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Lysine Biosynthesis in *Streptomyces lipmanii*: Implications in Antibiotic Biosynthesis

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Streptomyces lipmanii produces two β-lactam antibiotics, penicillin N and 7-(5-amino-5-carboxyvaleramido)-7-methoxycephalosporanic acid. Both antibiotics contain α-aminoadipic acid side chains. In similar antibiotics produced by certain fungi, the α-aminoadipoyl moiety is derived from an intermediate in lysine biosynthesis. Our findings indicate, however, that in *S. lipmanii*, lysine is synthesized via α, ε-diaminopimelic acid—an entirely different biosynthetic route. This finding suggests not only a unique mechanism for the derivation of α-aminoadipate, but also that the system may be particularly amenable to genetic manipulation.

The β-lactam-ring-containing antibiotics are a class of antibiotics of major commercial importance. The best known members of the group—penicillins and cephalosporins—are derived from taxonomically unrelated strains of true fungi (6, 9). It has recently been noted, however, that β-lactam antibiotics are also produced by several members of the genus *Streptomyces* (1, 18; I. M. Miller, E. O. Stapley, and L. Choilt, Bacteriol. Proc., p. 32, 1962).

Numerous studies have indicated that the basic structure of certain β-lactam antibiotics is a tripeptide consisting of α-aminoadipic acid (αAAA), cysteine, and valine (2, 22). Structure and labeling studies of the cephalosporins from *Streptomyces griseus* (1) and *Streptomyces clavuligerus* (24) have been consistent with that hypothesis.

The subject of this report is a portion of the current work with *Streptomyces lipmanii*, producer of 7-(5-amino-5-carboxyvaleramido)-7-methoxycephalosporanic acid (factor A) and penicillin N (factor B) (18). Before beginning work on antibiotic biosynthesis, it was necessary to establish a groundwork in related primary metabolism.

Lysine biosynthesis may occur by at least two entirely distinct pathways. In higher fungi, including *Penicillium* and *Cephalosporium*, lysine is synthesized via the homocitrate pathway in which αAAA is an intermediate. This provides the source of the antibiotic precursor (11, 16). The second pathway proceeds through diaminopimelic acid (DAP) and is related to synthesis of the branched-chain amino acids. The pathway from aspartic acid to DAP has been studied extensively in *Escherichia coli* by Gilvarg and his associates (8, 15, 19, 29). This pathway normally occurs in bacteria, a few groups of lower fungi, and higher plants (23). The phylogenetic position of the *Streptomyces* and the reports of DAP in streptomycete cell walls (4, 14) suggest that the DAP pathway would be operative in this genus.

It soon became apparent that lysine was indeed synthesized via the DAP pathway in *S. lipmanii*. This finding indicates a unique mechanism for the derivation of αAAA necessary for antibiotic synthesis in this organism.

In the following discussion, evidence will be presented which establishes the existence of the diaminopimelate pathway for lysine biosynthesis and provides the foundation for further studies on antibiotic biosynthesis.

MATERIALS AND METHODS

Organism. *S. lipmanii* (L100) and a lysine auxotroph derived from it (LA 423) were used throughout this study. The prototrophic wild type and mutation conditions employed for obtaining LA 423 have been described in an earlier paper (10).

Culture conditions. Complex vegetative and defined fermentation media were used. Compositions of the media per liter of distilled deionized water were as follows: (ii) vegetative medium—tryptone (5 g), glucose (5 g), yeast extract (5 g), dextrin (10 g), $MgSO_4 \cdot 7H_2O$ (2 g), and glycerol (1 ml); (ii) fermentation medium—NaCl (1 g), $MgCl_2 \cdot 6H_2O$ (1 g), K_2HPO_4 (1 g), $CaCO_3$ (4 g), trace salts (10 ml), maltose (20 g), glucose (5 g), glycerol (1 ml), DL-methionine (1 g), L-tryptophan (1 g), L-valine (1 g), and L-lysine (1 g). Trace salt stock solution consisted of: $FeSO_4 \cdot 7H_2O$ (0.01 g), $MnCl_2 \cdot 4H_2O$ (0.01 g),

$ZnSO_4 \cdot 7H_2O$ (0.01 g), and distilled deionized water (100 ml). Salts, carbohydrates, and amino acids were autoclaved separately and assembled aseptically after sterilization. Variations in lysine concentration shall be specified.

