acids of this family; the other is the reduction of aspartate semialdehyde to homoserine, mediated by homoserine dehydrogenase (EC 1.1.1.3), which controls the flow of carbon to methionine, threonine, and isoleucine. Two radically different systems of control have been described for these enzymes in different groups of bacteria.

In *Escherichia coli*, of which strains K-12 and B have been studied in detail, three different proteins catalyze the phosphorylation of aspartate (12, 17, 19, 22, 24, 25). Aspartokinases I and III are both allosterically inhibited and repressed by threonine and by lysine, respectively; aspartokinase II is repressed, but not inhibited, by methionine. In both strains, aspartokinase I is a multifunctional enzyme, which also carries homoserine dehydrogenase activity, likewise subject to repression and inhibition by threonine. In strain K-12, but not in strain B, aspartokinase II is multifunctional, carrying a second homoserine dehydrogenase activity subject to repression by methionine. Some of the characteristic features of this regulatory system can be established from the analysis of crude, cell-free extracts, notably the existence of the isofunctional aspartokinases. Neither threonine nor lysine alone can inhibit more than a certain fraction of the aspartokinase activity, irrespective of their concentration: when both are present at saturating levels, the inhibition observed is strictly additive but not total, since the activity of aspartokinase II remains unaffected. The detailed regulation of this pathway has not been studied in any other coliform bacterium; however, Freundlich (13) has shown that a lysine-sensitive and a threonine-

[1] Present address: Service de Physiologie Microbienne, Institut Pasteur, Paris 15e, France.

[2] John Simon Guggenheim Fellow, 1967-1968. Present address: Department of Bacteriology and Immunology, University of California, Berkeley, Calif. 94720.

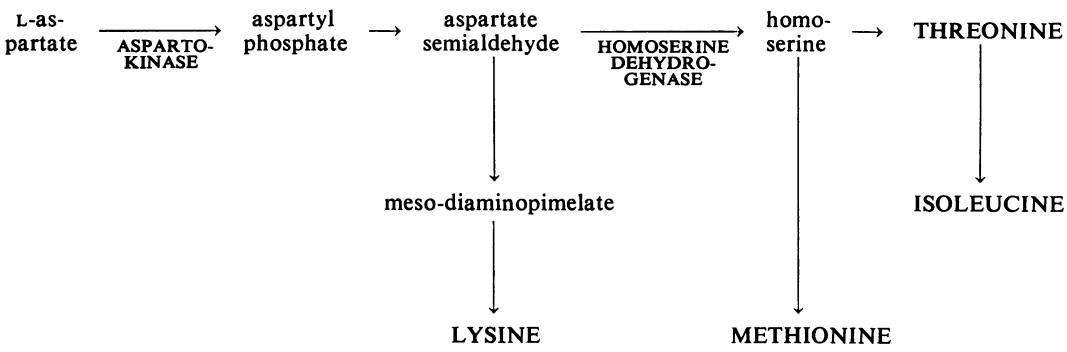

FIG. 1. *Biosynthetic pathways (in bacteria) leading from aspartate, showing the positions of the two primary sites of regulation, aspartokinase and homoserine dehydrogenase. Biosynthetic end products are capitalized.*

sensitive aspartokinase and a threonine-sensitive homoserine dehydrogenase are present in *Salmonella typhimurium*. More recently, he presented evidence for a methionine-repressible homoserine dehydrogenase in the same species (2).

The elements of a completely different control system governing the operation of this pathway have been revealed in certain purple bacteria and bacilli. In *Rhodopseudomonas capsulata* (9), and in *Bacillus subtilis* and *B. polymyxa* (20), there appears to be a single aspartokinase, which is little affected by either lysine or threonine singly but is almost totally inhibited by the two effectors together. This control mechanism has been termed "concerted feedback inhibition" by Datta and Gest (9). What has appeared to be a somewhat different type of control exists in *Rhodospirillum rubrum*, in which threonine is reported to be the only inhibitor of aspartokinase (8); however, as we have recently observed (*unpublished data*), this enzyme is in fact also subject to concerted feedback inhibition by threonine and lysine. The control of homoserine dehydrogenase has been examined only in *R. rubrum*, in which it is inhibited by threonine, as in *E. coli* (10); however, inhibition is released by isoleucine or methionine, a phenomenon not found in *E. coli*.

Jensen, Nasser, and Nester (15) recently made a comparative study of the feedback regulation of DAHP synthetase (EC 4.1.2.15), the first enzyme of the branching aromatic biosynthetic pathway, in bacteria belonging to a large number of different taxonomic groups. Their work shows that the mechanism of control of DAHP synthetase activity differs in different bacterial groups. This suggests that specific control mechanisms may prove to be important taxonomic and evolutionary markers among the bacteria. A similar general conclusion has been reached by Cánovas, Ornston, and Stanier (3) on the basis of a comparative study of the control mechanisms operative in a catabolic pathway. We have accordingly been prompted to make a systematic analysis of the primary regulation of the biosynthesis of amino acids belonging to the aspartate family in a number of different bacterial groups. In this paper, we shall discuss the regulation of aspartokinase and homoserine dehydrogenase activities in the two principal groups of gram-negative, rod-shaped, aerobic chemoheterotrophic bacteria: the coliform bacteria *sensu lato* (i.e., the members of the family *Enterobacteriaceae*) and the pseudomonads.

