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Partial purification, characterization and nitrogen regulation of the lysine ε-aminotransferase of *Streptomyces clavuligerus*

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The L-lysine ε-aminotransferase (LAT) of *Streptomyces clavuligerus* was partially purified and characterized. The 51.3-kDa enzyme exhibited optimal activity at pH 7.0–7.5 and 30°C. It catalyzed transfer of the terminal amino group of L-lysine or L-ornithine to α-ketoglutarate. Oxalacetate and pyruvate were also used as acceptors of the amino group but with very low efficiency. Increasing ammonium concentrations added to chemically-defined medium MM enhanced the formation of LAT and decreased production of cephalosporins by *S. clavuligerus*. In cultures grown in the absence of lysine, greater enhancement of LAT formation by ammonium and less repression of cephalosporin biosynthesis were observed. In the chemically-defined GSPG medium, ammonium ions decreased cephalosporin production without showing an effect on LAT formation.

Keywords: lysine ε-aminotransferase; *Streptomyces clavuligerus*; nitrogen regulation; β-lactam antibiotics; cephalosporins

Introduction

The biosynthesis of cephalosporins by *Streptomyces clavuligerus* begins with the conversion of L-lysine to 1-piperideine 6-carboxylate catalysed by the enzyme lysine ε-aminotransferase (LAT, EC 2.6.1.36). 1-Piperideine 6-carboxylate is then dehydrogenated to L-α-aminoadipic acid. The basic structure of β-lactam antibiotics is formed by condensation of three precursors, L-α-aminoadipic acid, L-cysteine and L-valine. The importance of LAT has been demonstrated by its designation as the rate-limiting enzyme of cephalosporin biosynthesis in *S. clavuligerus* NRRL 3585 [13,21]. We have recently shown that lysine is not only a substrate of LAT but also an inducer [14]. The mechanism of induction is unknown. It was, therefore, of great interest to characterize the LAT of *S. clavuligerus*.

It had been reported that the use of NH_4Cl as the sole nitrogen source or as an additive to asparagine depresses antibiotic production [2–4,15] in this microorganism. The negative effect is due to repression of L-α-aminoadipyl-L-cysteinyl-D-valine synthetase (ACVS), isopenicillin N synthase (cyclase), isopenicillin N epimerase and deacetoxycephalosporin C synthase (expandase) [3,4] The influence of NH_4^+ on LAT formation has never been reported.

We report here that LAT of *S. clavuligerus* is a very unstable α-ketoglutarate-dependent transaminase. LAT activity in *S. clavuligerus* cultures increases to a maximum during the growth phase and decreases throughout the stationary phase after antibiotic biosynthesis begins. We found that ammonium ions do not repress LAT and, in one of two media, even enhance formation of this important initial enzyme of cephalosporin biosynthesis.

Materials and methods

Microorganisms

Streptomyces clavuligerus NRRL 3585 (ATCC 27064) was used in this study. *Escherichia coli* ESS was the assay organism for cephalosporins. It is a mutant of *E. coli* W and is super-sensitive to β-lactam antibiotics [8].

Media and culture conditions

Spores of *S. clavuligerus* NRRL 3585 were maintained in 20% glycerol at –80°C; 0.5 ml of this suspension was used to inoculate 30 ml of seed medium which was incubated at 30°C and 220 rpm for 48 h. Two milliliters of this culture were inoculated into 30 ml of seed medium and incubated under the same conditions for 40–44 h. The resulting second-stage seed was inoculated into the fermentation media at 6% (v/v). All liquid cultures were incubated in 250-ml Erlenmeyer flasks containing 25 ml of medium, at 30°C on a rotary shaker at 220 rpm. The seed medium was Tryptic Soy Broth (Gibco Laboratories, Madison, WI, USA), adjusted to pH 7.0. Two chemically-defined fermentation media were used. Medium MM is that described by Fang *et al* [6], supplemented with 100 mM L-lysine. It contained (g L^{-1}): glycerol, 10; L-asparagine, 2; L-lysine · HCl, 18; 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, 21; $MgSO_4 \cdot 7H_2O$, 0.6; K_2HPO_4, 3.5; and trace salts solution (containing $FeSO_4 \cdot 7H_2O$, 1; $MnCl_2 \cdot 4H_2O$, 1; $ZnSO_4 \cdot H_2O$, 1; and $CaCl_2$, 1), 1 ml L^{-1}. The pH was adjusted to 6.8 before autoclaving. GSPG medium consisted of (g L^{-1}): glycerol, 15; sucrose, 20; proline, 2.5; glutamic acid, 1.5; NaCl, 5; K_2HPO_4, 2; $CaCl_2$, 0.4; $MnCl_2 \cdot 4H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.1; ZnCl, 0.05;

