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REVIEW

Lysine ε-Aminotransferase, the Initial Enzyme of Cephalosporin Biosynthesis in Actinomycetes

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Streptomyces clavuligerus, *Streptomyces lipmanii* and *Nocardia* (formerly *Streptomyces*) *lactamdurans* are Gram-positive mycelial bacteria that produce medically important β-lactam antibiotics (penicillins and cephalosporins including cephamycins) that are synthesized through a series of reactions starting from lysine, cysteine and valine. L-lysine ε-aminotransferase (LAT) is the initial enzyme in the two-step conversion of L-lysine to L-α-aminoadipic acid, a specific precursor of all penicillins and cephalosporins (Fig. 1). Whereas *S. clavuligerus* uses LAT for cephalosporin production, it uses the cadaverine pathway for catabolism when lysine is the nitrogen source for growth (7, 12, 33). Although the cadaverine path is present in all examined streptomycetes, the LAT pathway appears to exist only in β-lactam-producing strains (7, 12). Genetically increasing the level of LAT enhances the production of cephamycin. LAT is the key rate-limiting enzyme in cephalosporin biosynthesis in *S. clavuligerus* strain NRRL 3585 (17, 19). This review will summarize information on this important enzyme.

Temporal Nature of LAT Activity

LAT activity (7) has been detected in extracts of *S. clavuligerus* grown in complex and chemically-defined media (2a, 7, 12, 14, 17, 19, 25, 26, 29, 34). Most of LAT synthesis (in *S. clavuligerus* and *N. lactamdurans*) occurs during the growth phase (2a, 7, 12, 14) and then decreases rapidly, although significant activity is present throughout the idiophase. The timing of the peak LAT activity depends on the length of the growth phase and the medium used (14, 25, 26, 29).

Purification and Molecular Weight of LAT

LAT was partially purified from *S. clavuligerus* by 30~60% ammonium sulfate precipitation, Sephacryl S-200 gel filtration and ion-exchange DEAE-SPW HPLC with a yield of 33% (29). It has a molecular weight of 51.3 kDa. Partial purification of the recombinant *N. lactamdurans* gene product by ammonium sulfate precipitation and Sephadex G-75 chromatography revealed a molecular weight of 52.8 kDa in SDS-PAGE (3). Deduced sizes of 48.0 kDa (*S. clavuligerus*) and 48.8 kDa (*N. lactamdurans*) were determined by cloning the *lat* structural genes in *Escherichia coli* and *Streptomyces lividans*, respectively (3, 31, 34).

Characterization of LAT

All studies on enzyme kinetics were made with the enzyme purified by gel filtration (29). In Tris-maleate-NaOH buffer at pH values from 6.0 to 9.0, LAT of *S. clavuligerus* had a pH optimum of 7.2 and an optimal temperature of 30°C.

The enzyme has high specificity for L-lysine as substrate although L-ornithine can also be used. LAT is a very unstable enzyme which catalyzes transfer of the terminal amino group of the substrate to α-ketoglutarate. *cis*-Oxalacetate and pyruvate are also used as acceptors of the amino group but with very low efficiency (6.6 and 4%, respectively as compared to α-ketoglutarate). A 20% reduction of enzyme activity was observed when pyridoxal phosphate was omitted from the reaction mixture. The Km values of the aminotransferase for lysine and α-ketoglutarate are 3.2 mM and 3.6 mM, respectively (29). The characteristics of LAT from *S. clavuligerus* are very similar to those reported for the enzymes of *Flavobacterium* sp. (30) and *N. lactamdurans* (7).

The *lat* Gene Encodes Lysine ε-Aminotransferase

The gene governing LAT production (*lat*) is closely linked to other genes of the β-lactam biosynthetic pathway in *S. clavuligerus* (13) and *N. lactamdurans* (3). It lies upstream of and very close to *pcbAB* (encoding α-aminoadipyl-cysteinyl-valine synthetase, ACVS) and *pcbC* (encoding isopenicillin N synthase, IPNS, cyclase) in a cluster of early cephamycin biosynthetic genes. All three genes are transcribed in the same direction. The *lat* gene is approximately midway between *pcbC* and *cefE* (encoding deacetoxycephalosporin C synthase, DAOCS, expandase) (Fig. 2) (8, 13, 31). Almost identical DNA sequences of *lat*