MATERIALS AND METHODS

Biological materials. Most of the coliform bacteria and aeromonads studied were recent isolates, obtained through the kindness of L. Le Minor of the Institut Pasteur, in whose laboratory they had been identified (16). These strains will be referred to by designations under which they were received from him. They include: *Escherichia coli*, 35–67 and 36–67; *Aerobacter aerogenes*, 38–67; *A. cloacae*, 39–67; *Serratia marcescens*, 2–68 and 3–68; *Proteus vulgaris*, 3–66; *Providencia*, 7–67; *Edwardsiella tarda*; *Aeromonas shigelloides*, 2–62; and *A. hydrophila*, 3–66. A strain of *A. formicans* was received from I. P. Crawford of the Scripps Metabolic Clinic, La Jolla, Calif. *Erwinia aeroideae* EA144 and *E. carotovora* EC153 were received from the International Collection of Phytopathogenic Bacteria, University of California, Davis, Calif., through the kindness of the curator, M. P. Starr.

With one exception, all the aerobic pseudomonads examined originated from the culture collection of the Department of Bacteriology and Immunology, University of California, Berkeley, Calif., and had been subjected to taxonomic analysis by Stanier, Palleroni, and Doudoroff (23). They will be designated here by the names and strain numbers used by these authors (23). *Pseudomonas methanica* (strain of Dworkin and Foster) was obtained through the

courtesy of Dr. Antoniewski, Société Internationale de Recherches B. P., Epernon, France.

Growth conditions. The strains belonging to the genera *Escherichia*, *Aerobacter*, *Proteus*, *Providencia*, *Serratia*, *Erwinia*, *Aeromonas*, and *Edwardsiella* were grown in the mineral medium described by Cohen and Rickenberg (5) supplemented with 0.2% glucose. The temperature was 37 C, except for the strains of *Erwinia*, which were grown at 27 C. All the other strains used in this work were grown in the standard mineral base described by Cohen-Bazire, Sistrom, and Stanier (6), supplemented with 10^{-2} M sodium succinate. These strains were grown at 27 C. All cultures were strongly aerated. *P. maltophilia* cultures were supplemented with 5×10^{-4} M DL-methionine, *P. vulgaris* with nicotinic acid (100 μg/liter), and *E. tarda* with cysteine (150 μg/ml), methionine (100 μg/ml), and nicotinamide (2.2 μg/ml).

Preparation of cell-free extracts. All cultures were collected during exponential growth. After centrifugation, the pellets were suspended in the following washing buffer: 0.02 M potassium phosphate (*p*H 7.2) containing 1.5×10^{-1} M KCl, 2×10^{-3} M magnesium ethylenediaminetretraacetic acid (Mg Titriplex of E. Merck, AG, Darmstadt), 10^{-3} M DL-threonine, and 5×10^{-4} M L-lysine. After washing, the pellets were resuspended in the same buffer and the cells were disrupted in a 10-kc Raytheon sonic oscillator for periods which varied from 6 to 12 min, depending on the strain. The cell debris was removed by centrifugation at 4 C (23,000 $\times$ *g*). The extract was used directly for the determination of enzymatic activities. In a few cases, in which the activity was weak, the extract was fractionated with ammonium sulfate, and the fraction which precipitated between 25 and 50% saturation was collected and redissolved in a minimum amount of buffer.

Measurements of enzymatic activity. Aspartokinase was assayed by measuring formation of aspartohydroxamate as previously described (22). Homoserine dehydrogenase was determined from the rate of oxidation of either reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), at a concentration of 2.5×10^{-4} M, with aspartate semialdehyde (8×10^{-4} M) as the other substrate (4). It should be noted that both these assay conditions were developed for determination of enzyme activities in *E. coli* K-12. It is, therefore, by no means certain that they are optimal for all the different species of bacteria examined here. In certain strains examined, aspartokinase was extremely sensitive to concerted feedback inhibition by threonine and lysine. In such cases, the enzyme was precipitated by treatment of the extract with ammonium sulfate and the precipitate was resuspended in buffer devoid of threonine and lysine; the measurements of activity were made immediately, in order to avoid a possible desensitization of the enzyme.

All activities are expressed in nanomoles of aspartohydroxamate formed or of reduced pyridine nucleotide oxidized per minute per milligram of protein. Protein was estimated by the biuret method (14).

Growth studies. Growth was estimated turbidimetrically at 420 nm in a Zeiss spectrophotometer.

RESULTS

Range of coliform bacteria examined. Since we wished to determine whether the regulatory mechanisms established for *E. coli* are characteristic of the *Enterobacteriaceae*, strains representative of the whole family were examined. Our choice of strains was guided primarily by two characters which do not occupy a prominent place in the current classification of this family: the mean base composition [moles per cent guanine plus cytosine (G + C)] of the deoxyribonucleic acid (DNA), and the nature of the fermentative end products. These characters permit the recognition of three major groups (Table 1). Group A consists of organisms which have a mean base composition of the DNA in the range of 50 to 54 moles per cent G + C, and which perform a mixed acid fermentation of sugars. In terms of conventional generic subdivisions (11), it includes *Escherichia*, *Salmonella*, *Shigella*, *Citrobacter*, *Arizona*, and *Edwardsiella*. Group B consists of organisms which have a mean base composition of the DNA in the range of 50 to 58 moles per cent, and which perform a butanediol fermentation of sugars. It includes *Aerobacter* (*Enterobacter*), *Klebsiella*, *Serratia*, and, at least in part, *Erwinia*. Group C consists of organisms which have a mean base composition of the DNA of approximately 40 moles per cent, and which perform a mixed acid fermentation. It includes *Proteus* and *Providencia*.