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$MgSO_4 \cdot 7H_2O$, 1; at pH 7.0 [15]. For experimental purposes, NH_4Cl was added to both fermentation media at 20 mM or 120 mM as mentioned in the text. All fermentations were done at least twice and duplicate flasks were employed in each experiment.

Growth measurements

Growth was measured by absorbance using a Klett-Summerson colorimeter (Klett Manufacturing, New York, NY, USA) and converted to dry cell weight (DCW) as described by Fang *et al* [6].

Cell-free extracts

Between 25 ml and 100 ml of fermentation culture were sampled at the times described in the text and figures. Mycelia were harvested by centrifugation, washed twice with 0.85% NaCl, suspended in 0.2 M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) and disrupted by sonication in an ice/water bath with a Branson model 200 sonifier (Branson Ultrasonics Co, Danbury, CT, USA) using setting 3 and duty cycle 50%, 3×30 s. Cell debris was removed by centrifugation for 30 min at $26\,000 \times g$ and 4°C. The supernatant fluid was stored immediately at −80°C until used.

Lysine ε-aminotransferase assay

The assay of LAT activity was based on the procedure of Kern *et al* [9]. The incubation mixture consisted of cell extract (1.0 ml), 40 μmol of L-lysine, 40 μmol of α-ketoglutarate, and 0.15 μmol of pyridoxal phosphate in a final volume of 2 ml. The reaction mixture was incubated for 60 min at 37°C and 220 rpm and stopped by adding 1 ml of 5% trichloroacetic acid in absolute ethanol. Precipitated proteins were removed by centrifugation for 10 min at $20\,000 \times g$. The amount of 1-piperideine-6-carboxylate in 1 ml of the deproteinized reaction mixture was measured by adding 1.5 ml of 4 mM *o*-aminobenzaldehyde in 0.2 M phosphate buffer (pH 7.5). The mixtures were incubated at 37°C for 1 h and the yellow-orange color was measured at 465 nm. A solution containing 1 ml of phosphate buffer instead of the cell-free extract was used as blank. This solution was used to calibrate the spectrophotometer. The amount of 1-piperideine-6-carboxylate formed was calculated using the extinction coefficient of 2800 L mol^{-1} cm^{-1} reported by Fothergill and Guest [7]. One unit of enzyme activity is defined as that catalyzing formation of 1 nmol of product per min under the conditions used.

Protein in the cell-free extracts was determined with the Bio-Rad Protein Reagent Assay (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin was used as standard. The specific activity of LAT was expressed as units per mg of protein.

Antibiotic assay

Cephalosporins were determined by the agar plate diffusion assay with the assay strain seeded in LB agar. Cephalosporin C was used as standard and *E. coli* ESS as assay organism.

Enzyme purification

Cell-free extracts were prepared from GSPG cultures. Protamine sulfate (1%) was added slowly to the supernatant fluid to give a final concentration of 0.1%. Precipitated nucleic acids were removed by centrifugation at $20\,000 \times g$ for 15 min. The clear protamine sulfate supernatant fluid was precipitated with ammonium sulfate (30–60%). The active fraction after ammonium sulfate precipitation was redissolved in PP buffer (0.2 M K_2HPO_4/KH_2PO_4 buffer containing 10 μM pyridoxal 5′-phosphate, pH 8.0) and applied to a 1.6×46-cm Sephacryl S-200 column equilibrated with the same buffer at 4°C. The enzyme was eluted with PP buffer at a flow rate of 0.4 ml min^{-1}. The column was calibrated with standard proteins of known molecular weight (ribonuclease, 13.7 kDa; chymotrypsinogen A, 25 kDa; ovalbumin, 43 kDa; and bovine serum albumin, 67 kDa). Three milliliters of active sample were applied to a DEAE-SPW anion exchange HPLC column (Waters Española, SA, Madrid, Spain) equilibrated with PP buffer. The protein was eluted with a NaCl gradient (from 0 to 350 mM NaCl in the first 90 min, and from 350 mM to 500 mM NaCl, for an additional period of 10 min).