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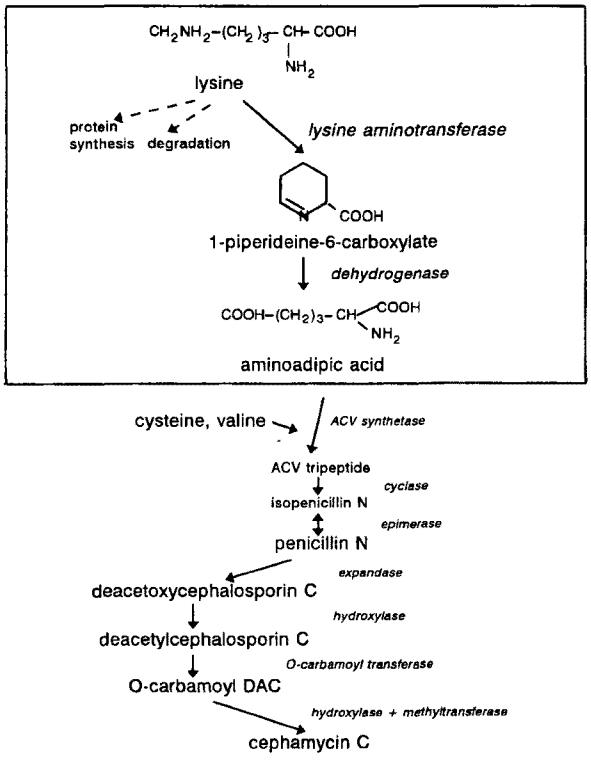

Fig. 1. Biosynthetic pathway of β-lactam antibiotics from lysine, cysteine and valine in *Streptomyces clavuligerus*.

from *S. clavuligerus* were reported by Tobin *et al.* (31) and Yu *et al.* (34). LAT from *S. clavuligerus* is derived from a single open reading frame (ORF) of 1374 nucleotides encoding a protein of 458 amino acids (31, 34). The *lat* gene of *N. lactamdurans* contains an ORF of 1353 nucleotides (71.4% G+C), encoding a protein of 450 residues (3).

Promoter probe analysis to locate the *lat* promoter(s) in *S. clavuligerus* indicated that a sequence promoting transcription was present in a 330 bp DNA fragment that extended from 227 bp upstream of *lat* to 103 bp inside the gene (22). The promoter showed modest activity in *S. lividans*, but had strong activity in *S. clavuligerus*. High-resolution S1 nuclease mapping of the 5' end of the *lat* transcript identified a T residue 88 bp upstream from the *lat* start codon as the transcription start point (22). The *lat* and *pcbAB* genes are separated by an intergenic region of 153 bp which lacks a transcriptional start site, promoter activity and recognizable promoter sequences (34). Based on the above observations, Petrich *et al.* (22) suggested that *lat* and *pcbAB* are cotranscribed. Yu *et al.* (34) found promoter activity also within the 3' terminal sequences of *lat* suggesting that transcription of *pcbAB* may originate within the *lat* ORF and that, under some conditions, the two genes may be expressed independently.

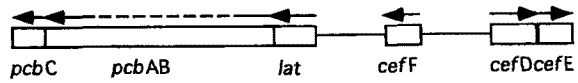

Fig. 2. Linear map of the β-lactam biosynthetic gene cluster in *Streptomyces clavuligerus*.

The open boxes represent the approximate sizes of each coding region. The genes and products are: *pcbC*, isopenicillin N synthase (IPNS); *pcbAB*, α-aminoadipyl-cysteinyl-valine synthetase (ACVS); *lat*, lysine ε-aminotransferase (LAT); *cefF*, deacetylcephalosporin C synthase (DACS); *cefD*, isopenicillin N epimerase (EPI); *cefE*, deacetoxycephalosporin C synthase (DAOCS). Solid arrows indicate directions of transcription. The transcript for *pcbAB* is represented as a dashed line since nt sequence has been obtained for the N- and C-terminal ends of the gene only. Based on Petrich *et al.* (11).

On the other hand, Petrich *et al.* (21) failed to detect such promoter activity for *pcbAB*.

Whereas Petrich and coworkers (22, 23) found no evidence for transcription termination downstream of *lat* or *pcbAB*, a GC-rich inverted repeat that may act as a transcription termination signal was found by others in the intergenic region (31, 34).

The importance of LAT in cephalosporin production is evident from the phenotypes of certain mutants producing little or no cephalosporin. Many of these mutants produce no LAT or abnormally low levels of the enzyme (24, 28). They are also pleiotropically affected in other enzymes of the pathway, especially *pcbAB* and *pcbC*, showing depressed levels of these enzymes and normal levels of later pathway enzymes. For example, *S. clavuligerus* mutant NP1 (15, 24, 34) produced 4% of wild-type levels of LAT and 17% ACVS and 17% IPNS whereas epimerase and expandase levels were normal. The culture produced only 1% of the wild-type cephalosporin titer. Feeding α-aminoadipate to NP1 cultures increased cephalosporin production to 17% (34). Cloning of wild-type DNA into mutant NP1 led to restoration of cephalosporin production (to 67% of wild-type level), LAT (to 72%) and increases in ACVS (to 39%) and IPNS (to 35%). The complementing DNA was found to contain the entire *lat* gene as well as the upstream 20% of *pcbAB* (34). Plasmid deletions which eliminated *lat* abolished complementation. Thus, it appears that NP1 is a *lat* mutant and that the activities of ACVS and IPNS are somehow dependent on LAT. The mechanism underlying the interdependence of *lat*, *pcbAB* and *pcbC* expression remains to be elucidated.

