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GENETICAL AND PHYSIOLOGICAL STUDIES OF LYSINE CATABOLISM AND IMPLICATIONS FOR β-LACTAM BIOSYNTHESIS IN STREPTOMYCETES

by

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To Amma and Nanna

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ABSTRACT

Biochemical and genetic analyses of lysine catabolism have revealed the presence of diverse lysine catabolic pathways in streptomycetes. The α-aminoadipate (AAA) pathway which generates a precursor, α-aminoadipate, for β-lactam biosynthesis is present only in β-lactam producers and is a secondary metabolic pathway not necessary for growth under any known conditions. The cadaverine pathway is present in all actinomycetes tested and is used to catabolize lysine when needed as a nitrogen source. S. venezuelae has an additional minor pathway for assimilation of lysine via D-lysine, pipecolate and AAA.

The cadaverine pathway enzyme, cadaverine aminotransferase (CAT), is induced by its substrate and is strongly depressed by well-metabolized carbon sources such as glycerol and starch, and mildly by nitrogen sources such as glutamate. Lysine ε-aminotransferase (LAT), the enzyme catalyzing the first step in the AAA pathway, is not induced by its substrate but is depressed by glycerol. There is a positive correlation between phosphate concentration and LAT activity.

The putative LAT gene is present in a cluster of genes encoding early enzymes governing β-lactam biosynthesis and is located between genes pcbC and cefE. In contrast, the cadaverine pathway genes are not adjacent to β-lactam biosynthesis genes. The two lysine catabolic pathways are apparently not interchangeable, consistent with the conclusion that the AAA pathway functions specifically in secondary metabolism. The sequence encoding LAT is present only in β-lactam producers; hence, the conversion of lysine to piperideine-6-carboxylate by LAT is the first step in β-lactam biosynthesis in Streptomyces.

ABBREVIATIONS AND SYMBOLS

g	grams
mg	milligrams
µg	micrograms
ng	nanograms
l	litres
ml	millilitres
µl	microlitres
cm	centimetres
mm	millimetres
h	hours
min	minutes
s	seconds
M	molar
mM	millimolar
µmol	micromoles
nmol	nanomoles
g/l	grams per litre
w/v	weight per volume
v/v	volume per volume
°C	degrees celsius
U	units
cfu	colony forming units
SDS	sodium dodecyl sulfate
TE	Tris-EDTA buffer
EDTA	ethylenediamine tetraacetic acid

Tris	tris-hydroxymethylaminomethane
TES	tris-(hydroxymethyl)methylaminoethanesulfonic acid
PEG	polyethylene glycol
DTT	dithiothrietol
ATP	adenosine 5'-triphosphate
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanidine 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
UV	ultra violet
AAA	α-aminoadipic acid
ACV	δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine
ACVS	ACV synthetase
IPNS	isopenicillin N synthetase
DAOCS	deacetoxycephalosporin C synthetase
6-APA	6-aminopenicillanic acid
cAMP	cyclic adenosine 5'-monophosphate
CAP	catabolite gene activator protein
HSD	homoserine dehydrogenase
DDPS	dihydrodipicolinic acid synthetase
AS	aspartate semialdehyde
AK	aspartate kinase
DAP	diaminopimelic acid
DNA	deoxyribonucleic acid
mRNA	messenger ribonucleic acid

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INTRODUCTION

Over 60 years ago the first β-lactam antibiotic was discovered by Alexander Fleming, yet β-lactams continue to elicit a great deal of interest, not only because of their clinical importance but also because of the biological and biochemical insights generated from studies on their production. β-Lactam antibiotics are produced by a wide variety of organisms, both procaryotes and eucaryotes; their antibacterial activity is due to their inhibition of murein cell wall biosynthesis. Because of the specificity of their mode of action, they continue to be among the most effective and safe antibiotics in chemotherapeutic use.

