Regulation of the Enzymes of Lysine Biosynthesis in *Bacillus sphaericus* NCTC 9602 during Vegetative Growth

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Enzymes were assayed in extracts of *Bacillus sphaericus* harvested late in the exponential phase from batch cultures in a minimal (acetate plus salts) medium. Aspartokinase was repressed and inhibited by threonine; lysine alone had no effect, though it increased the inhibition (but not the repression) by threonine. Aspartic β -semialdehyde dehydrogenase was slightly repressed by lysine. Dihydrodipicolinate synthase was inhibited non-competitively by lysine, and dihydrodipicolinate reductase was partly repressed by lysine. Diaminopimelate dehydrogenase (with tetrahydrodipicolinate as substrate) was inhibited by *meso*-diaminopimelate. Lysine did not repress diaminopimelate decarboxylase, and only slightly inhibited this enzyme. An auxotrophic mutant that required threonine and methionine excreted lysine after growth had stopped with a limited concentration of threonine.

INTRODUCTION

Bacillus sphaericus uses a distinctive pathway for lysine synthesis (Fig. 1). Neither succinylated nor acetylated intermediates occur after tetrahydrodipicolinate, and diaminopimelate epimerase is absent, so that diaminopimelate dehydrogenase plays an essential role (White, 1983). Regulation of this variant pathway has not previously been studied, although much is known from other organisms about control of the earlier steps (aspartate to tetrahydrodipicolinate) which are common to all bacteria that make lysine (Umbarger, 1978).

METHODS

Organisms. *Bacillus sphaericus* NCTC 9602 was maintained on slopes of nutrient agar, incubated overnight at 30 °C and stored at 2 °C. Auxotrophic mutants were derived from this strain by mutation with nitrosoguanidine and penicillin-selection (White, 1983), and were similarly maintained.

Medium. The chemically defined medium A3 (White & Lotay, 1980) was generally used for growth of liquid cultures. For some experiments, supplements of amino acids were added (as described later). The concentration of sodium acetate. 3H₂O was raised from 0.5% (w/v) to 1% (w/v) in some experiments on the excretion of lysine by mutant F28.

Conditions for growth. Cultures were grown on a mechanical shaker (200 r.p.m.) at 30 °C in liquid medium (500 ml per 2-l conical flask) and harvested in the late exponential stage of growth (before sporulation).

Preparation of extracts. This was done as described by Bartlett & White (1985). Protein was assayed by the Lowry method using crystalline bovine serum albumin as the standard.

Enzyme assays. All enzymic activities were measured at 30 °C. Every quoted activity is the mean of two or more measurements, none of which differed from the mean by more than about 10%. In cases where very scattered results were at first obtained (aspartokinase, aspartic β -semialdehyde dehydrogenase) the assays were modified (see below) and determinations were repeated until consistent values were obtained. Aspartokinase (EC 2.7.2.4) was assayed by the method of Kisumi *et al.* (1977), modified by adding NH₄Cl (final concentration 0.4 M) to the

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Fig. 1. The pathway of lysine biosynthesis in *B. sphaericus*. The enzymes are: 1, aspartokinase; 2, aspartic β -semialdehyde dehydrogenase; 3, dihydrodipicolinate synthase; 4, dihydrodipicolinate reductase; 5, diaminopimelate dehydrogenase; 6, diaminopimelate decarboxylase; 7, homoserine dehydrogenase; 8, dihydrodipicolinate dehydrogenase. Threonine, isoleucine and methionine are made by separate routes from homoserine, in which several other enzymes participate.