Control of aspartokinase activity in coliform

TABLE 1. *Assignment to biological groups of the strains of coliform bacteria examined*

Group	DNA base composition (moles % G + C)	Fermentative mechanism	Species[a]
A	50–54	Mixed acid	*Escherichia coli* (2) *Edwardsiella tarda* (1)
B	50–59	Butanediol	*Aerobacter aerogenes* (1) *A. cloacae* (1) *Serratia marcescens* (2) *Erwinia carotovora* (1) *E. aroideae* (1)
C	37–42	Mixed acid	*Proteus vulgaris* (1)

[a] The number in parentheses is the number of strains examined.

bacteria. As representatives of group A, we examined two recently isolated and typical strains of *Escherichia coli*, together with one strain of *Edwardsiella tarda*, which differs markedly in general phenotype from most other coliform bacteria of group A. All three strains show both lysine- and threonine-sensitive aspartokinase activity, and the effects of these two inhibitors are additive (Table 2). In the two strains of *E. coli*, about 10% of the total aspartokinase activity is retained in the presence of saturating concentrations of both inhibitors and probably represents the fraction contributed by a methionine-repressible aspartokinase II. Aspartokinase II accounts for an even smaller fraction of the total activity in *E. coli* K-12, for which data are also included in Table 2. In *Edwardsiella*, the inhibition of aspartokinase activity by threonine and lysine together is total, which may indicate the absence of an enzyme with the properties of aspartokinase II. However, since the *Edwardsiella* strain was grown in the presence of methionine, which is an essential nutrient for it, the experiment is not conclusive, since aspartokinase II would have been repressed under the conditions of cultivation used.

TABLE 2. *Aspartokinases[a] of coliform bacteria belonging to group A (Escherichia and Edwardsiella)*

Additions	*E. coli* 35-67	*E. coli* 36-67	*Edwardsiella tarda*	*E. coli* K-12
None	51.5	78.7	14.8	42
L-Lysine, 3×10^{-4} M			13.6 (8)	
L-Lysine, 5×10^{-3} M	34.8	28.5		
L-Lysine, 10^{-2} M	35.0 (32)	29.0 (63)	13.6 (8)	21 (50)
L-Threonine, 3×10^{-4} M			9.7 (34)	
L-Threonine, 5×10^{-3} M	29.4	62.6		
L-Threonine, 10^{-2} M	29.6 (42)	62 (21)	0.7 (95)	20 (52)
Both effectors, 10^{-2} M	4.8 (90)	7.9 (89)	0 (100)	1

[a] Expressed as nanomoles per minute per milligram. The values in parentheses represent the per cent inhibition of the activity.

Data on the aspartokinase activities of six strains belonging to group B are shown in Table 3. Here again, both lysine- and threonine-sensitive aspartokinases are clearly present, the inhibitory effects of the two amino acids being in all cases additive. In the *Serratia* and *Erwinia* strains, the fraction of activity attributable to aspartokinase II (i.e., not inhibitable by both effectors) is relatively small, as in *E. coli*. It is considerably larger in the two *Aerobacter* strains, in which it represents about 25% of the total activity. As shown in Table 4, this uninhibitable fraction is greatly reduced by growth of *A. cloacae* in the presence of 5×10^{-3} M L-methionine, a fact which further strengthens its identity as asparto-

TABLE 3. *Aspartokinases[a] of coliform bacteria belonging to group B (Serratia, Aerobacter, and Erwinia)*

Additions	*S. marcescens* 2-68	*S. marcescens* 3-68	*A. aerogenes* 38-67	*A. cloacae* 39-67
None	51.0	42.8	28.9	19.9
L-Lysine, 3×10^{-4} M	41.5 (18)	32.0 (25)		
L-Threonine, 3×10^{-4} M	45.5 (9.5)	34.5 (14)		
Both effectors, 3×10^{-4} M	38.0 (27)	30.0 (30)		
L-Lysine, 10^{-2} M	18.8 (63)	16.7 (61)	15.6 (46)	14.7 (27)
L-Threonine, 10^{-2} M	37.5 (26)	28.6 (33)	21.3 (26)	11.4 (43)
Both effectors, 10^{-2} M	5.4 (90)	4.8 (89)	6.4 (77)	4.5 (77)
	E. aroideae	*E. carotovora*		
None	24.5	22.3		
L-Lysine, 2×10^{-3} M		15.7 (29)		
L-Threonine, 2×10^{-3} M		12.9 (42)		
Both effectors, 2×10^{-3} M		4.9 (78)		
L-Lysine, 5×10^{-3} M	15.7 (36)			
L-Threonine, 5×10^{-3} M	20.8 (15)			
Both effectors, 5×10^{-3} M	10.8 (56)			
L-Lysine, 10^{-2} M	13.5 (45)	12.2 (45)		
L-Threonine, 10^{-2} M	14.7 (40)	11.6 (47)		
Both effectors, 10^{-2} M	3.7 (85)	3.8 (83)		

[a] Expressed as nanomoles per minute per milligram. The values in parentheses represent the per cent inhibition of the activity.

kinase II. It should be noted that the repression of aspartokinase II by growth with methionine causes a marked increase in the specific activity of aspartokinase III, a phenomenon also observed with some strains of *E. coli* (1).