Chemicals

o-Aminobenzaldehyde, α-ketoglutarate, L-lysine, pyridoxal 5′-phosphate and all other chemicals were of reagent grade, purchased from Sigma Chemical Co (St Louis, MO, USA).

Results

LAT activity and cephalosporin production in chemically-defined media

Cultures of *S. clavuligerus* grown in MM medium and GSPG medium were analyzed during the late growth and stationary phases to compare biomass, pH, LAT activity and cephalosporin production (Figure 1). *S. clavuligerus* grew well and reached maximum growth by 48 h of incubation in both media. An increase in pH was observed from 48 to 96 h of incubation in GSPG medium as shown previously by Romero *et al* [15]. No major change of pH occurred in MM medium. As had been observed with *Nocardia lactamdurans* [9] and with *S. clavuligerus* by other workers [12,13], we found that most of the synthesis of LAT occurs during the growth phase. LAT specific activity peaked at 48 h when both cultures reached maximum growth. LAT specific activity and cephalosporin production were higher in GSPG medium than in MM medium.

Partial purification and molecular weight determination of LAT

Since pyridoxal 5′-phosphate is required for maximal activity of LAT [9,17], all buffers used in the purification of the enzyme were supplemented with 10 μM pyridoxal 5′-phosphate. A 68% recovery was obtained after ammonium sulfate (30–60%) precipitation, but purification in this step was poor suggesting that enzyme inactivation had occurred. A single activity peak was obtained after Sephacryl S-200 gel filtration (Figure 2) with a specific activity of 0.12 units mg^{-1} protein and a recovery of 32.8%. Most of the enzyme was inactivated during gel filtration even in fast-flowing Sephacryl columns. Repeated attempts failed to improve preservation of enzyme activity during this step.