solution incubated during the phosphorylation reaction. As was found by Paulus & Gray (1964) with *Bacillus polymyxa*, a high concentration of ammonium ions was needed to measure consistent activities of aspartokinase. Aspartic β -semialdehyde dehydrogenase (EC 1.2.1.11) was assayed in the reverse of the biosynthetic direction (with aspartic β -semialdehyde as substrate), as described by Hegeman *et al.* (1970) but with the concentration of buffer doubled in the assay system. Dihydrodipicolinate synthase (EC 4.2.1.52; 'aspartic β -semialdehyde:pyruvate condensing enzyme') was assayed by the method of Yamakura *et al.* (1974). Dihydrodipicolinate reductase (EC 1.3.1.26) was assayed by the method of Kimura (1975) with substrate prepared by chemical reaction as described by White (1983). *meso*-Diaminopimelate D-dehydrogenase (EC 1.4.1.16) was assayed in the direction of synthesis of diaminopimelate at pH 7.5 (Misono & Soda, 1980) or in the reverse direction at pH 10.5 as described by Bartlett & White (1985). Diaminopimelate decarboxylase (EC 4.1.1.20) was assayed manometrically (White, 1971). Homoserine dehydrogenase (EC 1.1.1.3) was measured in both directions of reaction by the methods of Black & Wright (1955).

Chemicals. The preparations of aspartic β -semialdehyde, dihydrodipicolinate and L-2-amino-6-ketopimelate (tetrahydrodipicolinate) have been described before (White, 1983). *meso*-Diaminopimelic acid was isolated by fractional crystallization from synthetic material (Work, 1963); LL- and DD- isomers were obtained as described by Saleh & White (1976).

Identification of lysine. Mutant F28 (*met thr*) was grown in medium A3 plus L-methionine and L-threonine (both 40 mg l⁻¹). After incubation at 30 °C for 3 d the organisms were centrifuged and the supernatant liquid (containing about 100 mg lysine l⁻¹) was adjusted to pH 2 with HCl. This liquid (3 l) was passed through a column of Dowex 50 (H⁺ form; 3 × 13 cm) at a flow rate of about 3 ml min⁻¹. The column was washed with water, then eluted with 2.5 M-HCl while fractions (10 ml) were collected. Fractions that were positive in the assay (Gilvarg, 1958) for lysine were pooled and evaporated to dryness. The solid (0.4 g) was 70% (w/w) lysine by assay with ninhydrin (Work, 1957). The rather complex spectrum of the coloured product (over the range 340–750 nm) in this assay was identical to that given from authentic lysine. A single ninhydrin-positive spot in the same position and

with the same distinctive colour (dull brown) as a lysine marker was found after paper chromatography of an aqueous solution (1 mg ml^{-1}) of the solid with the solvent methanol/pyridine/ 11.6 M-HCl /water (80:10:2.5:17.5, by vol.; Rhuland *et al.*, 1955). Finally, the solid, dissolved to a concentration of 60 mg l^{-1} in medium A3, supported growth of the lysine-requiring mutant B3 (which lacks diaminopimelate decarboxylase; White, 1983) to a final density of $0.54 \text{ mg dry wt bacteria ml}^{-1}$ (assessed from turbidity) while authentic L-lysine (40 mg l^{-1}) yielded $0.56 \text{ mg dry wt bacterial ml}^{-1}$. Hence, the solid was about 65% (w/w) lysine. Mutant B3 responds to L- or to D-lysine, and so the configuration of the excreted lysine is uncertain.

RESULTS

Activities of the enzymes of lysine synthesis during exponential growth in batch culture

Bacillus sphaericus 9602 has a doubling time of about 2 h at 30°C in medium A3. If the organisms are assumed to contain 50% (w/w) protein (1/20 of this being lysine) and 10% (w/w) peptidoglycan (1/5 of this being lysine), then 1 g dry wt of bacteria will contain roughly 50 mg of lysine residues (0.4 mmol). Hence, the rate of lysine formation needs to be only $4\text{--}5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ to be compatible with the observed rate of growth. Measured enzymic activities in extracts were all much higher than this (Table 1), although conditions within the bacteria are unlikely to be optimal for every enzyme of the pathway to lysine. Aspartokinase and aspartic β -semialdehyde dehydrogenase must also supply homoserine, the precursor of methionine, threonine and isoleucine, and so the activities of these two enzymes need to be at least about $15 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