Data on the aspartokinase activities of two strains belonging to group C are shown in Table 5. In each case, both lysine- and threonine-sensitive aspartokinase activities are evident. In *Providencia*, aspartokinase activity is almost completely abolished by the two amino acids at concentrations of 10^{-2} M; aspartokinase II is accordingly either absent or present at a very low level in this strain. In *Proteus vulgaris*, on the other hand, a large fraction of the total activity appears to be attributable to aspartokinase II, as in the *Aerobacter* strains examined.

This survey accordingly shows that functional aspartokinases with allosteric properties like those of the aspartokinases I and III of *E. coli* (22) and *S. typhimurium* (13) are universal in coliform bacteria. With the possible exceptions of *Edwardsiella* and *Providencia*, the strains examined all contain as well a noninhibitable fraction of aspartokinase activity, which probably reflects the presence of a third enzyme having the properties of the aspartokinase II of *E. coli* K-12. The relative activities of the three types of aspartokinase differ greatly from strain to strain, even within a single species (see data for *E. coli* in Table 2).

Regulation of homoserine dehydrogenase activity in coliform bacteria. Like *E. coli* K-12 (18) and *S. typhimurium* (13), all strains examined contain a homoserine dehydrogenase partly inhibitable by threonine (Table 6); in most strains, activity is inhibited to the extent of 60 to 80% at a threonine concentration of 2×10^{-2} M.

TABLE 4. *Relative amounts of the three isofunctional aspartokinases of Aerobacter cloacae grown under different conditions*[a]

Addition to the growth medium	Aspartokinase I	Aspartokinase II	Aspartokinase III
L-Lysine, 10^{-3} M	19.5 (58)	9.8 (29)	4.4 (13)
L-Methionine, 5×10^{-3} M	12.5 (23)	4.9 (9)	35.2 (65)

[a] Aspartokinase I is the activity inhibited by 10^{-2} M L-threonine; aspartokinase III is the activity inhibited by 10^{-2} M L-lysine. Aspartokinase II is the activity which remains in the presence of threonine and lysine at the above concentrations. The results are expressed as nanomoles per minute per milligram. The values in parentheses represent the relative amounts (per cent) of each of the molecular species.

TABLE 5. *Aspartokinases*[a] *of coliform bacteria belonging to group C (Proteus and Providencia)*

Additions	*Proteus vulgaris* 3-66	*Providencia* 7-67
None	38.7	30.6
L-Lysine, 3×10^{-4} M	22.0 (43)	21.4 (30)
L-Threonine, 3×10^{-4} M	32.9 (15)	22.3 (27)
Both effectors, 3×10^{-4} M	14.7 (62)	14.0 (54)
L-Lysine, 10^{-2} M	18.6 (52)	17.4 (43)
L-Threonine, 10^{-2} M	32.9 (15)	13.8 (55)
Both effectors, 10^{-2} M	9.7 (75)	0 (100)

[a] Expressed as nanomoles per minute per milligram. The values in parentheses represent the per cent inhibition of the activity.

TABLE 6. *Homoserine dehydrogenase activities of coliform bacteria*

Organism	Specific activity with NADPH[a]	Ratio of specific activities (NADPH/NADH)	Inhibition by L-threonine	
			Molarity	Inhibition
				%
Group A				
Escherichia coli K-12	160	4.0	0.002	75
E. coli 35-67	107	3.6	0.001	86
E. coli 36-67	48	1.4	0.02	77
Edwardsiella tarda	78	2.0	0.02	45
Group B				
Aerobacter aerogenes 38-67	61	1.7	0.02	66
A. cloacae 39-67	69	2.7	0.02	85
Serratia marcescens 2-68	102	2.5	0.02	81
S. marcescens 3-68	105	3.7	0.02	77
Erwinia aroideae	127	2.2	0.02	56
E. carotovora	83	5.9	0.02	68
Group C				
Proteus vulgaris	137	2.0	0.01	66
Providencia 7-67	63	2.5	0.02	70

[a] Expressed as nanomoles per minute per milligram.

At the standard coenzyme concentration used (2.5×10^{-4} M), the homoserine dehydrogenase of all strains is active with both NADH or NADPH (Table 6). The activity with NADPH is always greater than with NADH: the ratios of activity vary between 1.5 and 6. In *E. coli* K-12, the ratio of activity is 4; however, the velocity maximum in this strain is identical with both pyridine nucleotides, so that the lower activity observed with NADH at a molarity of 2.5×10^{-4} M expresses the relatively low affinity of the enzyme for this pyridine nucleotide.

Range of aerobic pseudomonads examined. The fluorescent pseudomonads constitute the central taxonomic cluster of the genus *Pseudomonas*. Although they share many common phenotypic properties, a recent taxonomic analysis (23) has shown that they can be subdivided into three species, *P. aeruginosa*, *P. putida*, and *P. fluorescens*, of which the last two are further subdivisible on minor phenotypic traits into a number of different biotypes. It should be expressly noted that this system of classification is an extremely conservative one, relative to the current formal classification of coliform bacteria; the phenotypic differences between the three species of fluorescent pseudomonads are no smaller than those which differentiate between such genera as *Escherichia*, *Citrobacter*, *Salmonella*, and *Shigella*.

