Lysine Production by *Brevibacterium linens* and Its Mutants: Activities and Regulation of Enzymes of the Lysine Biosynthetic Pathway

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ABSTRACT. Activity and regulation of key enzymes of the lysine biosynthetic pathway were investigated in *Brevibacterium linens*, a natural excretor of lysine, its lysine-overproducing homoserine auxotroph (Hom-1) and its auxotrophic and multi-analogue-resistant high-yielding mutant (AEC NV 20r50). The activity of aspartate kinase (AK) and aspartaldehydate dehydrogenase (AD) was maximum during the mid-exponential phase of growth and decreased thereafter. The mutants showed 10 and 20 % more activity of AK and AD than the wild-type lysine excretor. *B. linens* (natural excretor) has a single AK and AD repressed and inhibited bivalently by lysine and threonine. Lysine slightly repressed and inhibited dihydrodipicolinate synthase (DS) and diaminopimelate decarboxylase (DD) of the wild type and of the mutant Hom-1. The mutant AEC NV 20r50 showed DS and DD to be insensitive to lysine inhibition and repression. Persistence of a major part of the maximal activity of these enzymes during the late stationary phase of growth allowed prolonged synthesis and excretion of lysine. Stepwise addition of resistance to the different analogues of lysine in the mutant AEC NV20r50 resulted in an increase of enzyme activity and reduced repressibilities of enzymes that contributed to the high yield of lysine.

In *Brevibacterium linens* strain 34C, lysine is synthesized via the diaminopimelate pathway. The variations of regulation of the enzymes of this pathway in different genera of bacteria have been reviewed by Umbarger (1978). The intracellular regulatory system in microbes is so precise that hyper-production of a particular metabolite is controlled by an automatic cut-off of the biosynthetic pathway. In rare instances, however, due to a disorder in the enzyme-forming system, organisms may escape from this stringent regulation and produce a metabolite in excess of what it needs. Strain 34C is such a natural excretor of lysine, isolated from Indian soil during a survey. Homoserine auxotroph (Hom-1) and auxotrophic plus lysine-analogue-resistant mutants (AEC NV 20r50) isolated from their wild-type strain that yields lysine up to 50 g/L of mineral salt medium.

Excretion of lysine in good amounts by *B. linens* strain 34C and its auxotrophic and regulatory mutants calls for an explanation of the reason for such overproduction. To explain the reason for lysine hyperproduction, every enzyme of the lysine biosynthetic pathway has been assayed in extracts of *B. linens* strain 34C, its homoserine auxotroph and auxotrophic plus lysine-analogue-resistant high-yielding mutant. Regulatory effects of lysine, methionine and threonine on these enzymes have been examined and are reported in this paper.

MATERIALS AND METHODS

Organisms. *Brevibacterium linens* strain 34C was isolated as a biotin-requiring lysine excretor by its ability to cross-feed a lysine auxotroph of *Escherichia coli* K12 (Chatterjee et al. 1978). The homoserine auxotroph (Hom-1) was isolated from *B. linens* 34C by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The auxotrophic plus multianalogue-resistant high-yielding mutant AEC NV20r50 was isolated by stepwise mutagenesis with MNNG, followed by screening with S-2-(aminoethyl)-L-cysteine (Ace) and norvaline (Nva). The wild-type excretor and the mutant strains were maintained on agar slopes of Medium A1 (White 1972) with required supplements.

Medium and growth of cultures. The minimal medium A1 (White 1972) with biotin (1 μg/L), homoserine or methionine plus threonine (0.5 mmol/L) was used. For enzyme assay the wild-type and the mutant strains were grown in a laboratory fermentor (BioFLO C-32, New Brunswick Scientific Co., USA) using the A1 medium supplemented with biotin and specific amino acids as required by the strains. Temperature was maintained at 30 °C and dissolved O2 in excess of 50 % saturation; the pH value was kept at 7.0 by addition of 2 mol/L NaOH. Growth was measured by measuring absorbance in an EEL (*Evans Electroelenium*, Halstead, UK) photoelectric colorimeter.
**Enzyme assay.** Bacterial cells at the late exponential phase of growth (30 h) were harvested by centrifugation (10,000 g, 30 min). The bacteria were then washed twice with 0.1 mol/L phosphate buffer (pH 7.2). The washed bacteria were resuspended in buffer appropriate for the enzyme assays in which the extract was subsequently used. Bacteria were disrupted by sonication (Soniprep 150, MSE, Sussex, England) for 20 s at 0 °C and the debris was removed by centrifugation (18,000 g, 30 min). Protein in the extract was measured by the Lowry method, using bovine serum albumin as standard. Extracts were dialyzed at 2 °C against buffer that was used for the particular enzyme assay.