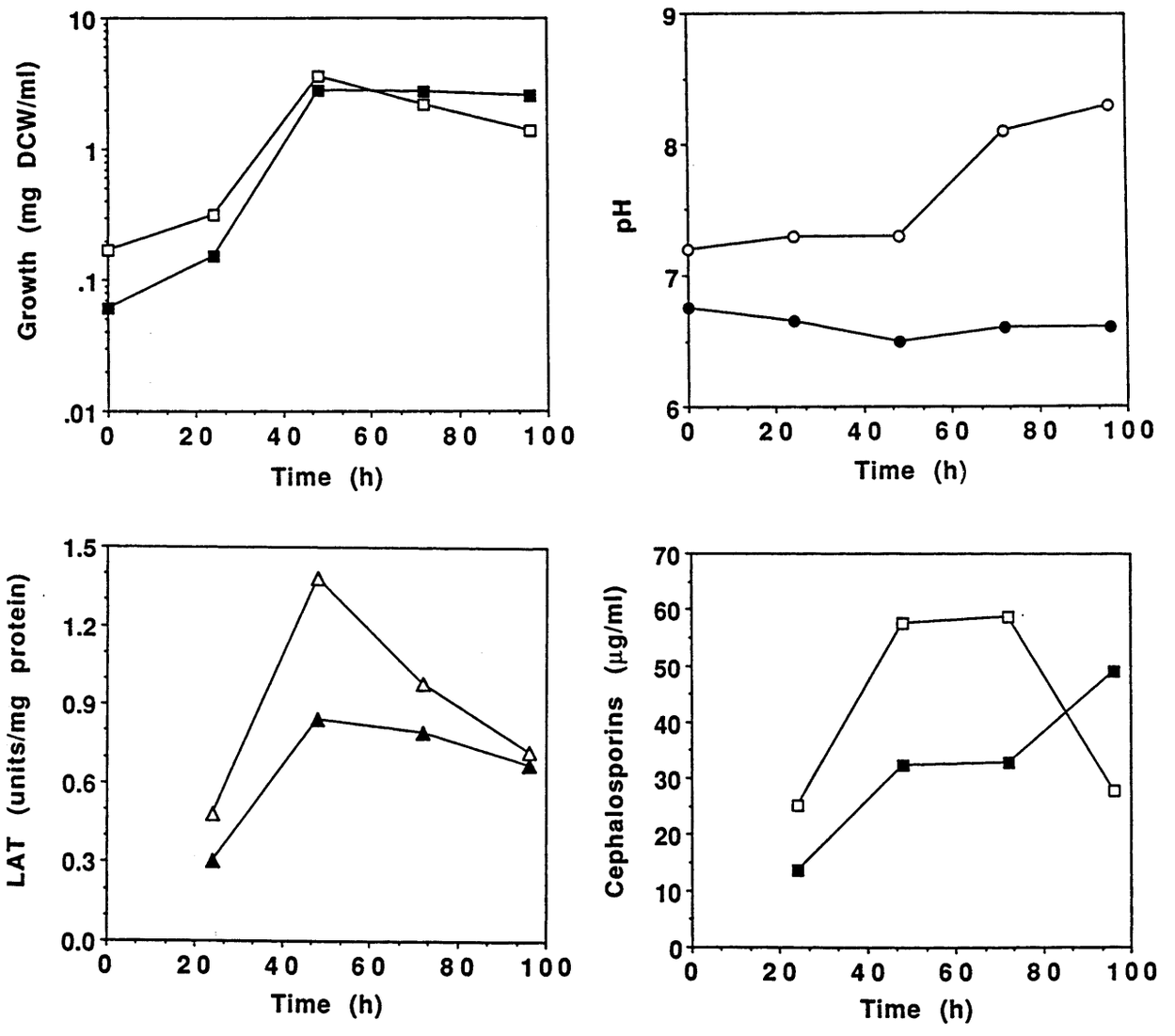

Figure 1 Growth, pH, lysine ε-aminotransferase (LAT) specific activity, and cephalosporin production in cultures of *Streptomyces clavuligerus* growing in MM medium (closed symbols) and GSPG medium (open symbols).

The molecular weight of the enzyme was determined by comparison with the elution profile of proteins of known molecular weight. LAT eluted as a single peak with a K_{av} value of 0.26 corresponding to a molecular weight of about 51.3 kDa (Figure 2, inset). This is close to the deduced size of 48.0 kDa obtained by cloning and expressing the *S. clavuligerus* gene in *Escherichia coli* [18]. The *N. lactamdurans* gene showed a molecular weight of 52.8 kDa and a deduced size of 48.8 kDa by cloning in *Streptomyces lividans* [15].

When the active fractions obtained from gel filtrations were applied to an ion-exchange DEAE-SPW HPLC column, fractionation of the protein into three major peaks was observed; one of these peaks showed a trace of LAT activity, but further purification could not be carried out due to inactivation of the enzyme.

Characterization of the enzyme

All studies on enzyme kinetics were made with the enzyme purified by gel filtration. A series of reaction mixtures were prepared in Tris-maleate-NaOH buffer at pH values from 6.0 to 9.0. LAT of *S. clavuligerus* has a pH optimum of 7.2 (Figure 3a). Although purification had been done at pH 8.0, Figure 3a shows that the activity at pH 8.0 is almost as high as at pH 7.2. Furthermore, the enzyme was equally unstable at pH 8.0 and 7.2. Incubation of standard enzyme reaction mixtures at 20–50°C showed an optimal temperature at 30°C (Figure 3b).

The enzyme showed a high specificity for L-lysine as substrate although L-ornithine could also be used. L-Arginine, L-citrulline, 4-aminobutyrate and glutamine (40 mM) did not inhibit LAT activity when L-lysine was used as substrate. Pyruvate, *cis*-oxaloacetate, α-ketobutyrate and α-