Markedly different from the fluorescent pseudomonads in many phenotypic respects are the two *Pseudomonas* spp. of the acidovorans group, *P. acidovorans* and *P. testosteroni*, which likewise share many common properties. The present analysis was accordingly centered on a collection of strains representative of the five species of *Pseudomonas* included in these two groups. In addition, we examined single strains representative of other *Pseudomonas* spp. which, on general phenotype, do not seem to belong to either the fluorescent or the acidovorans group.

Control of aspartokinase activities in fluorescent pseudomonads. The effects of lysine and threonine on the aspartokinase activity of strains representative of *P. aeruginosa*, *P. putida*, and *P. fluorescens* are shown in Table 7. There is a common pattern of inhibition in all three species, entirely different from that characteristic of coliform bacteria. The aspartokinase activity of these pseudomonads is subject to concerted inhibition by the two amino acids; each amino acid is, however, inhibitory alone when furnished at a high concentration. At a level of 10^{-2} M, either lysine or threonine can inhibit as much as 90% of the activity. Consequently, the concerted nature of the inhibition can be clearly shown only when relatively low concentrations of the effectors are used. At a level of approximately 3×10^{-4} M, inhibition by the single amino acids is negligible, whereas both together inhibit activity to the extent of 35 to 65%. As a rule, inhibition by the amino acids together becomes total at concentrations in the range of 10^{-3} to 10^{-2} M. Methionine is without effect on the aspartokinase activity, when added either alone or in combination with the two other amino acids. This characteristic pattern has been confirmed for additional strains belonging to all three species: *P. aeruginosa* 278, *P. putida* 5 and 98, *P. fluorescens* 2, 192, and 393. It is different in several respects from the mode of concerted feedback inhibition originally discovered in *Rhodopseudomonas capsulata* (9), in which the single amino acids have little effect on aspartokinase activity, even at high concentrations, and this concerted inhibition is never total.

Control of aspartokinase activity in the acidovorans group. The effects of lysine and threonine on the aspartokinase activity of four strains representative of the acidovorans group are shown in Table 8. Feedback inhibition is again

TABLE 7. *Concerted feedback inhibition of the aspartokinase activity[a] in P. aeruginosa, P. putida, and P. fluorescens*

Additions	*P. aeruginosa* 131	*P. putida* 90 (biotype A)	*P. fluorescens* 36 (biotype E)
None	26.8	16.5	13.1
L-Lysine, 10^{-2} M	14.2 (47)	2.4 (85)	8.5 (35)
L-Threonine, 10^{-2} M	6.5 (75)	1.5 (90)	5.9 (55)
Both effectors, 10^{-2} M	0 (100)	0 (100)	2.2 (83)
L-Lysine, 10^{-3} M	18.8 (29)	4.1 (75)	
L-Threonine, 10^{-3} M	9.5 (65)	2.7 (83)	
Both effectors, 10^{-3} M	2.6 (90)	0.5 (97)	
L-Lysine, 3×10^{-4} M	25.9 (3)	15.1 (9)	13.1 (0)
L-Threonine, 3×10^{-4} M	28.7 (0)	15.0 (9)	12.3 (6)
Both effectors, 3×10^{-4} M	9.0 (66)	9.4 (43)	8.7 (33)

[a] Expressed as nanomoles per minute per milligram. The values in parentheses represent the percent inhibition of the total activity.

TABLE 8. *Concerted feedback inhibition of the aspartokinase activity[a] in P. acidovorans and P. testosteroni*

Additions	*P. acidovorans* 14	*P. acidovorans* 114	*P. testosteroni* 25	*P. testosteroni* 78
None	62	31	27	43
L-Lysine, 10^{-2} M	48 (22)	22 (29)	27 (0)	44 (0)
L-Threonine, 10^{-2} M	54 (13)	23 (26)	27 (0)	29 (32)
Both effectors, 10^{-2} M	0 (100)	0.8 (97)	1.3 (95)	0 (100)
L-Lysine, 10^{-3} M	58 (6)			40 (7)
L-Threonine, 10^{-3} M	57 (8)			45 (0)
Both effectors, 10^{-3} M	15 (76)			5 (88)

[a] Expressed as nanomoles per minute per milligram. The values in parentheses represent the per cent inhibition of the total activity.

concerted and resembles that characteristic of *R. capsulata* rather than that characteristic of fluorescent pseudomonads. The single amino acids do not inhibit appreciably even at a concentration of 10^{-2} M; concerted inhibition is very marked at an amino acid concentration of 10^{-3} M, and becomes essentially complete at 10^{-2} M.

Control of aspartokinase activity in other Pseudomonas spp. Experiments with single strains of *P. multivorans*, *P. stutzeri*, and *P. methanica* show that the aspartokinase activity of these bacteria is likewise subject to concerted inhibition by lysine and threonine. The aspartokinase of *P. multivorans* and *P. stutzeri* resembles that of the fluorescent group, in the sense that it is severely inhibited by the single allosteric effectors at high concentrations (Table 9).