Repression and inhibition of enzymes of lysine synthesis

Aspartokinase. Growth in the presence of L-threonine repressed this enzyme (Table 2), whereas L-lysine on its own had no effect. Threonine at concentrations above 20 mg l^{-1} also delayed growth of *B. sphaericus* in medium A3; Lamb & Bott (1978) found inhibition of growth of *B. subtilis* by threonine, and White (1972) reported inhibition of *B. megaterium* by this amino acid. Threonine (50 mg l^{-1}) did not inhibit *B. sphaericus* 9602 when L-lysine and L-methionine (each 50 mg l^{-1}) were also present in medium A3, which suggests that inhibition of growth by threonine might result from restriction of the supply of lysine and methionine by repression and inhibition of aspartokinase (see below). Diaminopimelate was not tested in the growth medium because *B. sphaericus* cannot take up this amino acid (White, 1983).

L-Threonine (in the assay mixture) inhibited aspartokinase, and lysine with threonine caused a total loss of enzymic activity, even though lysine alone did not inhibit (Table 2). The aspartokinase made at a repressed level by organisms grown with threonine was still sensitive to threonine in the assay system.

Aspartic β -semialdehyde dehydrogenase. Because aspartic β -semialdehyde is easier to prepare than β -aspartyl phosphate, this enzyme is usually assayed in the reverse of the biosynthetic direction. Consequently, any apparent regulatory effects must be viewed cautiously.

Repeated measurements of the activity of a single extract in the assay system of Hegeman *et al.* (1970) were erratic; much more consistent results were found when the concentration of buffer was doubled in the assay mixture. All the reported activities (Tables 1 and 3) were determined in the modified system. Lysine slightly repressed the enzyme, but no inhibition was seen with lysine, methionine or threonine.

Dihydrodipicolinate synthase. Addition of lysine (50 mg l^{-1}) to medium A3 caused no repression. However, lysine inhibited when present in the assay mixture, whether or not the organisms had been grown with this amino acid (Fig. 2). The inhibition was non-competitive, and the K_i , determined from plots by the method of Dixon (1953), was 0.6 M-L-lysine (SD 0.1 with three estimates). Individual apparent K_m values for pyruvate (measured with $3.75 \text{ mM-aspartic } \beta\text{-semialdehyde}$ always present) and for aspartic β -semialdehyde (measured with 3.75 mM-pyruvate always present) were 9.0 mM (SD 4.3 with three estimates) and 5.1 mM (SD 1.4 with three estimates) respectively. These values were determined by Lineweaver-Burk plots, and are of the same order as those for this enzyme from *Bacillus licheniformis* (Halling & Stahly, 1976) and from

Table 1. *Enzymic activities in extracts of B. sphaericus 9602*

Enzymes were assayed (as described in Methods) in extracts of organisms grown in medium A3, and harvested during the late exponential phase.

Enzyme	Activity (nmol min ⁻¹ [mg protein] ⁻¹)
Aspartokinase	54 (14)
Aspartic β -semialdehyde dehydrogenase	43 (38)
Dihydrodipicolinate synthase	62* (20)*
Dihydrodipicolinate reductase	40 (29)
Diaminopimelate dehydrogenase	
pH 7.5 (ketoaminopimelate + NH ₃ + NADPH as substrates)	247 (474)
pH 10.5 (diaminopimelate + NADP as substrates)	452 (574)
Diaminopimelate decarboxylase	50 (77)

* Units (mg protein)⁻¹; a unit represents about 0.50 nmol min⁻¹. Values in parentheses are for organisms harvested during the stationary phase, but before sporulation (from White, 1983).

Table 2. *Activities of aspartokinase in B. sphaericus 9602 and in mutant F28*

Organisms were grown in medium A3 with various additions, and harvested in late exponential growth. Aspartokinase was measured in extracts, with various additions to the assay system.