Two milliliters of an overnight culture in the vegetative medium was diluted 1:1 in a solution of 20% glycerol and 10% lactose for storage in liquid nitrogen ampoules (13 by 100-mm screw-cap tubes). Immediately before use, an ampoule was quick-thawed at 43 C and inoculated into fresh vegetative medium (1% inoculum). Log-phase cells (16-h cultures) were washed once before inoculation into the fermentation medium.

Cultures were incubated at 26 C on a rotary shaker with a 5-cm stroke at 250 rpm.

Radioisotope quantitation. Chromatograms were scanned for radioactive peaks in a Packard model 7201 radiochromatogram scanner. The instrument has an efficiency of 24%.

Liquid counting was done in a Packard Tri-Carb liquid scintillation spectrometer model 3375 for 10 min by using the channels ratio method. Counting efficiency was 70%.

Separation of meso-DAP from a racemic mixture. The method of Work (28) was used to separate the meso-isomer from a mixture of the three isomers.

Isolation of [^{14}C]meso-DAP. *E. coli* ATCC 12408, a lysine auxotroph which excretes DAP, was grown in 1-liter lots under conditions similar to those described by Work and Denman (25). Minimal broth Davis (Difco) was prepared as directed except that the glucose concentration was increased to 0.5% and lysine was added to a final concentration of 10 μg/ml. Uniformly labeled glucose (15.91 mg of specific activity, 3.45 mCi/mmol) was added at 16 h to 1 liter of broth culture. The culture fluid was collected at 44 h by centrifugation. The pH of the broth was adjusted to 3.0 with concentrated HCl. The broth was heated to boiling and filtered through a Hyflo Super Cel pad. The filtrate was concentrated 10-fold in vacuo and passed through a carbon column (carbon 12 $\times$ 40; 3 by 30 cm) and eluted with 250 ml of water. The eluate was acidified to pH 0.6 with concentrated HCl and placed on a Dowex 50WX-8H$^+$ (50 to 100 mesh) column (3.5 by 40 cm). Six liters of 1.5 N HCl were used to elute the material. Fractions (500 ml) were collected and checked by paper chromatography (Rhuland solvent: methanol, water, 10 N HCl, and pyridine, 80:17.5:2.5:10, vol/vol/vol/vol) (20) for the presence of DAP. DAP is easily identified by its characteristic green color under the conditions described. Fractions containing DAP were dried and diluted in water to a concentration of 10 mg/ml. These were applied to Whatman 3MM chromatography paper and developed in Rhuland solvent (20). Chromatograms were scanned for radioactivity, and the portion corresponding to radioactive DAP was cut out. Material was eluted from the paper with 25 ml of water and filtered through a membrane filter (0.45μm pore size; Millipore Corp.). The operation was repeated and the filtrates were pooled. The sample was lyophilized and weighed. Specific activity of the material obtained was 0.91 μCi/mmol.

Harvesting and breaking of cells. Cultures were harvested by centrifugation and washed three times in physiological saline before breaking. After the final wash, cells were suspended in the buffer described by Grandgenett and Stahly (12) and broken in an "X-press" pressure cell. Debris was removed by centrifugation at 39,000 $\times$ *g* for 30 min at 0 C. The supernatant fluid was collected and kept on ice until used. Fresh enzyme preparations were used consistently.

Enzyme assay. Diaminopimelate decarboxylase (E.C. 4.1.1.20; meso-2, 6-diaminopimelate carboxylase) activity was measured by the specific colorimetric method of Work. The assay involves the reaction of ninhydrin under acidic conditions which make it specific for DAP (26). The standard reaction mixture contained: 1 mM 2,3-dimercaptopropan-1-ol (BAL); 0.2 mM pyridoxal phosphate; 5 mM meso-DAP; 0.1 to 0.5 ml of enzyme extract (~4 mg of protein); and 0.2 M sodium phosphate buffer, pH 7.3, in a final volume of 1.0 ml. The reaction was incubated in a 37 C water bath. Samples (0.1 ml) were removed periodically and plunged into a screw-cap tube containing 4.0 ml of glacial acetic acid and 0.4 ml of water. After all samples had been collected, 0.5 ml of ninhydrin reagent (250 mg of ninhydrin, 4.0 ml of 0.6 N H_3PO_4, and 6.0 ml of glacial acetic acid) was added to each tube. Tubes were capped and incubated at 37 C for precisely 90 min. A very slight color instability was noted (increasing color), and it was found that reproducible results could be obtained by keeping the time factor from addition of color reagent to reading of optical density strictly constant. Optical density was recorded at 435 nm on a Zeiss PMQII spectrophotometer.