β-Lactam antibiotics can be divided into several groups such as penams, cephems, oxapenams, carbapenems and monobactams, based on the component associated with the β-lactam structure. The penam-cephem groups are both synthesized from the tripeptide intermediate aminoadipylcysteinylvaline. The penams can be subdivided into a hydrophobic class of which penicillin G (benzyl penicillin) is an example, and a hydrophilic class represented by isopenicillin N. Cephems, which include cephalosporins and cephamycins, are also hydrophilic β-lactams. Cephalosporins differ from penicillins in having a six-membered dihydrothiazine ring instead of a five-membered thiazolidine ring. One effect of this difference in the ring stucture is to make them more resistant to bacterial β-lactamases. Hydrophobic β-lactams are produced only by certain fungi such as Penicillium chrysogenum and Aspergillus nidulans. Hydrophilic β-lactams are produced by fungi, such as Cephalosporium acremonium, and also by

prokaryotes, notably the genera Streptomyces, Nocardia and Flavobacterium.

β-Lactam antibiotics have been studied extensively and information is available not only on their biosynthetic pathways but also on their enzymology and on the genetics of their production. The role of primary metabolism in generating precursor molecules for β-lactam biosynthesis has also been subjected to detailed investigation; one of the aims of the latter studies has been to understand the relationship between primary and secondary metabolism. β-Lactam biosynthesis requires three precursors namely α-aminoadipic acid (AAA), cysteine and valine. Cysteine and valine are products of primary metabolism and are incorporated directly into penam and cephem rings. However, in bacteria AAA is a product of lysine catabolism and not a primary metabolite, whereas in eukaryotes it is an intermediate in lysine biosynthesis.

Since AAA is a limiting precursor in β-lactam biosynthesis (Inamine and Birnbaum, 1973; Hallada et al., 1975), efforts to improve pool concentrations of AAA in actinomycetes should lead to increased antibiotic production. Success in raising the AAA pool size through genetic approaches requires a knowledge of the function of AAA. Establishing a role for the AAA pathway of lysine catabolism in growth and secondary metabolism in actinomycetes therefore became the main focus of this investigation. Whether this or a different pathway is used for growth of Streptomyces on lysine as the nitrogen source and whether the AAA pathway is present in β-lactam nonproducers as well as in β-lactam-producing strains were questions addressed in the early part of the study.

There were three other objectives that developed from the initial investigation: 1) to examine the regulation of the AAA pathway and that of other lysine catabolic pathways in streptomycetes; 2) to clone the genes governing the AAA biosynthesis pathway and analyze the organization and location of these genes; and 3) to clone genes for the primary lysine catabolic pathway and to compare their location and organization with those of the AAA pathway. A fourth and practical objective of discovering whether cloned lat DNA included a putative piperideine-6-carboxylate oxidoreductase gene and allowed high levels of AAA to be made in S. clavuligerus thus boosting β-lactam levels, remains to be achieved.

REVIEW OF THE LITERATURE

In the following chapter, what was known when this work began about the biosynthesis of β-lactam antibiotics of the "classical" type from amino acid intermediates is summarized. I also briefly discuss current knowledge of the physiology, regulation and molecular biology of these antibiotics.

I. Biosynthesis

The penam and cephem groups of β-lactam antibiotics are assembled from α-aminoadipate (AAA), cysteine and valine. In the early steps of β-lactam biosynthesis, AAA, cysteine and valine are condensed to a tripeptide δ-(α-aminoadipyl)-cysteinyl-valine (ACV) by the enzyme ACV synthetase (ACVS). The tripeptide is then cyclized to isopenicillin N by a second enzyme, isopenicillin N synthetase (IPNS) (Fig. 1).

A. Biosynthesis of isopenicillin N

First observed in the intracellular pool of P. chrysogenum by Arnstein and Morris (1960) during isotope labelling experiments, , ACV was later identified as the tripeptide precursor of cephalosporin (Addlington et al., 1983). The valine component of ACV was in the D-configuration, even though the precursor amino acid was used in its L-form (Arnstein and Margreiter, 1958; Stevens and Delong, 1958). When cell extracts of C. acremonium were incubated with radiolabelled AAA, cysteine or valine, Addlington and coworkers (1983) found radioactivity not only in ACV but also in δ(α-aminoadipyl)cysteine and suggested that