Addition† to the assay system (1 mM unless stated otherwise)	Enzymic activities* after growth with stated additions† (50 mg l ⁻¹ unless shown otherwise)						
	Wild-type					Mutant F28	
	Control	Lys	Thr	Lys + Thr	Lys + Thr + Met	Thr (20 mg l ⁻¹) + Met (20 mg l ⁻¹) + Ile (20 mg l ⁻¹)	Thr (100 mg l ⁻¹) + Met (20 mg l ⁻¹) + Ile (20 mg l ⁻¹)
None	100	100	33	68	60	200	50
Thr	42	—	2	—	29	50	14
Lys	110	—	—	—	—	200	50
Thr + Lys	0	—	0	—	0	35	10
Thr + Met	64	—	—	—	—	52	14
Lys + Met	85	—	—	—	—	190	64
Met	—	110	—	—	—	150	50
Thr + Lys + Met	0	—	—	—	—	35	2
meso-Diaminopimelate	95	—	—	—	53	—	—
Thr + diaminopimelate (2 mM)	61	—	—	—	24	—	—

* Percentages of the specific activity [54 nmol min⁻¹(mg protein)⁻¹] of the enzyme from wild-type organisms grown and assayed with no additions. —, Not tested.

† All amino acids except diaminopimelate were L- isomers.

Table 3. *Activity of aspartic β -semialdehyde dehydrogenase in extracts of B. sphaericus 9602*

Organisms were grown and harvested as in Table 2. Additions to medium A3 were at 50 mg l⁻¹, and additions to the assay system were at 1 mM.

Addition to the assay system	Enzymic activities* after growth with stated additions		
	Control	Lys	Lys + Thr + Met
None	43	30	40
Lys	40	—	—
Thr	48	—	—
Met	43	—	—
Lys + Thr + Met	40	—	—

* nmol min⁻¹ (mg protein)⁻¹; —, not tested.

Fig. 2. Effect of lysine on dihydrodipicolinate synthase of *B. sphaericus*. ●, Organisms grown in medium A3; ○, organisms grown in medium A3 plus 0.3 mM-lysine.

Table 4. Activity of dihydrodipicolinate reductase in extracts of *B. sphaericus* 9602

Organisms were grown and harvested as in Table 2. Extracts were assayed at pH 6.1 except where shown otherwise. Additions to the assay system were at 1 mM, except where stated, and additions to the medium were at 50 mg l⁻¹.

Addition to the assay system	Enzymic activities* after growth with stated additions		
	Control	Lys	Lys + Thr + Met
None	41, 49†	30, 31†	33
Lys	41	—	—
Thr	41	—	—
Met	41	—	—
Dipicolinate	19	15, 27†	—
<i>o</i> -Phenanthroline (0.4 mM)	35	—	—

* nmol min⁻¹ (mg protein)⁻¹; —, not tested.

† Assayed at pH 7.2.

Bacillus subtilis (Yamakura *et al.*, 1974) but are more than ten times higher than those reported for *Bacillus megaterium* (Webster & Lechowich, 1970) or *Escherichia coli* (Yugari & Gilvarg, 1965).

Dihydrodipicolinate reductase. Lysine partly repressed the reductase (Table 4), but there was no inhibition by lysine, methionine or threonine. The effects of dipicolinic acid and *o*-phenanthroline were tested because these compounds are inhibitors in some species of *Bacillus* (Kimura *et al.*, 1978). Two types of enzyme have been distinguished; one (*B. subtilis* type) was inhibited completely by *o*-phenanthroline and partially by dipicolinate, and had a pH optimum of about 6, whereas the other (*B. megaterium* type) was sensitive to dipicolinate only, and had a pH optimum above 7. In *B. sphaericus*, the activity of dihydrodipicolinate reductase was slightly higher at pH 7.2 than at pH 6.1 (the pH value used for tests of the inhibitors), and dipicolinate was a stronger inhibitor than *o*-phenanthroline.