Malmberg *et al.* (17) used targeted gene insertion methodology to increase the dosage of *lat* in *S. clavuligerus*. They had earlier developed a structural kinetic model of cephalosporin biosynthesis in this organism (16, 18) which indicated that ACVS was the limiting enzyme in the pathway from the three amino acid precursors to the final products. They predicted that the formation of α-aminoadipate, the most limiting of the three

amino acid precursors, might play an important role in the flux of the pathway. They used a high-copy number plasmid containing *lat* to transform *S. clavuligerus* (17). The transformant, strain LHM100, contained one additional copy of *lat* adjacent to the corresponding wild-type chromosonal gene. LAT activity increased four-fold and cephalosporin production increased by 60% to 500% (17, 19). In the transformant, the level of intracellular α-aminoadipate was higher than in the wild-type until later in the fermentation. This accumulation is probably due to a bottleneck caused by a deficiency in the level of ACVS. The intracellular concentration of ACV was similar in both strains early in the fermentation but became higher in strain LHM100 later, presumably due to a limitation in one or more late enzymes of the pathway. The authors (19) concluded that early in the fermentation, LAT and ACVS are the limiting enzymes but later, one or more of the late pathway enzymes becomes limiting. Malmberg *et al.* (17) also found evidence for separate expression of *lat* and *pcbAB*. In *S. clavuligerus* strain LHM 100, they noted that while LAT activity was four-fold higher than the wild-type, ACVS activity was not altered. The protein encoded by the *lat* gene shows sequence similarity to ornithine 5-aminotransferases, and contains a pyridoxal phosphate-binding consensus amino acid sequence around Lys-304 (31).

Regulation of LAT

Induction by L-lysine. The stimulation of β-lactam production by L-lysine was first demonstrated by Mendelovitz and Aharonowitz (20). In their work, 10 mM and 20 mM lysine were found to stimulate β-lactam biosynthesis by 50~100%. Fang *et al.* (5) confirmed their findings but observed an additional 300% increase in β-lactam production upon raising exogenous L-lysine to 100~150 mM. It is not known why such a high concentration of lysine is needed to increase cephalosporin production. Perhaps it is due to poor uptake or to competition with lysine catabolism or anabolism. DL-lysine was almost as active as L-lysine, and D-lysine was less active than the DL- or L- forms. This suggests that *S. clavuligerus* has a lysine racemase or some other enzymatic means of converting D-lysine to L-α-aminoadipate. As mentioned above, genetically increasing the level of LAT enhances production of cephamycin C by *S. clavuligerus* (17, 19). Recently, we showed that L-lysine enhances antibiotic production, not only as a substrate of LAT (thus providing L-α-aminoadipic acid for synthesis of cephalosporins), but also as an inducer of LAT (26). Table 1 shows that the amount of LAT produced was tripled by addition of 150 mM L-lysine. Although Madduri *et al.* (14) and Bascarán *et al.* (2a) failed to observe lysine induction of LAT, they only tested lysine at 15 mM concentration. The table also confirms the results of Fang *et al.* (5), i.e., higher L-lysine concentrations yielded greater levels of antibiotic. The pH pattern of the fermentation was unaffected by lysine addition. We observed a linear correlation (r^2=0.99) between the L-lysine concentration added to the medium and the maximum volumetric cephalosporin production (26). Addition of 50 mM L-α-aminoadipic acid to the cultures enhanced cephalosporin production by *S. clavuligerus* to the same degree as 50 mM L-lysine, presumably by increasing the intracellular concentration of the precursor, but failed to induce LAT.

Effect of ammonium ions. Unlike some other enzymes of β-lactam biosynthesis, LAT is not repressed by ammonium ions (14, 29). The effect of increasing amounts of NH_4Cl on LAT and cephalosporin formation by *S. clavuligerus* was recently studied by us (29) in cells grown in two chemically-defined media, GSPG and MM, both of which had been used previously in studies of cephalosporin biosynthesis (26, 27). Whereas the specific activity of LAT was not affected by including NH_4Cl in GSPG medium, ammonium ions enhanced LAT activity in MM cultures (Table 2). Bascarán *et al.* (2a) also noted ammonium stimulation of LAT specific activity in medium SF, which is quite similar to medium MM except it lacks lysine and has a different concentration of inorganic salts. NH_4 repression of cephalosporin production was found in both GSPG and MM media. Culture pH was unaffected by NH_4Cl addition to MM medium but markedly dropped in GSPG medium.