The isotope assay was performed in a similar manner. Reaction mixtures contained: 0.25 ml of enzyme extract (~2 mg of protein); 0.2 mM pyridoxal phosphate; 42 mM ^{14}C-DAP (0.91 μCi/mmol); 1 mM BAL; and 0.2 M sodium phosphate buffer, pH 7.3, in a final volume of 0.5 ml. The reaction was carried out in a 37 C water bath. Samples (0.1 ml) were removed periodically, and the reaction was terminated by heating at 75 C for 10 min. After heat inactivation, the entire 0.1-ml sample was spotted on Whatman no. 1 chromatography paper for descending chromatography in the indicated solvent systems. Evolution of CO_2 was evaluated by trapping the gas in small cups (Kontes Glass K-882320 and K-882310) containing 0.2 ml of hyamine hydroxide. Cups were replaced at each sampling interval, and the used cup with its contents was placed in 10 ml of diatol scintillation fluid. No correction was made for CO_2 retention in the buffer.

Unless otherwise specified, assays were carried out on extracts from wild-type cells grown with 10 μg of lysine per ml and harvested at 48 h. One unit of enzyme activity is defined as 1 μmol of product formed per min per mg of protein.

Incorporation of label from ^{14}C-aspartic acid (UL) into DAP. LA 423 was grown in the fermentation medium with the lysine concentration reduced to 10 μg/ml. After 48 h of incubation, cells were concentrated approximately 10-fold by centrifugation at 4,000 $\times$ *g* for 5 min. Ten milliliters of the concentrated suspension was pipetted into a 50-ml Erlenmeyer flask. After 20 min of equilibration on a Dubnoff

reciprocal shaker at 25 C, shaking speed no. 5, 2.5 μCi of ^{14}C-aspartic acid (UL) (180 μCi/mmol) was added without carrier. Sampling was begun immediately. Samples (1 ml) were removed, filtered through a membrane filter (0.45 μm pore size; Millipore Corp.), and washed three times with cold distilled water. The entire pad was dropped into 5.0 ml of 70% ethanol, vortexed thoroughly, and placed at 50 C for 30 min to extract amino acids. Solids were removed by centrifugation. The supernatant fluid was dried in vacuo and dissolved in 0.1 ml of water. Samples (20 μliters) were used in chromatography. The following solvent systems were used: methanol-pyridine-water, 80:4:20; *n*-propanol-ammonia 7:3; *n*-butanol-acetic acid-water, 11:3:4, all vol/vol/vol, and water-saturated phenol.

Incorporation of label from ^{14}C-lysine (UL) into the antibiotic. To conserve isotope, culture size was reduced to 5 ml per 25-ml Erlenmeyer flask. LA 423 was grown in the standard fermentation medium (1 mg of lysine per ml). At 72 h, L-[^{14}C]lysine was added (5×10^5 dpm/ml of culture), zero-time sample was removed, and the flask was quickly returned to the shaker. Samples of approximately 0.1 ml were removed periodically with sterile Pasteur pipettes and kept on ice until used. These were sonicated briefly (3 min) to remove material adsorbed to the cell surface. Phase microscopy revealed no detectable cell breakage by this treatment. Samples (20 μliters) were spotted on Whatman no. 1 chromatography paper and developed ascending for 16 h in *t*-butanol-formic acid-water, 70:15:15 (vol/vol/vol). Radioactive peaks were correlated with ninhydrin-positive spots and biologically active zones.

Similar experiments were performed in which label was added at 24 h. All details were the same except that the level of lysine in the standard medium was reduced to 500 μg/ml. This was the lowest level to which lysine could be reduced without significant effect on antibiotic production. The reduction was necessary to minimize dilution of the label at such an early time period.

Accumulation studies. Levels of amino acids in intracellular pools were determined by extracting them from whole, washed cells with hot ethanol and water. Fresh cells were suspended in approximately 20 vol of 70% ethanol and boiled for 5 min. The suspension was filtered through Whatman 3MM disks. The mycelial pad was removed, and the entire operation was repeated once more with ethanol and three times with water. Filtrates were combined and lyophilized. The resulting solid extract was analyzed by the method of Brannon et al. (3), modified by the addition of a third buffer to obtain better resolution of all amino acids. The third buffer was 1.0 M NaCl and 0.2 M sodium citrate at pH 6.3 adjusted with concentrated HCl. Collection of samples and assay of ninhydrin-positive fractions followed their procedure.

