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Precursor Flux Control through Targeted Chromosomal Insertion of the Lysine ε-Aminotransferase (*lat*) Gene in Cephamycin C Biosynthesis

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Targeted gene insertion methodology was used to study the effect of perturbing α-aminoadipic acid precursor flux on the overall production rate of β-lactam biosynthesis in *Streptomyces clavuligerus*. A high-copy-number plasmid containing the lysine ε-aminotransferase gene (*lat*) was constructed and used to transform *S. clavuligerus*. The resulting recombinant strain (LHM100) contained an additional complete copy of *lat* located adjacent to the corresponding wild-type gene in the chromosome. Biological activity and production levels of β-lactam antibiotics were two to five times greater than in wild-type *S. clavuligerus*. Although levels of lysine ε-aminotransferase were elevated fourfold in LHM100, the level of ACV synthetase, whose gene is located just downstream of *lat*, remained unchanged. These data strongly support the notion that direct perturbation of α-aminoadipic acid precursor flux resulted in increased antibiotic production. This strategy represents a successful application of metabolic engineering based on theoretical predictions of precursor flux in a secondary metabolic pathway.

A critical component of understanding the basic biological processes of secondary metabolism involves dissecting the molecular mechanisms that control carbon flow from primary to secondary metabolic pathways (7). Although the biosynthesis of β-lactams represents one of the most thoroughly studied antibiotic pathways, there remains a fundamental lack of knowledge about the control of carbon flow into the synthesis of these metabolites. Our approach to understanding the control of β-lactam biosynthesis has been to use a two-stage strategy in which (i) a kinetic model is constructed to predict an essential rate-limiting enzymatic step and how this step is controlled by precursor flux and (ii) the model is tested by engineering a novel biosynthetic pathway that enhances precursor flux or kinetic parameters of the predicted key rate-limiting enzyme.

The wealth of information available on specific biosynthetic steps, including enzyme kinetic data, has provided unique opportunities to analyze rate-limiting reactions in construction of β-lactam antibiotics from δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) tripeptide and its amino acid precursors (5) (Fig. 1). Previously, we have developed a structured kinetic model describing the production of cephalosporins in *Cephalosporium acremonium* and *Streptomyces clavuligerus* (18, 19, 21). Simulation showed that predicted time profiles of the specific production rate during batch culture fermentation paralleled those of experimental observation. Sensitivity analysis indicated that nonribosomal condensation of ACV tripeptide from α-aminoadipic acid (α-AAA), valine, and cysteine is the rate-limiting step in the pathway. It also predicted that precursor flux, which affects the formation of ACV tripeptide, may play an important role in controlling rate of cephamycin C biosynthesis (18).

A limiting factor for precursor flux appears to involve the biosynthesis of α-AAA, a branched pathway from primary to secondary metabolism. Interestingly, the metabolic origin of α-AAA differs between fungi and actinomycetes. In fungi, this product is an intermediate of the lysine pathway (29), whereas in actinomycetes, α-AAA is a catabolic product of lysine (12, 13) (Fig. 1). Studies have shown significant effects of primary metabolites on the production levels of β-lactam antibiotics. Addition of lysine in the fermentation culture of *Penicillium chrysogenum* depressed the level of antibiotics; this depression was attributed to the feedback inhibition of homocitrate synthetase by lysine (16, 22). In contrast, addition of lysine and DL-*meso*-diaminopimelic acid in *S. clavuligerus* stimulated cephamycin C production (23), possibly by providing a larger precursor pool for biosynthesis of α-AAA or as a result of activation of aspartokinase, the first enzyme involved in lysine biosynthesis via the aspartate pathway. Furthermore, mutants resistant to the lysine analog, *S*-(2-aminoethyl)-L-cysteine, were shown to be deregulated in the lysine pathway, since aspartokinase became insensitive to concerted feedback inhibition of lysine and threonine (24). These mutants produced increased levels of cephamycin C compared with wild-type (wt) *S. clavuligerus*. Further studies of these mutants indicated that aspartokinase and DL-*meso*-diaminopimelic acid decarboxylase may be the key regulating or rate-controlling enzymes in the aspartate pathway to lysine; by relaxing the primary metabolic regulation of aspartokinase, carbon flux to the biosynthesis of β-lactams was enhanced (2). These findings along with our theoretical kinetic analysis suggest that biosynthesis of α-AAA from the aspartate pathway represents a key secondary metabolic regulatory step in carbon flux involving this important class of molecules.

To investigate whether precursor flux at the level of secondary metabolism plays an important role in determining levels of β-lactam antibiotics in *S. clavuligerus*, we decided to use chromosomal integration of a key biosynthetic gene to augment carbon flux on production of cephamycin C. Our strategy involved a unit increase in copy number of the gene (*lat*) that encodes lysine ε-aminotransferase (LAT), the first enzyme