Control of homoserine dehydrogenase activity in aerobic pseudomonads. In general, the homoserine dehydrogenase activity of the aerobic pseudomonads is extremely sensitive to inhibition by threonine (Table 10). Methionine has no effect on activity, alone or in the presence of threonine. The homoserine dehydrogenase of most aerobic pseudomonads is almost totally inhibited by threonine at a concentration of approximately 4×10^{-3} M. The activity of *P. testosteroni* appears to be markedly less sensitive than that of other *Pseudomonas* spp.: in the two strains examined, considerable activity was retained in the presence of 2×10^{-2} M threonine.

As also shown in Table 10, the homoserine dehydrogenases of the aerobic pseudomonads vary widely with respect to their pyridine nucleotide specificity. In this respect, the acidovorans group is distinctive, since the homoserine dehydrogenase of these pseudomonads shows an absolute specificity for NADH. In all the other strains examined, NADPH is more effective than

TABLE 9. *Concerted feedback inhibition of the aspartokinase of some Pseudomonas species*[a]

Additions	*P. multivorans* 104	*P. stutzeri* 221	*P. methanica*[b]
None	68.0	21.0	
L-Lysine, 10^{-2} M	26.5 (61)	4.0 (81)	
L-Threonine, 10^{-2} M	32.6 (52)	0 (100)	1.35
Both effectors, 10^{-2} M	5.7 (88)	0.6 (97)	
L-Lysine, 3×10^{-4} M	57.8 (15)	15.3 (27)	1.2 (11)
L-Threonine, 3×10^{-4} M	54.4 (20)	14.3 (32)	0.9 (33)
Both effectors, 3×10^{-4} M	26.5 (61)	5.6 (73)	0.11 (92)
L-Lysine, 2×10^{-4} M		21.0 (0)	
L-Threonine, 2×10^{-4} M		21.0 (0)	
Both effectors, 2×10^{-4} M		16.8 (20)	

[a] Expressed as nanomoles per minute per milligram. The values in parentheses represent the per cent inhibition of the activity.

[b] Owing to the low activity present in *P. methanica* extracts, the assay was done with an ammonium sulfate fraction; the activity was not measurable in the crude extracts.

TABLE 10. *Homoserine dehydrogenase activities of aerobic pseudomonads*

Organism	Specific activity[a]		Ratio of specific activities, NADPH/NADH	Inhibition by L-threonine	
	NADPH	NADH		Molarity	Inhibition
					%
Fluorescent group					
P. aeruginosa 131	74	49	1.5	0.02	80
P. aeruginosa 278	93	65	1.4	0.002	100
P. putida A 90	82	64	1.3	0.002	85
P. putida A 5	95	42	2.3	0.002	88
P. putida B 98	29	5	5.8	0.002	96
P. fluorescens A 192	91	0		0.02	94
P. fluorescens B 2	49	13	3.9	0.002	100
P. fluorescens C 18	70	0		0.02	93
P. fluorescens D 393	47	13	3.6	0.02	88
P. fluorescens E 36	19	7	2.7	0.002	100
Acidovorans group					
P. acidovorans 14	0	321	0	0.02	89
P. acidovorans 114	0	68	0	0.004	100
P. testosteroni 78	0	158	0	0.004	4
P. testosteroni				0.02	29
P. testosteroni 25	0	148	0	0.005	55
P. testosteroni				0.02	64
Other species					
P. multivorans 104	48	0		0.005	96
P. stuzeri 221	84	56	1.5	0.0002	100
P. maltophilia 70	101	31	3.3	0.002	95
P. methanica[b]	5	1.3	3.8	0.002	100

[a] Expressed as nanomoles per minute per milligram.

[b] Owing to the low activity of *P. methanica* extracts, the assay was done on an ammonium sulfate fraction; the activity was not measurable in the crude extracts.

NADH. For the most part, the ratios of activities with the two coenzymes lie in the range observed with coliform bacteria; however, two of the fluorescent strains (both belonging to *P. fluorescens*) and the single strain of *P. multivorans* contain homoserine dehydrogenases that appear absolutely specific for NADPH.

Effects of amino acids of the aspartate family on the growth of P. acidovorans. We have not systematically studied the physiological effects of amino acids on the growth of the aerobic pseudomonads. However, in order to ascertain whether the inhibitions demonstrated in cell-free extracts were physiologically significant, one series of growth experiments was performed with *P. acidovorans*. At 30 C in a mineral medium with succinate as the carbon and energy source, the mean generation time of strain 14 is 83 min. With the addition of 5×10^{-3} M DL-threonine, it falls to 180 min, and, with the further addition of 10^{-3} M L-lysine, to 265 min. Neither lysine nor methionine, added singly, affects the growth rate. The depression of the growth rate caused either by threonine alone or by a mixture of threonine and lysine can be largely reversed by the addition of 5×10^{-5} M DL-methionine: the mean generation time is 83 min in the presence of threonine, lysine, and methionine, and 116 min in the presence of threonine and methionine.

Since threonine alone does not appreciably inhibit the aspartokinase of *P. acidovorans*, its effect on growth must be ascribed to its ability to cause a pronounced inhibition of homoserine dehydrogenase, which results in methionine deficiency. This deficiency is further increased by the presence of both threonine and lysine, as a result of their concerted action on aspartokinase. The reversal of both effects by methionine supports this interpretation.