Assay of antibiotic activity. Antibiotic production was assayed by the standard disk plate technique described by Dennen et al. (7) by using *Salmonella gallinarium* and *Pseudomonas solanacearum* as assay organisms. Activity could be measured simultaneously or differentially by selectively destroying penicillin N with penicillinase (Riker Laboratories). Qualitative determination could be made by descending paper chromatography (*n*-propanol-pyridine-acetic acid-acetonitrile-water, 40:30:9:40:36, vol/vol/vol/vol/vol) and plating the chromatograms on plates seeded with one of the test organisms. The test plates were incubated overnight, *P. solanacearum* at 30 C and *S. gallinarium* at 37 C.

One unit of activity is equal to 0.3 μg of the purified antibiotic.

Chemicals. A standard of meso-DAP was kindly supplied by D. P. Stahly. A mixture of the three DAP isomers and BAL was obtained from Sigma Chemical Co. Pyridoxal phosphate was obtained from Calbiochem. L-[^{14}C]aspartic acid (UL), 180 mCi/mmol, was obtained from Schwarz-Mann. L-[^{14}C]lysine (UL), 220 mCi/mmol, D-[^{14}C]glucose (UL), 3.45 mCi/mmol, and diatol scintillation fluid were obtained from New England Nuclear Corp. Amino acids were shown to be chromatographically pure. All other chemicals were of analytical reagent grade.

RESULTS

Accumulation. Auxanographic studies on the *lys* mutants obtained have been described elsewhere (10). LA 423, the mutant described in Materials and Methods, showed no growth response on any compound other than lysine. The mutant accumulated large quantities of DAP in its free amino acid pools when starved for lysine (Fig. 1). Maximal DAP accumulation was observed when the organism was grown in the fermentation medium with the lysine concentration reduced to 10 μg/ml. This concentration was chosen, therefore, as the low level of supplementation in all succeeding studies. These findings are consistent with observations under similar conditions with a number of other *lys* mutants showing similar growth responses.

Accumulation of DAP was repressed when LA 423 was grown at 1 mg of lysine per ml and was not noted under either condition of lysine supplementation (10 μg/ml or 1 mg/ml) in the wild type (Fig. 1). No significant accumulation of any other ninhydrin-positive products of the aspartic acid family was noted.

Complete scans (not illustrated) indicated that a variety of amino acids were present in the pools of L100 and of LA 423 grown with 1 mg of lysine per ml. However, under derepressed conditions in the mutant, the DAP peak was essentially the only one found—a dramatic change in the metabolism of the cell.

Figure 2 indicates a sequence of events when LA 423 was grown with 1 mg of lysine per ml. Examination of the broth revealed that exogenous lysine was depleted in a fairly linear manner. Intracellular lysine pools increased through 72 h, and DAP was not present. How-

ever, as available lysine supplies began to be exhausted, DAP began to accumulate. This represents an apparent derepression or "turning on" of the pathway. By 120 h, a significant amount of DAP was present in the free amino acid pools, although it did not compare with the levels produced under starvation conditions (Fig. 1).

When lysine was limited (10 μg/ml), neither strain accumulated αAAA in the intracellular pools (Table 1). Both strains, however, produced such a pool when they were fed 1 mg of lysine per ml. The pool also appeared when 1 mg of DAP per ml was added to the low lysine broth in an L100 culture. Antibiotic was produced by either strain, but only when it was supplied high levels of lysine. Adding DAP did not allow detectable antibiotic production in either organism. These observations are in accord with the hypothesis that αAAA is a catabolic product of lysine.

Enzymology. Diaminopimelate decarboxylase catalyzes the conversion of DAP to lysine and CO_2. The colorimetric assay described in Materials and Methods measured the disappearance of substrate, without regard to how it was metabolized. The products of the reaction in *S. lipmanii* were identified in the isotope assay. Radioactive CO_2 was collected in hyamine hydroxide and quantitated by scintillation counting. Lysine was identified by examination of the reaction mixtures in several paper chro-

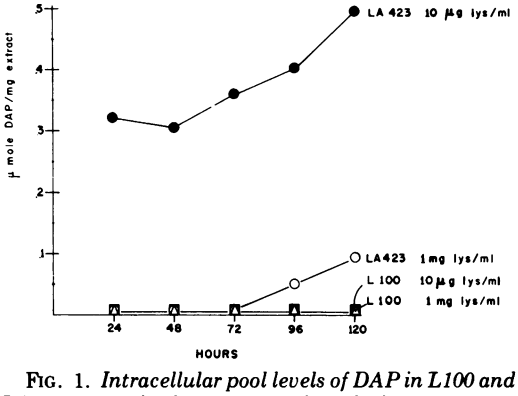

FIG. 1. *Intracellular pool levels of DAP in L100 and LA 423 grown in the presence of two lysine concentrations.*