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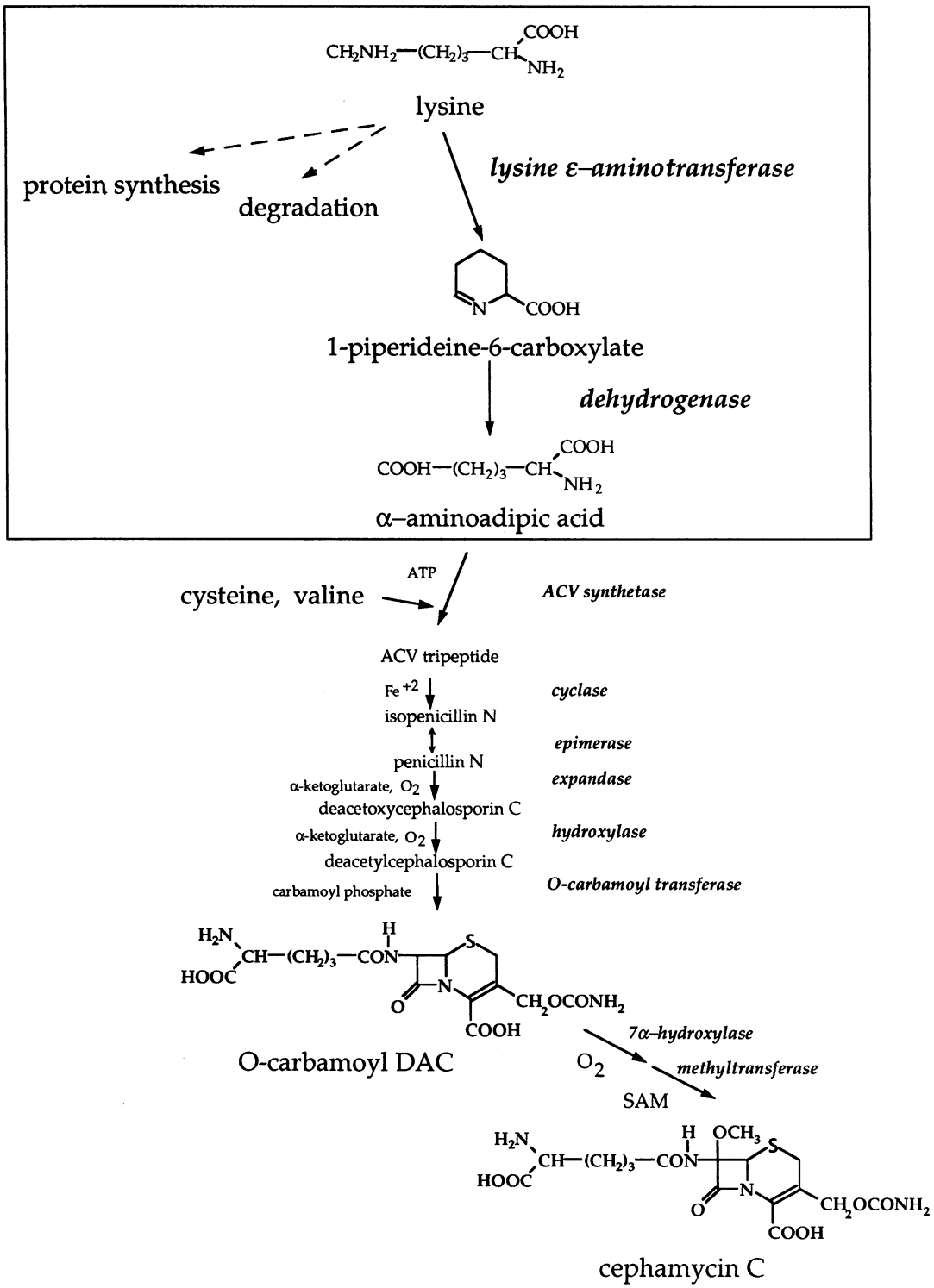

FIG. 1. Biosynthetic pathway of cephamycin C from lysine, cysteine, and valine in *S. clavuligerus*. O-carbamoyl DAC represents *O*-carbamoyl deacetylcephalosporin C.

involved in the conversion of lysine to α-AAA, which is the first dedicated secondary metabolic step in the β-lactam pathway. A targeted insertion strategy was used to generate a recombinant strain of *S. clavuligerus* that showed a number of important properties. First, production levels of cephamycin C and *O*-carbamoyl deacetylcephalosporin C increased fivefold over levels observed in the wt strain. Significantly, the production ratio of these two compounds is identical to that of the wt, which is consistent with an early-step perturbation in the biosynthetic pathway. In addition, assay of LAT revealed a

fourfold average increase in enzyme activity, whereas levels of ACV synthetase, whose structural gene is located just downstream of *lat*, remained essentially unchanged. Significantly, increased cephamycin C production was correlated with both LAT activity and extracellular accumulation of α-AAA. This is consistent with our previous theoretical prediction that precursor flux limits cephamycin C production (18). Our experimental findings indicate that increasing precursor flux is an effective strategy for enhancing biosynthesis of cephamycin C.

MATERIALS AND METHODS

Microorganisms and plasmids. All experiments were conducted with *S. clavuligerus* NRRL 3585 (ATCC 27064). *Escherichia coli* ESS, a strain supersensitive to β-lactam antibiotics, was a gift of A. L. Demain (Massachusetts Institute of Technology, Cambridge). *Streptomyces lividans* 66 (John Innes Institute strain 1326) and plasmid pIJ702 were kindly provided by D. A. Hopwood (John Innes Institute, Norwich, United Kingdom). Plasmid pDQ302 was generously provided by C. Stuttard (Dalhousie University, Halifax, Nova Scotia, Canada).

Medium and culture conditions. Spores of *S. clavuligerus* were produced on tomato-oatmeal agar (10 g of tomato paste, 10 g of oatmeal, 12.5 g of Bacto Agar, 500 ml of water, adjusted to pH 6.8) and stored at −20°C in 50% glycerol. About 10^9 spores were inoculated into a 100-ml seed culture containing the chemically defined medium of Aharonowitz and Demain (1) supplemented with 0.1% Bacto Yeast Extract and 0.1% NH_4Cl and grown at 30°C and 250 rpm for 40 h. Thirty milliliters of seed culture was inoculated into 1.5 liters of chemically defined medium without 3-(*N*-morpholino)propanesulfonic acid (MOPS). Batch fermentations were carried out in a 2-liter Multigen F-2000 fermentor (New Brunswick Scientific Co.) and were maintained at pH 6.9 with 5 N KOH and 5 M HCl at 30°C, 250 rpm, and an air sparging rate of 1.5 liters/min; foaming was prevented by adding approximately 20 ml of 10% polypropylene glycol 2000 (Dow Chemicals, Ltd.) at the beginning of fermentation. Shake flask fermentations were conducted in 2-liter baffled Erlenmeyer flasks containing 500 ml of chemically defined medium at 30°C and 250 rpm. The chemically defined medium consisted of the following (per liter): glycerol, 10 g; L-asparagine, 2 g; K_2HPO_4, 3.5 g; $MgSO_4 \cdot 7H_2O$, 1.23 g; MOPS, 20.9 g, pH 6.9; and trace salt solution, 1 ml (0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2$ per 100 ml of water).

DNA isolation and manipulation. Plasmids from *S. lividans* were obtained by the alkaline lysis method (9). Plasmids from *S. clavuligerus* were isolated by preparing and lysing protoplasts by the alkaline lysis procedure. Total chromosomal DNA from the *Streptomyces* species was obtained by the Kirby procedure (9). DNA manipulations in *E. coli* were performed as described by Sambrook et al. (26), and those in the *Streptomyces* species were performed as described by Hopwood et al. (9).