Effect of carbon source. LAT formation and biosyn-

Table 1. Induction of LAT by L-lysine*.

L-Lysine (mM)	L-α-Amino-adipate (mM)	Maximum growth (mg DCW/ml)	Maximum LAT (units/mg protein)	Maximum cephalo-sporins (μg/ml)
0	0	3.1	1.0	26.6
50	0	3.3	1.7	57.6
100	0	4.0	1.7	77.1
150	0	3.7	3.0	114.9
0	50	3.7	0.83	62.3

*Data from experiments of reference 26.

Table 2. Lack of repression of LAT by NH_4Cl*.

Medium	Added NH_4Cl (mM)	Maximum growth (mg DCW/ml)	Maximum LAT (units/mg protein)	Maximum cephalo-sporins (μg/ml)
MM	0	2.8	0.84	49.0
	20	3.2	1.2	20.6
	120	1.9	1.4	4.0
GSPG	0	3.6	1.4	58.7
	20	1.3	1.3	10.3
	120	2.1	1.5	4.7

*Data from experiments of reference 29.

Table 3. Effect of carbon source on LAT formation*.

Carbon source	Concentration (%)	Maximum growth (mg DCW/ml)	Maximum LAT (units/mg protein)	Maximum cephalosporins (μg/ml)
Glycerol	1	3.1	2.5	74.3
	3	4.5	1.7	30.9
Starch	1	3.6	2.3	66.4
	3	5.1	5.7	35.3

*Data from experiments of reference 25.

thesis of cephalosporins by *S. clavuligerus* are influenced by the type of carbon source used and its concentration (2, 6, 10, 14, 25, 27, 32, 35). Cephalosporin production is negatively regulated by high concentrations of carbon source. We found that the antibiotic production of cells grown on 3% glycerol or 3% starch decreased to about 40% and 50%, respectively, of the level in cultures containing 1% of the respective carbon source (Table 3) (25). Madduri *et al.* (14) had reported that mycelia grown in a 1% glycerol medium had substantially lower LAT specific activities than did mycelia from 1% starch-grown cultures. We found that at this concentration, LAT formation was about the same in glycerol vs. starch (25). However at the 3% level, starch more than doubled LAT production, while glycerol repressed LAT by 40% (Table 3). The pH pattern was not influenced by the high carbon source concentration because the MOPS buffer had been increased in concentration to 200 mM.

Effect of inorganic phosphate. Madduri *et al.* (14) reported that increasing the phosphate concentration from 6 mM to 100 mM in 1% starch cultures of *S. clavuligerus* progressively increased the specific activity of LAT. We did not observe an enhancement (Table 4), i.e. increasing the concentration of potassium phosphate from 20 mM to 100 mM in 1% glycerol cultures had no effect on LAT but reduced antibiotic production by 85~90% (25) as expected (1, 9, 11).

Promoters and terminator. As mentioned earlier in this review, *lat* and *pcbAB* are adjacent to each other on the *S. clavuligerus* chromosome with an intervening region of 153 bp. Although it appears that a promoter exists upstream of *lat* which possibly regulates transcription of *lat*, *pcbAB* and *pcbC* (21-23) and a second promoter exists in the C-terminius of *pcbAB* which regulates only *pcbC* (23), a controversy exists with respect to the possibility of a third promoter in the C-terminal portion of *lat* which would control transcription of *pcbAB* (and possibly also *pcbC*), but not that of *lat* (4, 21, 22, 34). The findings described above on nutritional regulation of LAT support the third promoter concept of Yu *et al.* (34) since control of LAT and ACVS are very different in the following ways: (i) ammonium ions repress ACVS (36) but not LAT (29); (ii) a high concentration of glycerol represses LAT (14, 25) but has no effect on formation of ACVS (35); (iii) a high concentration of phosphate represses ACVS (37) but not LAT (14, 25).

Table 4. Lack of LAT repression by inorganic phosphate*.

K_2HPO_4 (mM)	Maximum growth (mg DCW/ml)	Maximum LAT (units/ mg protein)	Maximum cephalosporins (μg/ml)
20	3.2	0.90	107.8
100	3.3	1.0	11.9

*Data from experiments of reference 25.

The highly GC-rich inverted repeat reported by two groups (31, 34) in the *lat-pcbAB* intergenic region, downstream of the controversial third promoter region, could form a stable hairpin structure with a stem of 15~17 bp and a loop of 3 bp with a calculated ΔG value of -54.4 kcal/mol, resembling a transcription terminator. A transcription terminator in this location could allow selective *lat* expression under certain environmental conditions, without preventing expression of *pcbAB* and *pcbC* downstream (34). Under conditions where cephalosporin synthesis would be desirable to the microorganism, expression of distal genes could be permitted by an antitermination mechanism. It should be noted, however, that no evidence of transcription termination downstream of *lat* was found in transcript mapping experiments (23).

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