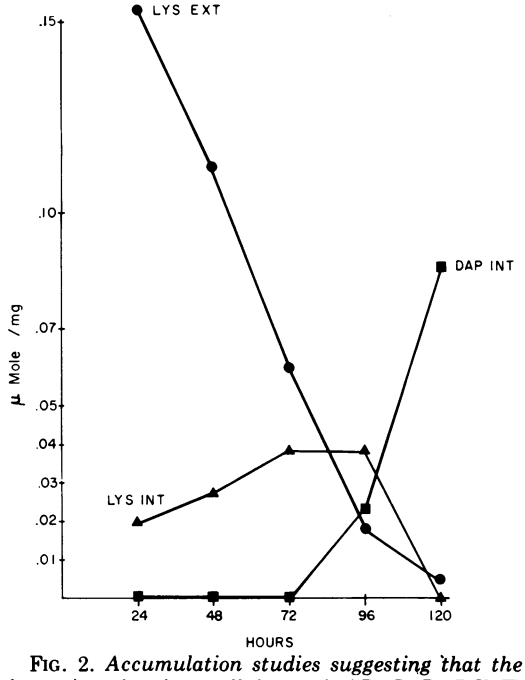

FIG. 2. *Accumulation studies suggesting that the formation of an intracellular pool of DAP (DAP INT) may be dependent on lysine pools. Intracellular lysine pools (LYS INT) apparently form as extracellular lysine (LYS EXT) is depleted.*

TABLE 1. *Relationship of lysine and DAP to αAAA or antibiotic formation*[a]

Strain	Amino acid (mg/ml)		Hours									
			24		48		72		96		120	
	Lysine	DAP	αAAA	Fac A	αAAA	Fac A	αAAA	Fac A	αAAA	Fac A	αAAA	Fac A
L100	1.0	0	.081		.044	64	.048	97	.087	149	.013	120
	0.01	0										
	0.01	1.0	.004		.012		.007		ND[b]	ND	ND	ND
LA 423	1.0	0	.095		.038	70	.077	119	.047	140	.011	123
	0.01	0										
	0.01	1.0							ND	ND	ND	ND

[a] αAAA is expressed in micromoles per milligram of intracellular pool extract. Antibiotic formation is expressed in units per milliliter of Factor A (Fac A) excreted into the broth.

[b] ND, Not determined.

matography systems. In each, the new peak corresponded to the position of authentic lysine (Table 2). No additional peaks were formed even upon incubation for as long as 24 h.

The production of radioactive CO_2 was essentially constant during the first 2 h, but diminished sharply thereafter. In Fig. 3, the rates of CO_2 and lysine formation are compared. The significant point is that the two curves rise over the same period of time and level off together. This indicates the correlation between production of the products.

Figure 4 illustrates the rate of conversion of diaminopimelate to lysine as measured by the colorimetric assay. To normalize data obtained with this technique, results were expressed as product formed per milligram of protein, assuming an equimolar ratio of substrate and product. In most cases, specific activity was calculated from the reading taken at 20 min.

Enzymatic activity exhibited a pH optimum of 7.3. Activity was somewhat more stable at basic than at acidic pH. Approximately 50% activity was retained at pH 8.05, whereas it was not detectable at pH 6.0.

Activity was significantly decreased by deletion of either of the cofactors, pyridoxal phosphate or BAL. Omission of pyridoxal phosphate or BAL led to 50 or 16% losses in activity, respectively.

Induction by substrate. L100 was grown with 10 μg of lysine per ml and 1 mg of DAP per ml in an effort to determine whether the specific activity of the enzyme could be increased. Extracts were assayed at 24-h intervals over a period of 72 h under otherwise standard conditions. Pool levels of DAP were significantly higher under these conditions than when grown with 10 μg of lysine per ml alone. No increase in specific activity was noted.

Inhibition of activity. Two known inhibitors of pyridoxal phosphate-dependent enzymes were used in the standard reaction mixture.