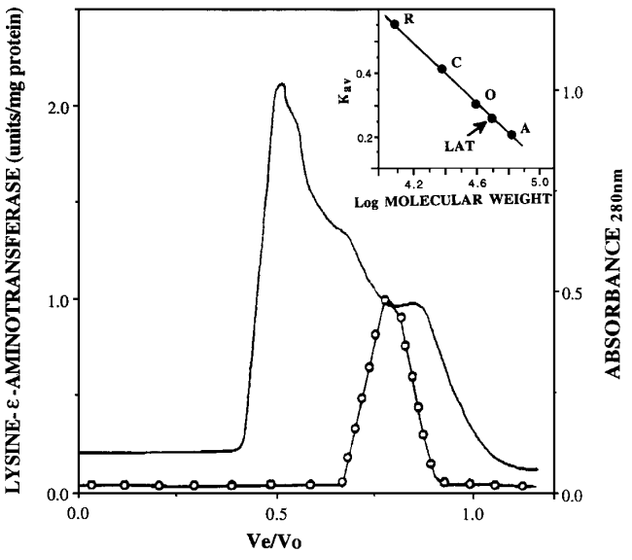

Figure 2 Sephacryl S-200 gel filtration of LAT. LAT activity (○); total protein as A_{280} (continuous line). V_e/V_o, elution volume/void volume. Inset, K_{av} value and determination of molecular weight of LAT. R, ribonuclease; C, chymotrypsinogen A; O, ovalbumin; A, bovine serum albumin. LAT, L-lysine ε-aminotransferase (arrow).

ketoisovalerate (40 mM) were also tested as amino group acceptors. Only *cis*-oxaloacetate and pyruvate could replace α-ketoglutarate but with very low efficiency (6.6 and 4%, respectively). A 20% reduction of enzyme activity was observed when pyridoxal phosphate was omitted from the reaction mixture.

The K_m values of the aminotransferase for lysine and α-ketoglutarate were determined with enzyme purified by gel filtration. The apparent K_m value for lysine was 3.2 mM ($V_{max} = 0.023$ units mg protein^{-1}) and for α-ketoglutarate was 3.6 mM ($V_{max} = 0.026$ units mg protein^{-1}).

Regulation of LAT formation by ammonium

Addition of NH_4Cl resulted in a clear reduction in the biosynthesis of cephalosporins by *S. clavuligerus* in both media (Figure 4). When MM medium was supplemented with 20 mM and 120 mM NH_4Cl, antibiotic production decreased to 42% and 8%, respectively, of the levels in the control culture. No significant effect of NH_4Cl on the pH pattern was observed. Cells grown in GSPG medium with 20 mM or 120 mM NH_4Cl suffered a drastic reduction of cephalosporin production in parallel with a strong pH decrease, from 7.2 to 4.4, due to GSPG being an unbuffered medium.