Diaminopimelate dehydrogenase. *meso*-Diaminopimelate is easier to obtain than is ketoamino-pimelate (tetrahydrodipicolinate), and so this enzyme was first assayed in the direction of oxidation of diaminopimelate (at pH 10.5). There was no repression or inhibition by lysine (Table 5). Assays in the direction of reductive amination (at pH 7.5) showed that *meso*-diaminopimelate inhibited, while the LL- or DD- isomers had less effect. Competitive inhibition

Table 5. *Activity of diaminopimelate dehydrogenase in extracts of B. sphaericus 9602 and mutant B3*

Organisms were grown and harvested as in Table 2. Additions to the assay system were at 1 mM, except where stated, and additions to the medium were at 50 mg l⁻¹.

(a) *Oxidative deamination of meso-diaminopimelate, pH 10.5*

Addition to the assay system	Enzymic activities* after growth with stated additions	
	Control	Lys
None	450	530
Lys	520	—
Met	630	—
Thr	550	—
Lys + Thr + Met	590	450

(b) *Reductive amination of 2-amino-6-ketopimelate, pH 7.5*

Addition to the assay system (all 1.67 mM)	Enzymic activities* after growth with stated additions			
	Control	Lys	Thr	Lys + Thr + Met
None	250	190, 3600†	240	190
<i>meso</i> -Diaminopimelate	30	—, 400†	—	—
LL-Diaminopimelate	—	—, 1500†	—	—
DD-Diaminopimelate	—	—, 800†	—	—

* nmol min⁻¹ (mg protein)⁻¹; —, not tested.

† Purified enzyme (as in Chatterjee & White, 1982) from mutant B3 (grown with lysine).

by LL- or DD-diaminopimelate has been found (Misono & Soda, 1980) when the *meso*- isomer was substrate in the oxidative reaction catalysed by this enzyme. In *B. sphaericus*, diaminopimelate epimerase is absent (Bartlett & White, 1985), so that the LL- and *meso*- isomers are not interconvertible. However, at pH 7.5 *meso*-diaminopimelate might be quickly broken down by diaminopimelate decarboxylase. Hence, the inhibition of the dehydrogenase by *meso*-diaminopimelate was also examined in the mutant B3, which lacks diaminopimelate decarboxylase (White, 1983). The dehydrogenase of mutant and wild-type showed similar sensitivity to *meso*-diaminopimelate (Table 5).

Diaminopimelate decarboxylase. In many bacteria, lysine represses this enzyme (White *et al.*, 1964). However, with *B. sphaericus* 9602, Grandgenett & Stahly (1971) found no repression by lysine, and very little inhibition (about 10%) when lysine (10 mM) was added to the assay system. We confirmed these results, and found a slightly increased inhibition (30%) when methionine and threonine were both present with lysine.

Excretion of lysine by an auxotroph of B. sphaericus

The phenotype of mutant F28, which requires methionine and threonine (but not lysine) for growth in medium A3, is indicative of the loss of homoserine dehydrogenase (see Fig. 1). Nevertheless, this enzyme was present (assayed in either direction of reaction) in crude extracts of the mutant. Possibly there are two blocks (both after homoserine) in the auxotroph, since production of multiple mutations by nitrosoguanidine is well known. One block should be in threonine synthesis and one in methionine synthesis; these presumed blocks have not been identified.

Organisms were grown in medium A3 (with amino acids, see below) and cultures were sampled at intervals. The bacteria were centrifuged, and the supernatant fluid was assayed for lysine (Work, 1957). Ornithine will react in this procedure, but the coloured product absorbs more strongly at 515 nm than at 440 nm, while the opposite is true of lysine. Every test was

therefore read at both of these wavelengths; the absorbance was always higher at 440 nm. In addition, lysine was isolated and characterized from a culture filtrate (see Methods).

The concentration of threonine needed by the mutant was decreased on adding isoleucine, of which threonine is a precursor. When mutant F28 was given a limiting supply of L-threonine (20 mg l⁻¹), with L-methionine and L-isoleucine (both 20 mg l⁻¹), about 200 mg lysine l⁻¹ was produced. Increasing the concentration of threonine to 60 mg l⁻¹ diminished the yield of lysine to 30 mg l⁻¹.