TABLE 2. *Relative positions of ninhydrin-positive spots and radioactive peaks*

Solvent[a]	*m*-DAP	[^{14}C] *m*-DAP	Lysine	Unknown
1	1.0[b]	1.0	1.90	1.90
2	1.0	1.03	2.36	2.39
3	1.0	.95	2.59	2.59

[a] Compositions of the solvent systems (all vol/vol) were as follows: (1) methanol-water-10 N HCl-pyridine, 80:17.5:2.5:10; (2) *n*-propanol-pyridine-acetic acid-acetonitrile-water, 45:30:9:40:36; (3) *n*-propanol-ammonium hydroxide, 7:3.

[b] R_{DAP} values.

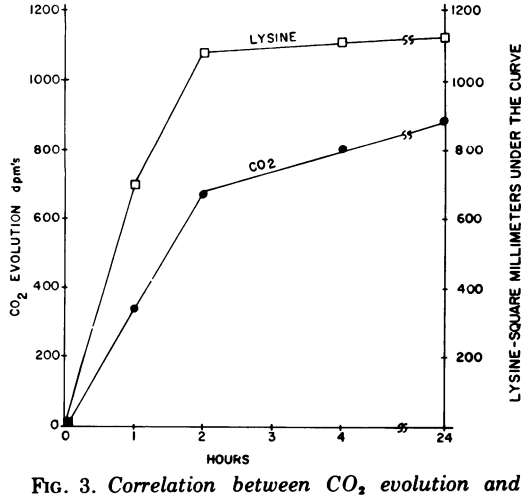

FIG. 3. *Correlation between CO_2 evolution and lysine peak formation in the isotope assay.*

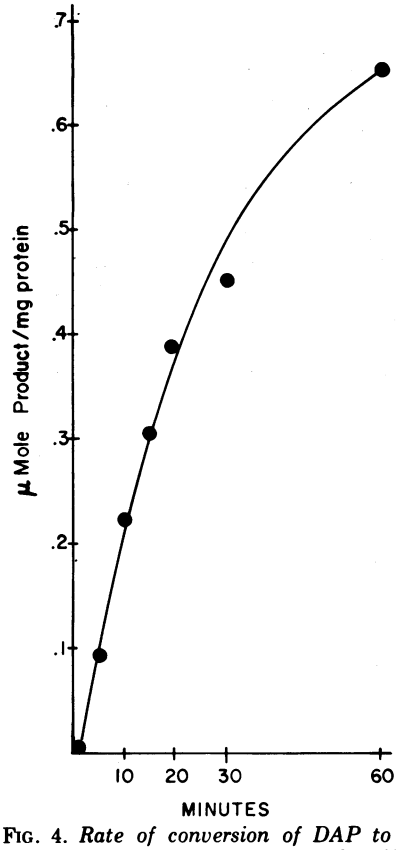

FIG. 4. *Rate of conversion of DAP to lysine as measured by the colorimetric assay. Specific activity (micromoles of product per minute per milligram of protein) calculated at 15 min was 0.020, and that at the 20-min sample was 0.019.*

These were hydroxylamine and D-penicillamine.

The reaction of hydroxylamine proved to be too slow for application here. The untreated control lost so much activity during the incubation period (70 min at 30 C) that it was not possible to gain useful information.

D-Penicillamine, however, did produce inhibition of treated samples only. The compound is presumed to combine with the coenzyme to form a thiazolidinecarboxylic acid (12). Inhibition of activity ranged from 7% at 1 mM penicillamine to 35% at 3 mM. An excess of pyridoxal phosphate (6 mM) added at the same time as the inhibitor completely prevented inhibition. Similar results were described by Grandgenett and Stahly in *Bacillus licheniformis* (12).

Effect of lysine on enzyme activity. To determine whether lysine repressed enzyme synthesis, specific activities of the enzyme from L100 grown at 1 mg of lysine per ml and at 10 μg of lysine per ml were compared. Cultures were harvested, washed, and broken at 24-h intervals during a 96-h fermentation. Despite the fact that free lysine pool levels were greatly changed under the two growth conditions, there was no significant difference in the specific activity of the DAP decarboxylase.

Lysine added directly to the reaction mixture was inhibitory, however, Although there was some variation between experiments, Table 3 illustrates representative rates of inhibition noted. D-Lysine produced less than one-third the inhibition of the L-isomer, but the possible presence of a racemase was not excluded.

Enzyme activity in LA 423. There was no detectable DAP decarboxylase activity in LA 423 under any condition used for L100. Conversion of DAP to lysine could not be demonstrated in either the colorimetric or isotope assays. The genetic block appears to be complete since there was no measurable activity at all. However, the sensitivity of the assays described is such that minor leakiness would not be detected.