Fermentative pseudomonads (genus Aeromonas). The rod-shaped, gram-negative bacteria with polar flagella include a number of facultative anaerobes that perform either a mixed acid or a butanediol fermentation of carbohydrates. These bacteria are most commonly placed in the genus *Aeromonas*, of which the type species, *A. hydrophila*, performs a butanediol fermentation; other generic names (*Kluyvera*, *Fergusonia*) have been suggested for the aeromonads that perform a mixed acid fermentation. In addition to their polar flagellation, the aeromonads possess a second character that is absent from all coliform bacteria, but common (though not universal) in aerobic pseudomonads: they are oxidase-positive. The base content of the DNA of aeromonads lies in the range of 52 to 60 moles per cent (G + C). The taxonomic position of the aeromonads is accordingly uncertain, since they have properties that could permit assignment either to the pseudomonads or to the coliform group. The great weight traditionally assigned to the mode of flagellar insertion in bacterial taxonomy has hitherto led to their exclusion from the coliform group. The absolute and clear-cut differences between coliform bacteria and aerobic pseudomonads with respect to the regulation of aspartokinase activity revealed by our work on these two groups accordingly prompted us to extend our study to the aeromonads. Single strains of three different species were investigated. *A. hydrophila* performs a butanediol fermentation, whereas *A. shigelloides* and *A. formicans* perform a mixed acid fermentation.

Control of aspartokinase activity in aeromonads. In all three strains, the inhibition of aspartokinase activity conforms to the coliform pattern: the effects of lysine and threonine are additive (Table 11). In *A. hydrophila* and *A. shigelloides*, about 10% of the total aspartokinase activity is not inhibited by either effector, whereas in *A. formicans* this fraction is much larger (approximately 40%). The large uninhibitable fraction of the aspartokinase activity of *A. formicans* disappears completely when the organism is grown in the presence of 10^{-3} M L-methionine and can thus be attributed to a methionine-repressible aspartokinase of type II (Table 12). The lysine-inhibitable fraction of the aspartokinase activity of *A. formicans* is substantially reduced by growth in the presence of 5×10^{-3} M L-lysine, a response characteristic of the aspartokinase III of *E. coli*.

Homoserine dehydrogenase activity of aeromonads. In all three strains, the activity of homoserine dehydrogenase is partly inhibited by 10^{-2}

TABLE 11. *Isofunctional aspartokinases[a] of Aeromonas hydrophila, A. shigelloides, and A. formicans*

Additions	*A. hydrophila* 3-66	*A. shigelloides* 2-62	*A. formicans*
None	45.0	26.5	74.5
L-Lysine, 3×10^{-4} M	15.8 (65)	15.9 (40)	
L-Threonine, 3×10^{-4} M	40 (11)	24.0 (9.5)	
Both effectors, 3×10^{-4} M	9.9 (78)	14.5 (45)	
L-Lysine, 10^{-2} M	12.0 (73)	13.5 (49)	47.0 (37)
L-Threonine, 10^{-2} M	35.5 (21)	16.1 (39)	60.1 (18)
Both effectors, 10^{-2} M	4.0 (90)	3.2 (88)	32.5 (56)

[a] Expressed as nanomoles per minute per milligram. The values in parentheses represent the per cent inhibition of the activity.

to 2×10^{-2} M L-threonine (Table 13). The enzyme is active with either NADPH or NADH, the former being somewhat more effective at the standard concentration used.

DISCUSSION

The conclusion drawn by Jensen, Nasser, and Nester (15) from a comparative study of the primary regulation of the aromatic biosynthetic pathway in bacteria has been confirmed by the present work: specific mechanisms of regulation are stable group characters, shared by the members of large taxa for which a common evolutionary origin can be postulated on other independent grounds.

Specifically, we have found that primary regulation of the biosynthesis of the amino acids of the aspartate family by means of isofunctional aspartokinases subject to different end-product controls, a regulatory mechanism discovered and explored in detail in *E. coli* K-12 (12, 17, 19, 22, 24, 25) is shared by the members of all branches of the family *Enterobacteriaceae*. This control mechanism does not exist among the aerobic pseudomonads, which appear to have a single aspartokinase, subject to concerted feedback inhibition by lysine and threonine. Control of aspartokinase activity by concerted feedback inhibition, first discovered in *Rhodopseudomonas capsulata* (9) and also found in *Bacillus* spp. (20), appears to be far more widespread among procaryotic organisms than the coliform system of control through isofunctional enzymes; we have also found it in a variety of non-sulfur purple bacteria, a unicellular blue-green alga, *Desulfovibrio desulfuricans*, and *Mycobacterium rhodochrous* (*unpublished data*).

The regulation of aspartokinase activity by concerted feedback inhibition can take two somewhat different forms, both of which are exemplified among the aerobic pseudomonads. In the species of the acidovorans group, aspartokinase activity is either not inhibited, or only slightly so, by either lysine or threonine alone, even at high concentrations; the same behavior is shown by the aspartokinase of *R. capsulata* (9), in which this control mechanism was first analyzed. In the fluorescent group and in the other aerobic pseudomonads, aspartokinase activity is severely inhibited by high concentrations of either lysine of threonine, and the concerted nature of the inhibition becomes evident only when the concentrations of the two amino acids are lowered to a level at which individual inhibitory effects become negligible. In such organisms, the interaction of the two effectors can easily be overlooked, unless experiments are performed with a wide range of concentrations.