DNA labeling and Southern hybridization. Labeling of DNA was performed by nick translation with [α-^{32}P]dCTP, DNase I, and DNA polymerase I. DNA fragments were blotted onto MagnaGraph 0.45-μm-pore-size nylon membranes (Micron Separations, Inc.). Nylon membranes were incubated in prehybridization buffer (1% nonfat dry milk, 1 mM EDTA, 0.5 mM sodium phosphate [pH 7.2], 7% sodium dodecyl sulfate [SDS]) at 65°C for 20 min. After the addition of the denatured and labeled probe, hybridization was carried out at 65°C. The filters were washed three times for 10 min in 0.5% nonfat dry milk–1 mM EDTA–40 mM sodium phosphate–5% SDS at 65°C and three times for 20 min in 1 mM EDTA–20 mM sodium phosphate–1% SDS at 65°C.

Transformation of *S. clavuligerus*. The culture condition for preparation of protoplasts was a modification of the method of Bailey and Winstanley (3). About 10^8 spores were inoculated into 50 ml of tryptic soy broth in a 250-ml baffled flask with a coiled spring and grown at 26°C for 48 h. Two milliliters of this culture was then inoculated into a new flask containing 20 ml of tryptic soy broth and 30 ml of YEME (9) supplemented with 3 mM $MgCl_2$ and 0.5% (wt/vol) glycine.

After 24 h, mycelia were harvested and protoplasts were prepared according to the method of Leskiw et al. (14). Transformation of protoplasts and regeneration were performed by a modification of the procedures of Hopwood et al. (9), Leskiw et al. (14), and Bailey and Winstanley (3). Twenty-five milliliters of culture was collected by centrifugation at 3,000 × *g* for 15 min and washed twice with 10.3% sucrose. The pellet was resuspended in 2 ml of 1-mg/ml lysozyme in P buffer containing 0.3 M sucrose, 0.57 mM K_2SO_4, 25 mM MOPS (pH 7.2), 1 ml of trace elements [0.04 g of $ZnCl_2$, 0.2 g of $FeCl_3 \cdot 6H_2O$, 0.01 g of $CuCl_2 \cdot 2H_2O$, 0.01 g of $MnCl_2 \cdot 4H_2O$, 0.01 g of $Na_2B_4O_7 \cdot 10H_2O$, 0.01 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per liter], and 1% (wt/vol) bovine serum albumin (BSA) and then incubated at 30°C for 15 min. After trituration three times with a pipette before and after dilution with 2.5 ml of P buffer, the protoplast suspension was filtered through a sterile cotton plug. The filtered protoplasts were centrifuged at 1,000 × *g* for 10 min, washed three times with P buffer, and counted in a hemacytometer. Approximately 10^9 protoplasts were centrifuged and suspended in the drop of P buffer left after the supernatant was decanted. The protoplasts were then incubated in a 42°C water bath for 10 min to inactivate the restriction system in *S. clavuligerus* (3). The heated protoplasts were transformed with 1 μg of DNA, immediately mixed with 0.5 ml of 25% (wt/vol) polyethylene glycol 1000 (NBS Biologicals, Hatfield, United Kingdom) in P buffer without BSA, and then triturated once with a P1000 pipetman. After 1 min at room temperature, the protoplasts were diluted with 2.5 ml of P buffer, centrifuged at 2,000 × *g* for 10 min, and finally resuspended in 1 ml of P buffer. The transformed protoplasts were plated on regeneration plates with R2YEG (171 g of sucrose, 20 g of Bacto Agar, 5.0 g of Bacto Yeast Extract, 11.0 g of monosodium glutamate, 1 g of Casamino Acids, 0.1 g of K_2SO_4, 0.05 g of $MgSO_4$, 10 ml of glycerol, 2 ml of trace elements per liter) and incubated at 26°C. After 42 h, the plates were overlaid with 2.5 ml of R2YEG with 0.6% agar and 50 μg of thiostrepton per ml. Thiostrepton-resistant colonies became visible after 4 days; transformants were transferred to thiostrepton-containing tomato-oatmeal sporulation plates after 14 days.

Determination of cell growth. Cell growth was monitored by the optical density of a suspension of broken mycelia at 595 nm (OD_{595}), as modified from the procedure by Brana et al. (4). A total of 0.5 ml of cell culture was added to a tube containing 0.5 ml of 2.5 M HCl and 3 ml of water. The mixture was homogenized by ultrasonification for 30 s. The OD of the resulting suspension was then measured. Dilution was taken to maintain an OD_{595} of less than 0.6, at which a linear correlation between dry cell weight and OD_{595} was observed. One OD_{595} unit was equivalent to 0.59 mg of dry cell weight per ml.

Bioassay of β-lactam antibiotics. Antibiotics were determined by the agar plate diffusion assay, with *E. coli* ESS as an indicator microorganism seeded in nutrient broth with 0.8% agar. Cephalosporin C was used as the standard. One unit of β-lactam produces an inhibition zone equivalent to that formed by 1 g of cephalosporin C.

Identification and quantitation of antibiotics by HPLC. The high-performance liquid chromatography (HPLC) system was