TABLE 3. *Rates of inhibition by free lysine in the reaction mixture*[a]

Enzyme	Concn (mM)	% Activity	% Inhibition
L-Lysine	1	97	3
	3	58	42
	5	49	51
	7	34	66
	10	34	66
D-Lysine	5	87	13
	10	81	19
Control		100	0

[a] Enzyme mixtures described in Materials and Methods were varied only as indicated above. Rates of inhibition of the disappearance of DAP were calculated at 20 min. Specific activity of the control was 0.02 μmol per min per mg of protein.

Incorporation of aspartic acid into DAP. Cells of LA 423 derepressed for lysine biosynthesis rapidly took up aspartic acid and accumulated it in a compound which was chromatographically indistinguishable from DAP in four solvent systems. A representative scan is illustrated in Fig. 5. Both meso- and LL-isomers were present. The peak corresponding to the LL-isomer was larger at early time periods, whereas that corresponding to the meso-isomer became the larger at later intervals. This observation is in accord with the expected reaction sequence in which LL-DAP formation precedes epimerization to the meso-isomer (15). Radioactive DAP was detected in cell extracts within 10 min. It continued to accumulate during the assay period (2 h) and was not excreted into the broth. There was no evidence of conversion to lysine. In similar experiments performed with L100, neither DAP nor lysine accumulated.

The first radioactive compound detected in the amino acid pool extracts (labeled ASP X in Fig. 5) failed to migrate with aspartic acid in any of four chromatography systems. Examination of the broth revealed that the amino acid remained unchanged outside the cell. This may indicate that aspartic acid is activated on entrance into the cell—possibly to the phosphate ester. When the material corresponding to the unknown peak was eluted from the chromatogram and reacted with a reagent specific for phosphate esters (70% perchlorate-1 N HCl-ammonium molybdate-water, 5:1:25:60) (5), results were positive. However, the lability of the compound prevented further identification. Additional handling resulted in a com-

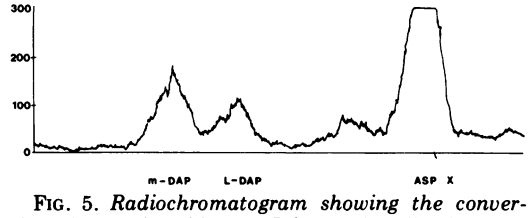

FIG. 5. *Radiochromatogram showing the conversion of aspartic acid to DAP by whole cells of LA 423 under derepressed conditions. Free amino acid pools were extracted after 30 min of incubation with the labeled aspartic acid. Chromatographic solvent was methanol-pyridine-water, 80:4:20 (vol/vol/vol).*

pound which once more behaved like aspartic acid.

Incorporation of ^{14}C-lysine (UL) into the antibiotic. Incorporation of label from lysine into the antibiotic supported the hypothesis that lysine catabolism yields an antibiotic component. The times chosen for addition of label were the log phase of production of each antibiotic factor—24 h for penicillin N and 72 h for the cephalosporin (Fig. 6). The results under both sets of conditions were the same. Figure 7 is a scan developed with a sample removed 24 h after the addition of the label. New peaks labeled as antibiotic factors corresponded both to ninhydrin spots and bioautograms on sensitive organisms produced with authentic antibiotic factors.

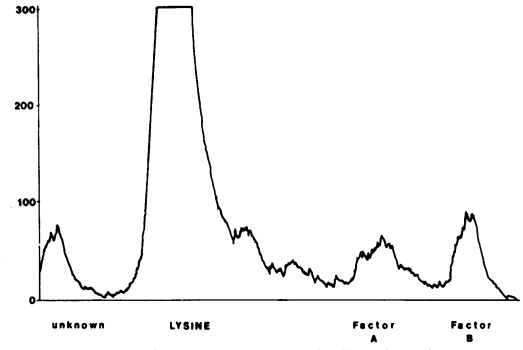

FIG. 7. *Radiochromatogram indicating the conversion of lysine to both antibiotic factors and one unknown compound. Peaks were correlated to both ninhydrin positive spots and biologically active zones produced by authentic antibiotic factors.*

DISCUSSION

LA 423, the lysine mutant described previously, was shown to be defective in diaminopimelate decarboxylase. The organism failed to show measurable activity under any condition used for the wild type. The inability of the organism to grow without lysine, its accumulation of DAP under derepressed conditions, and its lack of DAP decarboxylase activity establish that this reaction is a necessary step for lysine biosynthesis in *S. lipmanii*.