Increasing the concentration of sodium acetate.3H₂O in the medium to 1% (w/v) led to greater excretion of lysine (about 300 mg l⁻¹) with limiting threonine. L-Aspartate (100–500 mg l⁻¹) had little effect on lysine formation, and growth with the maximum tolerated concentration of benzylpenicillin (1 µg l⁻¹) was also ineffective. The rate of formation of lysine has not been accurately measured; the amino acid accumulated over a period of about a day, after turbidity had reached its maximum but before many spores had appeared in the culture. In no case was any lysine detectable in the culture filtrate before growth had caused obvious turbidity.

Since the regulatory effect of threonine (in the wild-type) occurs on only aspartokinase, this enzyme was examined in extracts of the mutant (Table 2). Threonine caused marked repression; the enzyme that was formed at the repressed level was still inhibited by threonine. When the mutant was grown with a minimal concentration of threonine, aspartokinase had a specific activity double that of the wild-type (grown without threonine, with or without methionine plus isoleucine). The pattern of effects of inhibitors on the derepressed enzyme of the mutant was generally similar to that seen with the enzyme from the wild-type.

DISCUSSION

The means of regulation of lysine synthesis by *B. sphaericus* generally resemble those found in other bacteria. Thus, aspartokinase and dihydrodipicolinate synthase are major targets for feed-back inhibition, and threonine, lysine and diaminopimelate are the principal effectors.

Without evidence from enzyme purification or genetics, it is impossible to say whether there is more than one type of aspartokinase in these organisms. The observed pattern of repression and inhibition of aspartokinase can be explained by the presence of a single enzyme, partly repressed by threonine, and subject to moderate inhibition by threonine alone, and to greater inhibition when lysine is present with threonine. No other species examined in the genus *Bacillus* shows precisely the same pattern: *B. stearothermophilus* (Kuramitsu & Yoshimura, 1971), *B. subtilis* (Rosner & Paulus, 1971) and *B. cereus* (Hoganson *et al.*, 1978) all have two aspartokinases, one of which is inhibited by meso-diaminopimelate; *B. brevis* (Hitchcock *et al.*, 1980) has two enzymes, one repressed and inhibited by lysine, the other repressed by threonine and inhibited by threonine plus lysine; *B. licheniformis* (Gray & Bernlohr, 1969), *B. polymyxa* (Biswas & Paulus, 1973) and *B. colistinus* (Ito *et al.*, 1969) all have one aspartokinase, sometimes repressed by lysine (but not by threonine) and inhibited by lysine or lysine plus threonine.

Inhibition by lysine of dihydrodipicolinate synthase (the first enzyme of the lysine-branch) has been found in many bacteria, though numerous cases of an insensitive enzyme have also been reported. If an organism needs diaminopimelate for incorporation into its peptidoglycan, then the inhibition by lysine must not be complete so that wall synthesis may go on in a medium that contains lysine. *B. sphaericus* makes a vegetative peptidoglycan without diaminopimelate (Hungerer & Tipper, 1969), but the peptidoglycan of its spore includes diaminopimelate

Lysine does seem unable alone to shut off its own synthesis completely, because a mutant that lacks threonine will excrete lysine. However, the excretion takes place after growth has stopped, and enzymic activities have not been measured at this stage. Possibly the inhibition of dihydrodipicolinate synthase by lysine might become less, or more of the enzyme might be formed, as a preparation for sporulation, when dipicolinate and diaminopimelate are needed even when lysine may be present.

There may be an advantage to the organism in being able to decrease the activity of diaminopimelate dehydrogenase, because ammonia and NADPH will be conserved, even

though carbon may flow through the pathway up to tetrahydrodipicolinate, and perhaps may accumulate as this compound. Whether *meso*-diaminopimelate (substrate at pH 10.5 in the oxidative deamination reaction) and tetrahydrodipicolinate (substrate at pH 7.5 in the reductive amination reaction) compete at the same binding site on the enzyme is unknown. Certainly each compound can be bound, and the presence of one may affect the reaction of the other.

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