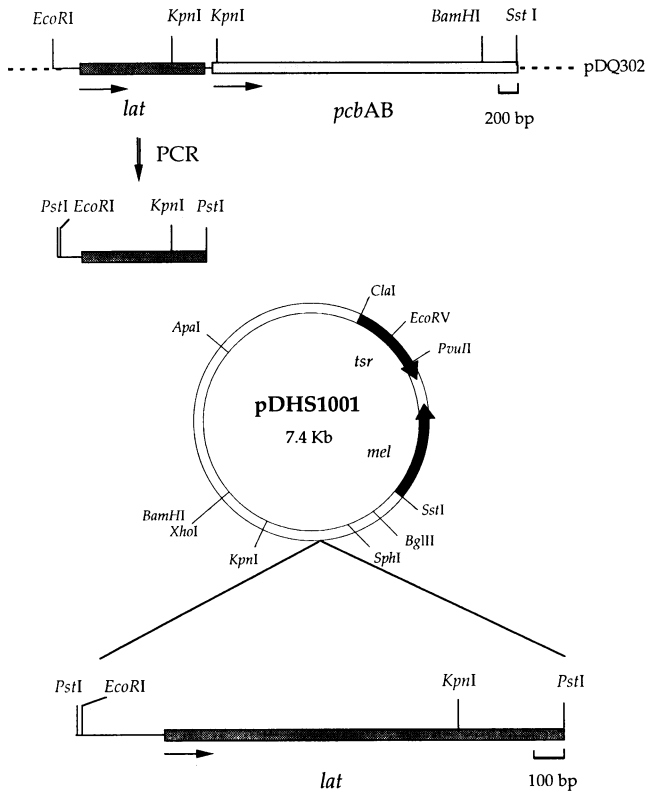

FIG. 2. Construction of pDHS1001. The top portion shows the partial restriction map of the 4.7-kb *Eco*RI-*Sst*I DNA fragment of *S. clavuligerus* cloned in pDQ302 that contains *lat* and part of the *pcbAB* genes. A 1.6-kb PCR fragment containing the *lat* gene was ligated into the *Pst*I site of pIJ702 to create pDHS1001. The shaded boxes indicate open reading frames. The arrows show the direction of transcription. *lat* and *pcbAB* encode LAT and ACV synthetase, respectively.

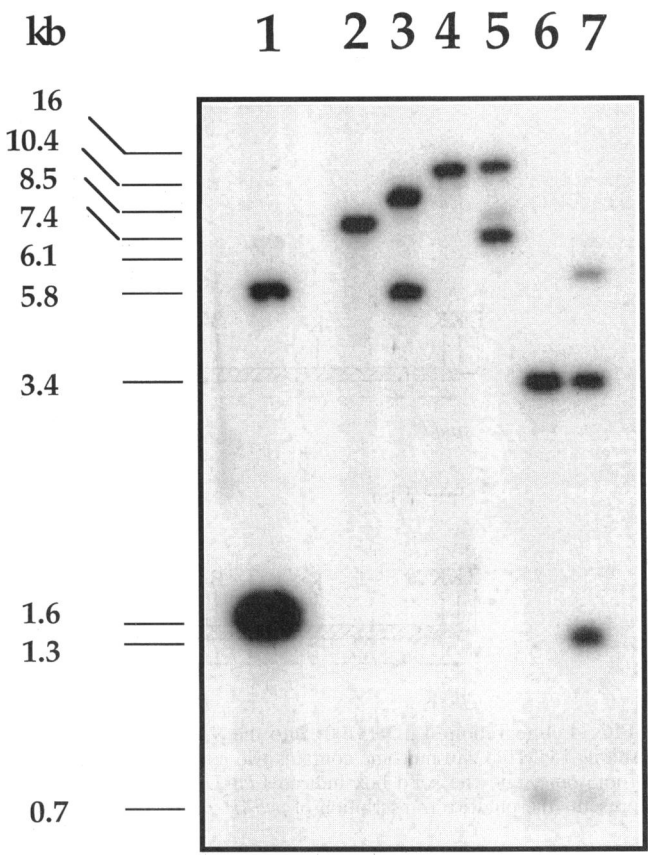

FIG. 3. Southern blot analysis of recombinant strain LHM100 (lanes 3, 5, and 7) and its parent, strain NRRL3585 (lanes 2, 4, and 6). Total DNA from these strains, digested with *Bam*HI (lanes 2 and 3), *Eco*RI (lanes 4 and 5), or *Kpn*I (lanes 6 and 7), was loaded on a 0.7% agarose gel. pDHS1001 was digested with *Pst*I, resulting in two separate fragments (lane 1). The 5.8-kb fragment contains pIJ702, and the 1.6-kb fragment contains the *lat* gene. The filter was probed with a 1.6-kb *Pst*I fragment isolated from pDHS1001.

a Rainin liquid chromatograph with an HPXL solvent delivery system, Rheodyne model 7125 sample injector, and Dynamax absorbance UV-D detector (detected wavelength, 254 nm). Twenty-microliter fermentation samples were injected on an Alltech Econosil C_{18} 10-μm column (25 cm by 4.6 mm) and eluted at a flow rate of 1 ml/min. The two mobile phases used were A (14 mM sodium phosphate buffer containing 3.5 g of tetrabutylammonium hydrogen sulfate per ml adjusted to pH 6.5 with NaOH) and B (methanol) (8). Elution was carried out with a linear gradient over 30 min from 0% B in A to 5% B in A. The peaks corresponding to β-lactam antibiotics were identified and quantified by comparing chromatograms of authentic samples of deacetylcephalosporin C, deacetoxycephalosporin C, *O*-carbamoyl deacetylcephalosporin C, and cephamycin C. The antibiotic standards were gifts of J. R. Miller (Eli Lilly and Co., Indianapolis, Ind.) except cephamycin C, which was provided by J. V. Heck (Merck and Co., Rahway, N.J.).

Preparation of cell extracts. Twenty-five to 50 ml of sample culture was harvested at the times noted in Fig. 8. Mycelia were washed twice with 0.05 M MOPS-KOH (pH 7.5) containing 0.1 M KCl and suspended in an equal volume of cold 0.1 M MOPS-KOH buffer (pH 7.5) containing 20 mM EDTA and 50% glycerol (vol/vol) and were disrupted by sonication in an ice-water bath with a Branson sonifier (30% strength for 1 min). Cell debris was removed by centrifugation for 25 min at 14,000 × *g* and 4°C. The supernatant fluid was stored at −70°C and later used in assays of LAT and ACV synthetase activities.

LAT assay. Cell extracts were desalted by passage through a Sephadex G-25 column (P-10; Pharmacia) with an elution buffer containing 0.2 M K_2HPO_4-KH_2PO_4 buffer (pH 7.5). The desalted cell extract was assayed immediately for LAT on the basis of the method of Kern et al. (12).

ACV synthetase assay. The cell extract was desalted by passage through a Sephadex G-25 column (P-10; Pharmacia) with an elution buffer containing 0.1 M MOPS-KOH (pH 7.5). The desalted cell extract was used immediately for the ACV synthetase assay developed by Zhang et al. (30). The ACV concentration was estimated by comparing the peak areas with those of ACV standards, provided by S. E. Jensen (University of Alberta, Edmonton, Alberta, Canada). For both LAT and ACV synthetase assays, one unit of enzyme is defined as the amount producing 1 μmol of product per min. Protein in cell extracts was determined by the method of Lowry et al. (15), using BSA as a standard.