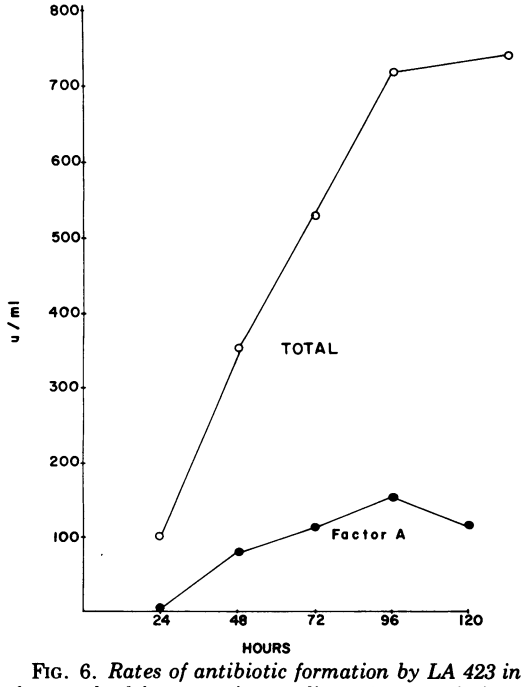

FIG. 6. *Rates of antibiotic formation by LA 423 in the standard fermentation medium. Assay technique is described in Materials and Methods.*

The fact that DAP was accumulated only when the mutant was starved for lysine suggests that lysine may exert some control over an earlier step(s) in the pathway. It is unlikely, however, that the pathway could be completely turned off since DAP is itself necessary for cell wall biosynthesis. Although data are not presented in this paper, it has been verified that DAP is present in the cell wall mucopeptide of both L100 and LA 423. Further work on cell wall synthesis and structure is to be the subject of a later paper.

Very basic characterization of the enzyme in extracts from the wild type was done. Results indicated that the enzyme from this organism is similar to those described in other bacteria. Physical properties such as the pH and temperature optima, stability, and requirement for cofactors are in accord with the findings of others (12, 27). Grandgenett and Stahly (13) surveyed the effect of lysine on diaminopimelate decarboxylase in a number of *Bacillus* species. They found that the enzyme was inhibited by L-lysine in all cases and repressed in 9 of 14 species. Although the rates of inhibition we are reporting are higher than theirs, the conditions of the assay (specifically product and substrate concentrations) were not the same. Repression was not observed in *S. lipmanii* despite the fact that free lysine did accumulate to significantly higher levels in the free amino acid pools. The highest initial lysine concentration tested was 1 mg/ml.

The lack of enzyme induction by substrate is similar to the findings of Work (27). It must be recognized, however, that the inability to demonstrate induction is far from conclusive proof that it does not occur. The difficulty in cases such as this stems from the fact that it is not

possible to completely deprive the cell of the inducer in question or even to strictly regulate the levels present. Even with these considerations in mind, however, one might reasonably expect some difference in activity from an inducible enzyme in view of the changed pool levels of DAP.

The incorporation of label from ^{14}C-aspartic acid into DAP concludes the evidence for the operation of the diaminopimelate pathway for lysine biosynthesis in *S. lipmanii*. This finding is unique to a producer of a β-lactam antibiotic with an αAAA side chain.

Accumulation studies support the hypothesis that αAAA is a catabolic product of lysine in this system. The production of either the αAAA pool or antibiotic is dependent upon lysine being supplied. If αAAA were an intermediate, the reverse effect would be expected. In *Penicillium chrysogenum*, for example, lysine has been shown to repress antibiotic synthesis (11, 16). Repression is thought to be the result of feedback inhibition of an early enzyme(s) in the lysine pathway. This, in turn, would prevent synthesis of αAAA, an intermediate in that organism's pathway. Without that source of precursor, antibiotic is not synthesized.

The simple experiment showing L-[^{14}C]lysine (UL) incorporation into the antibiotic is in no way intended to prove the lysine catabolism theory correct. It simply provides an early indication that such a theory might prove true and a rationale for continuing this approach to antibiotic biosynthesis. Similar observations were made by Whitney et al. (24) in labeling studies with the methoxycephalosporin produced by *S. clavuligerus*. They noted that DL-lysine-*1*-^{14}*C* was incorporated into the antibiotic and that radioactivity resided largely in the side chain. There are known catabolic pathways for lysine which contain αAAA or closely related compounds (17, 21). The possible presence of these pathways or of an entirely new pathway will be the subject of subsequent work.

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