LAT specific activity was measured during and after

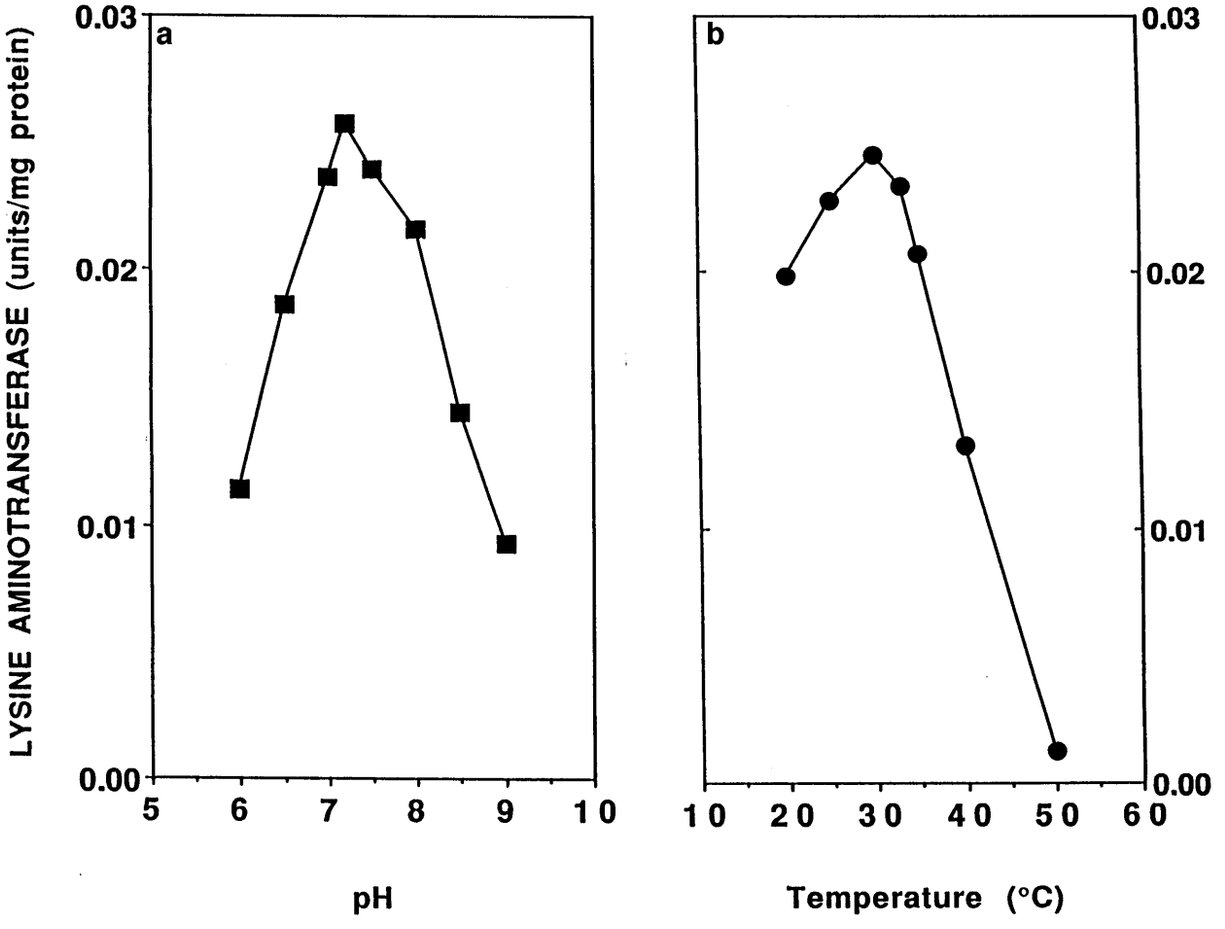

Figure 3 Effect of pH (a) and temperature (b) on activity of LAT.

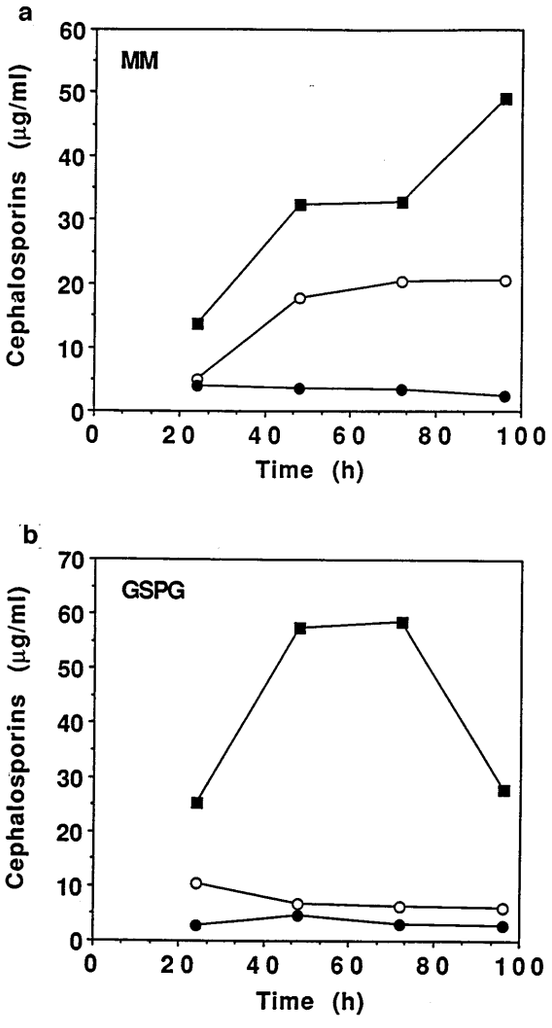

Figure 4 Effect of ammonium chloride on biosynthesis of cephalosporins by *Streptomyces clavuligerus* in (a) MM medium and (b) GSPG medium. Additions: None (■); 20 mM NH_4Cl (○); 120 mM NH_4Cl (●).