Chemicals. *o*-Aminobenzaldehyde, α-ketoglutarate, lysine, α-aminoadipic acid, and pyridoxal-5-phosphate were purchased from Sigma Chemical Co. (St. Louis, Mo.). Monobromobimane (Thiolyte reagent), valine, and cysteine were from Calbiochem (San Diego, Calif.). An ion-pairing reagent, tet-

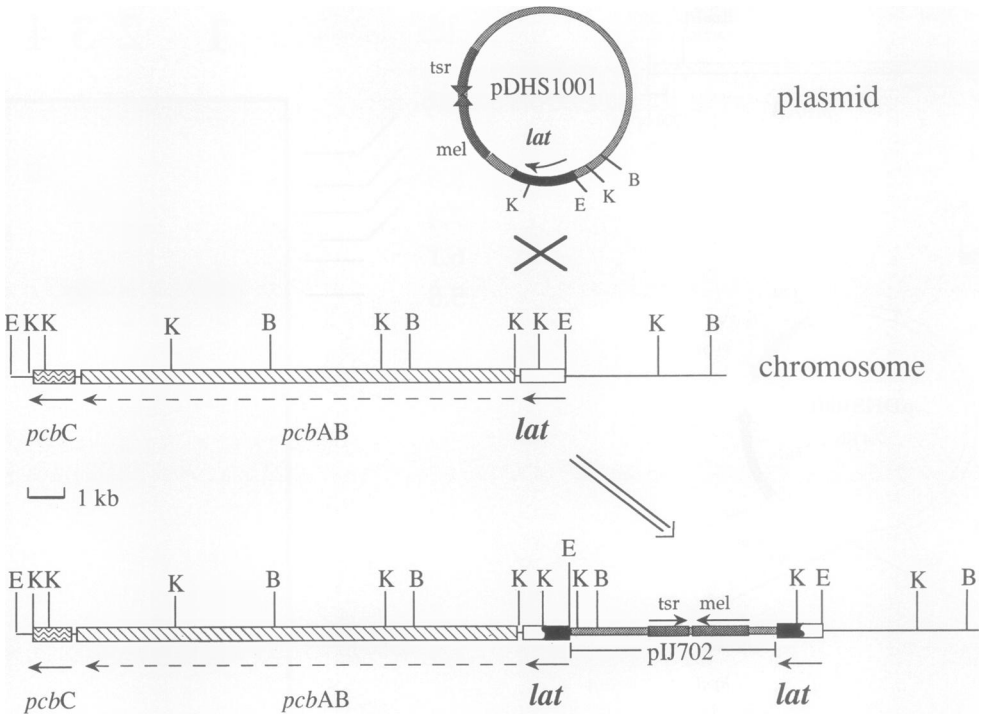

FIG. 4. Integration of pDHS1001 into the *S. clavuligerus* chromosome. pDHS1001 was inserted into the *lat* gene by a single crossover. The resulting LHM100 chromosome contains two copies of *lat* separated by plasmid pIJ702. The open box indicates the resident *lat* DNA from the chromosome, and the solid box indicates *lat* DNA from pDHS1001. The solid arrows show the direction of transcription. The dashed line represents the putative transcription of *pcbAB*. *pcbC* codes for isopenicillin N synthase.

rabutylammonium hydrogen sulfate, was purchased from Kodak (Rochester, N.Y.). Restriction endonucleases and other DNA-modifying enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). All other fine chemicals were of reagent grade and were purchased from Sigma Chemical Co.

RESULTS

Construction of *lat* integration vector. In order to investigate the effect of increasing precursor flux of α-AAA on cephamycin C production in *S. clavuligerus*, we generated a construct containing the *lat* gene (1.4 kb) and an additional 228 bp upstream of the translational start codon in the high-copy-number plasmid pIJ702 (see Fig. 2). The upstream region was used to ensure inclusion of the *lat* promoter, which had been inferred through determination of the *lat* mRNA transcriptional start point (10). Two oligonucleotide primers, CAAC CTGCAGTCAGACGCTCTCGGCGACCG and GAGTCTG CAGGAATTCCCCTGAACACGAAG, were used to generate the 1.6-kb DNA product comprising *lat* and the upstream region. The polymerase chain reaction (PCR)-generated DNA was designed to include *Pst*I sites at both ends of the fragment to facilitate subsequent cloning (Fig. 2). pDQ302, an *E. coli-Streptomyces* shuttle vector which includes a 4.7-kb *Sst*I-*Eco*RI fragment from *S. clavuligerus*, contains the *lat* gene (and has been shown to express LAT activity in *S. lividans* and *E. coli*) (17) and was used as a template for PCR (Fig. 2). The amplified fragment was subsequently ligated into the *Pst*I site of pIJ702. Transformation was performed first in *S. lividans*, and pDHS1001 was identified by colony hybridization with the *lat* PCR product as a hybridization probe (Fig. 2). *S. clavuligerus* protoplasts were then transformed with pDHS1001 isolated from *S. lividans*, using thiostrepton to select transformants containing the desired plasmid.

Characterization of pDHS1001 transformants in *S. clavuligerus*. pDHS1001 transformants of *S. clavuligerus* were collected and maintained on 5-μg/ml thiostrepton tomato-oatmeal agar sporulation plates. Two morphologically distinct populations developed following subsequent rounds of propagation on agar plates. Population A (LHM101) grew poorly, did not form white mycelium, and produced high levels of melanin, presumably by expression of the *mel* gene in pDHS1001. Population B (LHM100) grew normally, formed white mycelium, and lacked visible melanin production. LHM100 remained stable after multiple rounds of liquid culture in the absence of thiostrepton and was stable on agar medium with or without selection by thiostrepton. pDHS1001 was recovered from LHM101, but it was absent from plasmid preparations of LHM100. To address whether pDHS1001 had integrated into the *S. clavuligerus* chromosome, Southern hybridization of genomic DNA from wt *S. clavuligerus* and the Ts^r strain LHM100, lacking autonomous plasmid, was performed. Initially, the genomic DNA from wt *S. clavuligerus* and LHM100 was probed with pIJ702 and showed clearly that pIJ702 was present in the chromosome of strain LHM100 and absent from the wt strain of *S. clavuligerus* (data not shown). The observed integration had presumably occurred by homologous recombination between *lat* in pDHS1001 and the *S. clavuligerus* chromosome. To determine the site of integration and the copy number of pDHS1001, genomic DNA of the wt and strain LHM100 was digested with selected restriction enzymes and probed with the 1.6-kb *Pst*I fragment containing *lat* (Fig. 3). On the basis of the hybridization patterns and intensities of each band from the wt and strain LHM100, one copy of pDHS1001 was inserted into