Aspartate kinase (EC 2.7.2.4) was assayed in a triethanolamine buffer (pH 8.0) using the method of Truffa-Bachi and Cohen (1970). Asparaginyl-enzyme dehydrogenase (EC 1.2.1.11) was assayed by recording the reduction of NADP+ in the reaction mixture similar to that of Hegeman et al. (1970). Dihydrodipicolinate synthase (EC 4.2.1.52) was assayed by measuring at 540 nm the purple adduct formed by dihydrodipicolinate with 2-aminobenzaldehyde in reaction mixture of Shedlarsky (1971). One unit of enzyme is defined as the amount that catalyzes an increase of absorbance of 0.001/min, when a steady state was reached. Dihydrodipicolinate reductase (EC 1.3.1.26) was assayed following anaerobic dehydrogenation of NADPH using the method of Farkas and Gilvarg (1965). The dihydrodipicolinate synthase required to generate dihydrodipicolinate was purified from *E. coli* K12 (Shedlarsky 1971). Tetrahydrodipicolinate N-acetyltransferase (EC 2.3.1.89) was assayed using the method of Chatterjee and White (1982). 2-Acetamido-6-oxopimelate aminotransferase was assayed in the reverse of the biosynthetic direction by measuring the decrease of L-2,6-diaminopimelic acid following the method of Sundhardas and Gilvarg (1967). N-Acetyl-L-2,6-diaminopimelate deacetylase (EC 3.5.1.47) was assayed using the method of Salch and White (1979), L-N-acetyl-2,6-diaminopimelate required for this assay was synthesized from L-2,6-diaminopimelate by the method of Ward (1975). Diaminopimelate epimerase (EC 5.1.1.7) was assayed manometrically by the method of White et al. (1971). Diaminopimelate decarboxylase (EC 4.1.1.20) was assayed colorimetrically using the method of White (1971). Diaminopimelate dehydrogenase (EC 1.4.1.16) was assayed by recording the reduction of NADP+ following the method of Misono et al. (1979), using a glycine–KCl–KOH buffer (pH 10.5). Homoserine dehydrogenase (EC 1.1.1.3) was assayed following the L-4-aspartaldehydate-dependent dehydrogenation of NADH (Black and Wright 1955b).

**Chemicals.** DL-4-Aspartaldehyde was synthesized by ozonolysis of DL-allylglycine (Black and Wright 1955). The molarity of the compound was standardized enzymically using homoserine dehydrogenase purified from yeast (Ward 1975). Acetyl-CoA was prepared from CoASH (Stadman 1957). Succinyl-CoA was prepared following the method of Salch and White (1976). L-2,3,4,5-Tetrahydrodipicolinate was prepared from meso-2,6-diaminopimelic acid using diaminopimelate dehydrogenase (Misono et al. 1979) purified from Bacillus sphaericus strain NCTC 9602, following the conditions described by Chatterjee and White (1982).

**RESULTS AND DISCUSSION**

*Activities of aspartate kinase and asparaginyl-enzyme dehydrogenase during different phases of growth*

To select the time for harvesting bacteria for enzyme assays, aspartate kinase (AK) and asparaginyl-enzyme dehydrogenase (AD), the two enzymes common for the aspartate family of amino acids, were assayed in bacteria harvested at intervals during growth of the wild type *B. linens* 34C.

Activities of AK and AD, however, reach a maximum after 30 h, during the mid-exponential phase of growth. Activities of these enzymes remain more or less at the same level up to 48 h and declined afterwards. The organism retains more or less 60% of the maximum activities of enzymes till the late stationary phase (66–72 h).

*Activities of enzymes of the lysine pathway and effect of lysine*

To see whether lysine has any effect on the enzymes pertaining to lysine biosynthesis, all the enzymes of the lysine pathway were assayed in *B. linens* 34C, its auxotrophic mutants (Hom−1, requiring homoserine or methionine plus threonine) and auxotrophic and multianalogue-resistant high-yielding mutant (AEC NV 20′50), grown in a medium without and with L-lysine (5 mmol/L).

Assay of the enzymes of the lysine biosynthetic pathway revealed (Table I) that mutant Hom−1 showed about 10% more activity of almost all the enzymes than the wild type. The high-yielding mutant AEC NV 20′50 has 20% more activity of almost all the enzymes than the wild type.