growth in the absence or presence of NH_4Cl (20 mM and 120 mM) in both media. Since LAT activity of these cultures did not peak at the same time point, samples were taken throughout the fermentation course and the maximum values are given in Table 1. The effect of NH_4Cl on LAT formation depended on the medium used but enzyme repression was not observed in either case. There were no major differences in maximum LAT activity between cultures of *S. clavuligerus* growing in GSPG with and without ammonium. On the other hand, LAT specific activity was enhanced by the addition of NH_4Cl to MM medium. To determine whether the enhancement was dependent on the lysine content of MM medium, *S. clavuligerus* was cultivated in MM medium without lysine. The enhancement of LAT activity by ammonium ions under these conditions was even higher than that observed in lysine cultures. Maximum LAT specific activity of cultures supplemented with 120 mM NH_4Cl was 220% of the activity in control unsupplemented cultures; cephalosporin production was reduced to 37% of the control value (data not shown).

The direct effect of ammonium chloride on LAT activity was also examined by adding ammonium chloride to the cell-free assay system; 80 mM NH_4Cl produced a 25–30% decrease of LAT activity.

Table 1 Effect of ammonium chloride on lysine ε-aminotransferase (LAT) activity in two different media

Growth medium	Maximum LAT activity (units mg^{-1} protein)	% of control
MM	0.84	100
MM + 20 mM NH_4Cl	1.16	138
MM + 120 mM NH_4Cl	1.42	169
GSPG	1.38	100
GSPG + 20 mM NH_4Cl	1.30	94
GSPG + 120 mM NH_4Cl	1.52	110

Discussion

S. clavuligerus converts L-lysine to 1-piperideine-6-carboxylate, with the enzyme L-lysine α-ketoglutarate ε-aminotransferase; the piperideine-6-carboxylate is further converted to L-α-aminoadipic acid. No role of α-aminoadipic acid has been described in *Streptomyces* except for antibiotic production [11,12] It seems, therefore, that the main role of LAT is to provide α-aminoadipic acid, a direct precursor of β-lactam antibiotics. The involvement of LAT in providing α-aminoadipic acid for antibiotic production is further supported by the finding that some mutants impaired in cephamycin biosynthesis show very low levels of LAT [16].

Production of β-lactam antibiotics by *S. clavuligerus* has been reported in complex and chemically-defined media. Here we employed two chemically-defined media, GSPG and MM, which had been used previously in cephalosporin biosynthesis studies [6,15]. *S. clavuligerus* exhibited higher LAT specific activity and cephalosporin production in GSPG medium than in MM medium. Sucrose present in GSPG medium is utilized very slowly [15] and mycelia grown with a slowly assimilated carbon source give substantially higher LAT specific activities and antibiotic production than do mycelia from cultures with carbon sources which are rapidly assimilated such as glycerol [1,12,20].

The negative effect of ammonium ions on β-lactam antibiotic production by *S. clavuligerus* has been reported previously [2,3,15] We observed a higher degree of NH_4 repression of cephalosporin production in GSPG medium than in MM cultures. Unlike some other enzymes of β-lactam biosynthesis [2–4,15], LAT was not repressed by ammonium ions. Whereas the specific activity of LAT was not strongly affected by including NH_4Cl in GSPG medium, ammonium ions enhanced LAT activity in MM cultures. To make sure that the enhancement of LAT activity was due mainly to the added ammonium ions, MM cultures without lysine were examined. An even greater enhancement of LAT production by ammonium was obtained in cultures grown in the absence of lysine, while the formation of cephalosporin by these cultures was less repressed by ammonium ions.

The specificity of LAT of *S. clavuligerus* for substrate

amino acids and amino group acceptors is very similar to those reported for the enzymes of *Flavobacterium* [17] and *Nocardia lactamdurans* [9]. The stimulation of LAT activity by pyridoxal phosphate resembles the effect of this cofactor on isopenicillin N epimerase of *S. clavuligerus* [19] and *N. lactamdurans* [10].

LAT formed an *o*-aminobenzaldehyde reactive compound from L-ornithine, suggesting that L-ornithine might also be used as substrate. However we have evidence that an L-ornithine-5-aminotransferase exists in *S. clavuligerus* (Liras P, Romero J and Martín JF, unpublished results). The relationship between these two enzymes is of interest with respect to antibiotic biosynthesis.

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