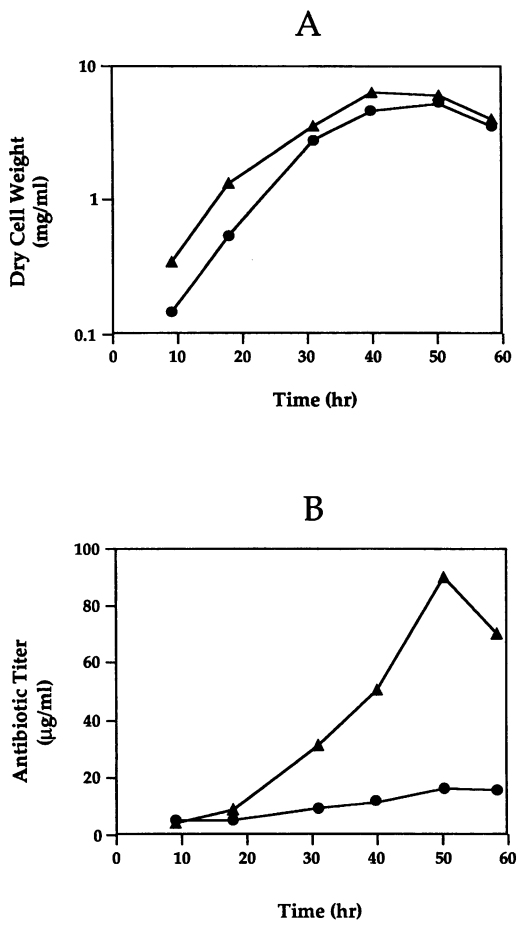

FIG. 5. Growth (A) and antibiotic production (B) of LHM100 (▲) and the wt (●). Five hundred-milliliter cultures were grown in 2-liter baffled shake flasks with seed medium at 30°C and 250 rpm. The antibiotic concentration was determined by HPLC and expressed as the sum of *O*-carbamoyl deacetylcephalosporin C and cephamycin C.

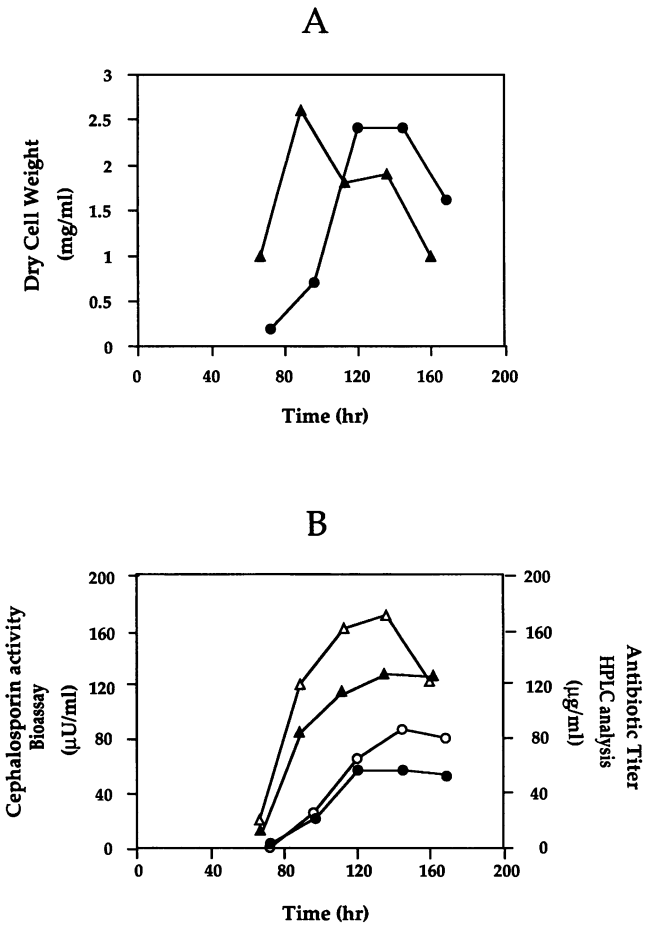

FIG. 6. Growth (A) and antibiotic production (B) of LHM100 (▲) and the wt (●). Cultures of 1.5 liters each were grown in a 2-liter fermentor with defined medium and 1 μg of thiostrepton per ml in the LHM100 culture. Open symbols represent the cephalosporin activity determined by bioassay, and solid symbols represent the antibiotic concentration measured by HPLC analysis.

a site within the cephamycin C gene cluster by single crossover into the resident *lat* gene (Fig. 4). Although the precise crossover point between resident and plasmid-borne *lat* genes cannot be determined, it is clear that both copies of the gene are transcribed from right to left, as shown in Fig. 4, and are separated by the vector pIJ702. The relative positions of the *mel* and *tsr* genes are shown, although the direct influence that these or other genes contained within pIJ702 may have on transcription of each *lat* gene is not clear.

LHM100 produces two- to fivefold-higher levels of cephamycin C than wt *S. clavuligerus*. To examine the effect of an additional copy of *lat* on production of cephamycin C and its immediate precursor *O*-carbamoyl deacetylcephalosporin C, production levels of each antibiotic in liquid cultures of the wt and recombinant LHM100 strains were determined. Fermentations in shake flasks and 2-liter fermentors indicated that LHM100 produced significantly higher levels of cephamycin C than wt *S. clavuligerus* (Fig. 5 and 6). Shake flask fermentations of LHM100 consistently gave five times greater antibiotic production than wt *S. clavuligerus* while maintaining essentially identical growth kinetics (Fig. 5 and 6). In the 2-liter fermentor, both bioassay and HPLC analysis of the culture broth revealed that LHM100 produced a twofold-higher level of cephamycin C than the wt (Fig. 6). The disparity of these two fermentation runs may be due to differences in the culture medium used and, in particular, the use of thiostrepton selection in the 2-liter growth medium. Significantly, HPLC analysis of β-lactams showed that the production ratio of the two major β-lactam antibiotics, *O*-carbamoyl deacetylcephalosporin C and cephamycin C, remained invariant between the wt and recombinant (LHM100) strains (Fig. 7).