TABLE 12. *Aspartokinase activity[a] of Aeromonas formicans*

Addition to the growth medium	Total aspartokinase	Aspartokinase I	Aspartokinase II	Aspartokinase III
None	74.5	13.4 (18)	33.6 (45)	27.5 (37)
L-Lysine, 5×10^{-3} M	47.0	9.4 (20)	32.5 (69)	5.1 (11)
L-Methionine, 10^{-3} M	42.6	3.9 (9)	0	38.7 (91)

[a] Aspartokinase I is the activity inhibited by 10^{-2} M L-threonine; aspartokinase III is the activity inhibited by 10^{-2} M L-lysine. Aspartokinase II is the activity which remains in the presence of threonine and lysine at the above concentrations. The results are expressed as nanomoles per minute per milligram. The values in parentheses represent the relative amounts in per cent of each of the molecular species.

TABLE 13. *Homoserine dehydrogenase activities of aeromonads*

Organism	Specific activity with NADPH[a]	Ratio of specific activities (NADPH/NADH)	Inhibition by L-threonine	
			Molarity	Inhibition
				%
Aeromonas formicans	211	1.9	0.02	87
A. hydrophila 3-66	130	2.0	0.02	78
A. shigelloides 2-62	88	4.0	0.01	64

[a] Expressed as nanomoles per minute per milligram.

The data of Jensen et al. (15) concerning the regulation of DAHP-synthetase activity in aerobic pseudomonads revealed a biochemical dichotomy between the acidovorans and fluorescent groups. In fluorescent pseudomonads, DAHP-synthetase is inhibited by tyrosine but not by phenylalanine, whereas in *P. testosteroni* and *P. acidovorans* it is inhibited by both amino acids in less than cumulative fashion. These findings, coupled with our own, suggest that the acidovorans group may diverge considerably from other aerobic pseudomonads with respect to its regulatory mechanisms. These two groups of pseudomonads also diverge in other biochemical respects, since they possess different pathways for the dissimilation of tryptophan and aromatic acids (23).

Once the difference between two major taxonomic groups with respect to a particular regulatory mechanism has been established, it becomes possible to use this character as an independent criterion for assessing the taxonomic allocation

of organisms the affinities of which are uncertain or disputed. In the context of the coliform bacteria and the aerobic pseudomonads, the aeromonads pose such a problem, since they have some gross phenotypic characters characteristic of coliform bacteria, and others characteristic of aerobic pseudomonads. We have shown that three different *Aeromonas* spp. possess separately regulated isofunctional aspartokinases and thus fall, on the basis of this criterion, into the coliform category. One of the species examined, *A. formicans*, has been studied previously in other biochemical respects by Crawford and his collaborators, who showed that β-galactosidase and the enzymes of tryptophan biosynthesis in this strain are similar to the corresponding enzymes of *E. coli* (7, 21). Furthermore, they found that the regulation of tryptophan biosynthesis in *A. formicans* is virtually identical with that in *E. coli* (7), and different from that characteristic of *Pseudomonas putida*. With respect to this species, our findings accordingly confirm and extend the conclusion of Crawford et al. (7), namely, that the genotype of *A. formicans* is closer to that of the coliform bacteria than to that of the aerobic pseudomonads. Our observations on *A. hydrophila* and *A. shigelloides* suggest that the same conclusion may apply to other members of the genus. The positive oxidase reaction, one of the two "pseudomonas-like" characters of the aeromonads, is not a universal property of aerobic pseudomonads, being absent from *P. maltophilia* (23) and certain members of the fluorescent group, so that the primary justification for associating the genus *Aeromonas* with other pseudomonads really rests on a single character, the mode of flagellar insertion. Although this particular morphological trait has been assigned major importance in the classification of eubacteria for over half a century, a reappraisal of its significance now seems necessary. Since the aeromonads fit into the coliform group in terms both of the DNA base content and of many biochemical properties, an enlargement of this group to include polarly flagellated forms appears unavoidable.

With respect to the regulation of homoserine dehydrogenase, there appears to be little difference between coliform bacteria and aerobic pseudomonads. In both groups, homoserine dehydrogenase is inhibited by threonine; although the inhibition is as a rule much more severe in aerobic pseudomonads, the variations within each group in this respect are too great to endow it with taxonomic significance. Nor does the pyridine nucleotide specificity of this enzyme permit a differentiation between coliforms and aerobic pseudomonads. Within the aerobic pseudomonads, however, the absolute specificity of homoserine dehydrogenase for NADH that is characteristic of the acidovorans group provides yet another biochemical criterion for distinguishing the two species of this group from all other members of the genus examined.

In *E. coli* K-12 and *E. coli* B, aspartokinase I is a multifunctional enzyme, which also carries the threonine-sensitive homoserine dehydrogenase activity. Unfortunately, we could not systematically examine the possible physical association between aspartokinase and homoserine dehydrogenase in a large number of strains, since this is a property determinable only after some degree of enzyme purification. However, the problem has been subsequently studied in this laboratory for *P. testosteroni* and *P. putida* by F. Richaud and M. Gero-Robert, respectively (*personal communication*). In extracts of both these *Pseudomonas* species, the aspartokinase and homoserine dehydrogenase activities can be readily separated from one another, and are therefore associated with different proteins. Accordingly, this is yet another character of the pathway which may eventually prove to be of taxonomic significance.

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