In contrast to LHM100, LHM101, which contains pDHS 1001 at high copy number as an autonomous plasmid, was found to have no biological activity against the *E. coli* ESS indicator strain. HPLC confirmed that the strain failed to produce the antibiotics *O*-carbamoyl deacetylcephalosporin C and cephamycin C (data not shown). Significantly, *S. clavuligerus* transformed with pIJ702 alone showed 20% lower levels of antibiotic production. Such plasmid effects have been observed previously with other antibiotic-producing streptomycetes (27). Further analysis of LHM101 will be required to determine why antibiotic production was completely abrogated.

LHM100 exhibited fourfold-higher LAT activity than wt *S. clavuligerus*. To examine whether an additional copy of *lat* resulted in higher activity of LAT in LHM100 than in the wt, enzyme activities produced by these two strains during fermen-

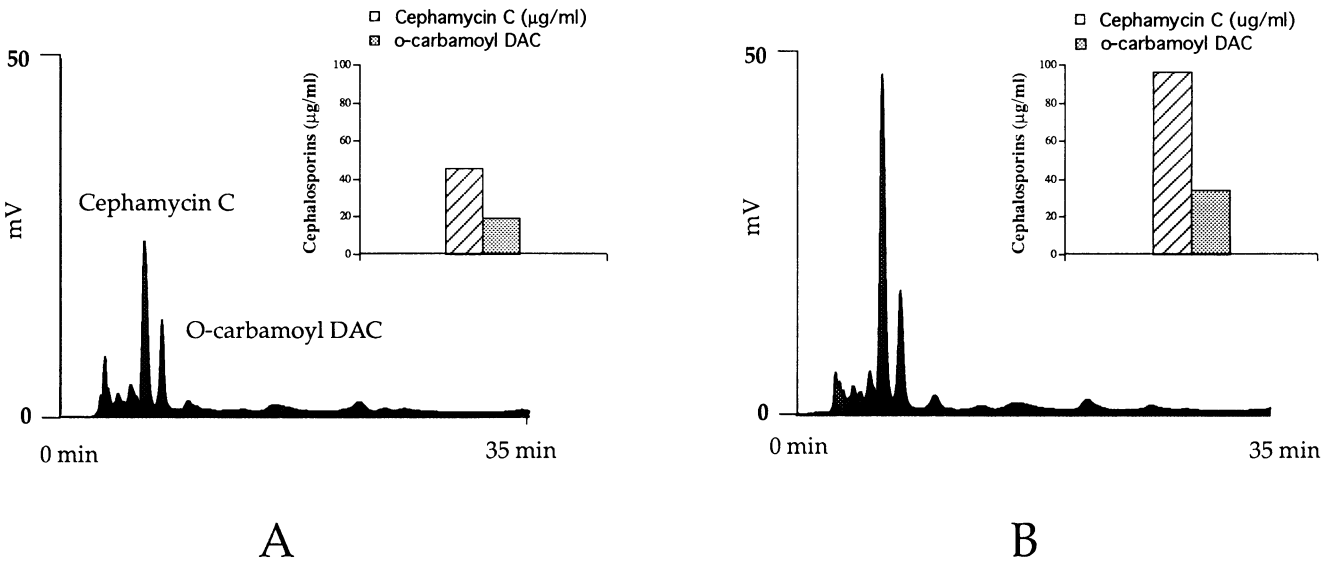

FIG. 7. HPLC chromatograms of antibiotics in the culture broth. Samples were taken from the fermentation culture of the wt strain (A) at 120 h and LHM100 (B) at 138 h as shown in Fig. 6.

tation were determined. LHM100 showed consistently higher LAT activity than wt *S. clavuligerus* (Fig. 8A). This increase ranged from two- to ninefold during the culture growth and was considerably greater than expected from one additional copy of *lat* in the cephamycin C gene cluster.

To assess whether transcriptional activation of the presumed *lat/pcbAB* operon had occurred from a vector promoter in LHM100, with a subsequent increase in ACV synthetase activity, cell extracts of the wt and LHM100 strains were also assayed for ACV synthetase activity. HPLC analysis of the cell extracts containing ACV synthetase activity from the wt and LHM100 showed no increase in enzyme level in LHM100 (Fig. 8B). It seems quite likely that transcriptional activation of *lat* from a promoter within pIJ702 occurred; however, this activation does not increase the level of ACV synthetase, which is encoded by a gene just downstream of *lat* (Fig. 4).

DISCUSSION

A critical physiological parameter that influences the level of production of antibiotics by microorganisms is carbon flux from primary to secondary metabolism. In order to increase production levels of specific metabolites by rational design, it is essential to determine inefficient steps in the pathway which may be targeted and improved through molecular genetic manipulation. This report describes the second part of a two-part strategy to enhance the production level of cephamycin C in *S. clavuligerus*. The first part involved kinetic analysis and simulation of the pathway to predict potential rate-limiting enzymatic steps (18, 19, 21). The second part, described in this report, involved enhancing the identified rate-limiting step through designed pathway construction at the genetic level.

This work describes an application of a metabolic engineering strategy involving the effect of an additional copy of *lat* on production of *O*-carbamoyl deacetylcephalosporin C and cephamycin C in *S. clavuligerus*. This perturbation resulted in a fivefold increase in cephamycin C production, which appears to have been caused by a parallel increase in the intracellular level of LAT. The evidence described in this report, therefore, strongly suggests that deamination of lysine is one of the key rate-limiting steps in the biosynthesis of cephamycin C. This is consistent with our earlier theoretical analysis that precursor pools and ACV synthetase are the predicted controlling parameters modulating antibiotic production levels. We propose that increasing LAT activity resulted in a metabolic shift of lysine from charging lysine-tRNA for protein synthesis to synthesis of α-AAA. A higher conversion rate from lysine corresponds to a larger α-AAA precursor pool for conversion to ACV tripeptide, thereby increasing antibiotic production. This interpretation was supported further by showing that the stimulatory effect of lysine on production of cephamycin C, which was observed during fermentation of the wt strain, did not occur in LHM100 (data not shown).

The role of carbon flow in supplying amino acid precursors for antibiotic biosynthesis in *S. clavuligerus* was first investigated by Aharonowitz et al. (2). They showed that aspartokinase-deregulated mutants produced five times more cephamycin C than did the wt. An additional effect of deregulating the aspartate pathway was an increased intracellular concentration of diaminopimelic acid, which was shown to be stimulatory to antibiotic synthesis. However, the fundamental basis for a higher antibiotic titer by such deregulation, for example, increased metabolic flux for α-AAA, was not investigated. It would be interesting to determine whether manipulation of both aspartokinase (regulation) and LAT (copy number) results in increased levels of intracellular α-AAA.

Our strategy for increasing the copy number of *lat* by chromosomal insertion was chosen because it was least likely to cause pleiotropic effects on the cephamycin C biosynthetic pathway or on the developmental life cycle of *S. clavuligerus*. Indeed, control experiments with pIJ702 alone resulted in consistently lower levels (20%) of antibiotic production (data not shown). Plasmid effects are common in antibiotic-producing streptomycetes and often lead to lower overall yields of secondary metabolites (27). Although pIJ702 is not known generally to undergo chromosomal integration or mediate homologous recombination in *Streptomyces* spp., an earlier study suggested that such an event can occur in the *S. clavuligerus* chromosome (6). It remains unclear why a single additional copy of *lat* leads to a corresponding fourfold average

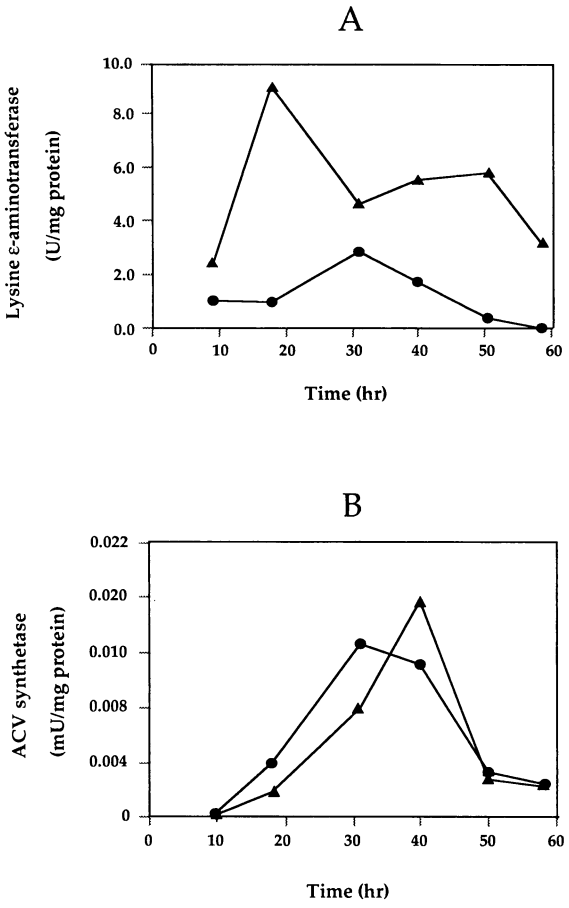

FIG. 8. Specific activity of LAT (A) and ACV synthetase (B) from LHM100 (▲) and the wt (●) during fermentation. Cell extracts were taken from the same culture as for Fig. 5.

increase in LAT activity. On the basis of the location of *lat* within the integrated copy of pDHS1001 (in LHM100), it is conceivable that considerable transcriptional read-through occurs from the pIJ702 *rep* region that is adjacent to the *Pst*I cloning site chosen for this work (11). Alternatively, a potential monocistronic transcript corresponding to the upstream *lat* mRNA may have enhanced stability and thus an increased rate of translation.

The presumed *pcbAB* gene encoding ACV synthetase in *S. clavuligerus* is located adjacent to *lat* and is separated by a 125-bp noncoding region of DNA (28). Analysis of the DNA sequence in this region showed that an inverted repeat (28), corresponding to a potential transcriptional terminator, separates the two genes. The significance of this repeat has not been determined, and there is contradictory evidence as to whether *lat/pcbAB* comprises a single polycistronic message (10, 25). This was potentially significant to our expression strategy because transcriptional activation of a *lat/pcbAB* polycistronic message could have led to the concomitant increase in both LAT and ACV synthetase activities. This would have complicated our analysis concerning the role of precursor flux in determining cephamycin C production, since ACV synthetase is predicted to be a key rate-limiting step as well. However, our analysis has shown that the contribution from ACV synthetase is insignificant since there was no increase in the level of this enzyme in LHM100.

LHM100 was shown to accumulate 80% more α-AAA in the fermentation broth than wt *S. clavuligerus* (20), indicating that the intracellular concentration of α-AAA may be higher in LHM100. The addition of lysine to the LHM100 fermentation medium did not stimulate the antibiotic production rate, suggesting that the intracellular pool of α-AAA in LHM100 may be in excess. Specifically, the intracellular concentration of α-AAA exceeds its K_m value. Therefore, the addition of lysine will not increase the production of cephamycin C when ACV synthetase is saturated with α-AAA. On the basis of this observation and our previous analysis showing ACV synthetase as a predicted rate-limiting enzyme in the cephamycin C secondary metabolic pathway, we propose the following hypothesis. In the wt strain, both LAT and ACV synthetase are rate-limiting enzymes; however, in LHM100, ACV synthetase alone becomes the primary rate-limiting enzyme. In order to improve further the biosynthesis of cephamycin C in LHM100, the metabolic flux from precursors to ACV tripeptide must be relieved by increasing ACV synthetase activity. Thus, it is now critical to examine the effect of increasing ACV synthetase activity in both the wt and LHM100 strains of *S. clavuligerus*. Augmentation of ACV synthetase activity will allow us to evaluate the relative control of precursor flux and ACV synthetase activity on the biosynthesis of cephamycin C.

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