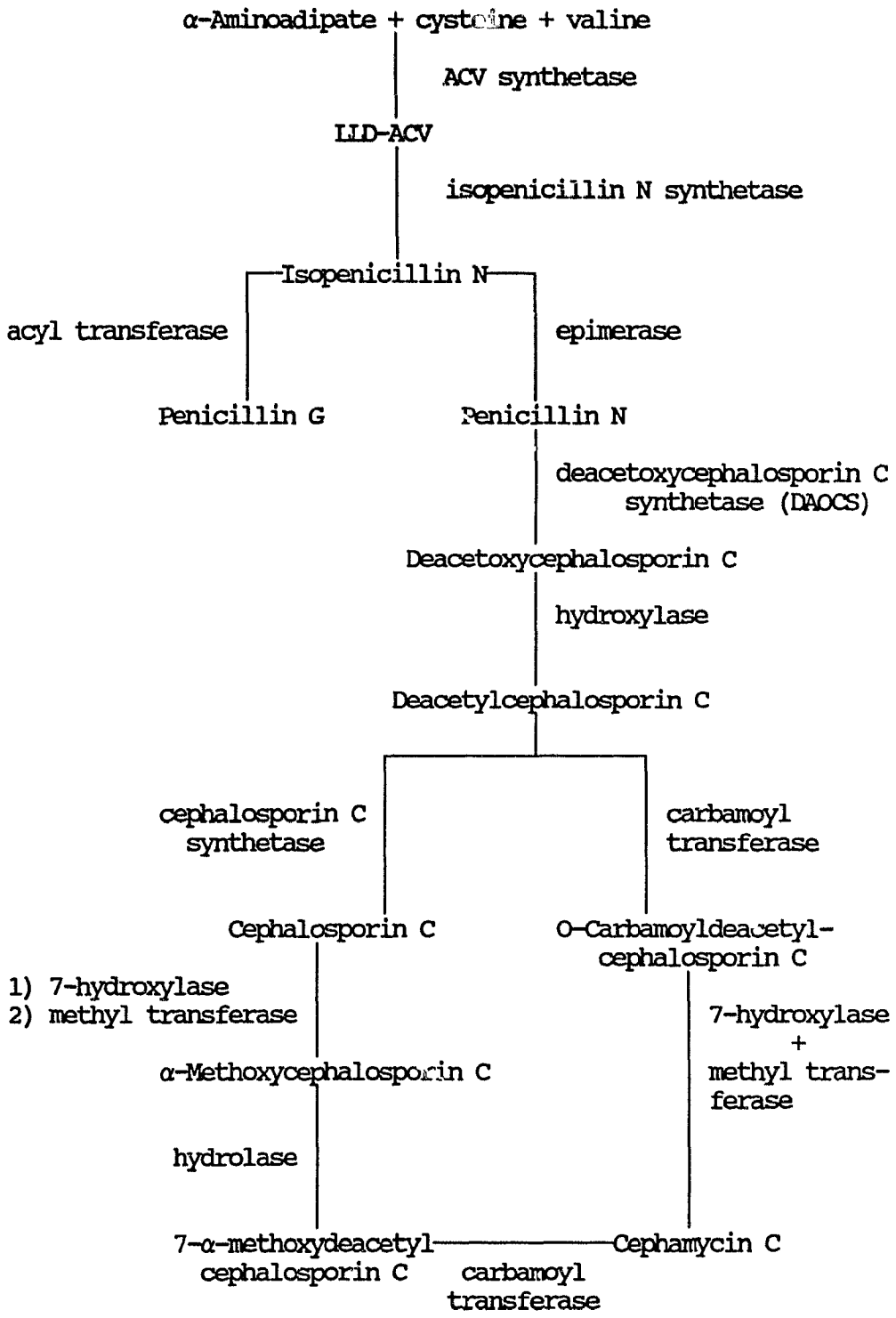


Fig. 1. Pathway for the biosynthesis of β-lactam antibiotics (modified from Demain, 1983).

the dipeptide was an intermediate in tripeptide formation. However, Banko et al. (1987) subsequently observed that ACV synthesis was much faster from individual amino acids than from δ-(α-aminoadipyl)cysteine and valine, indicating that the dipeptide was not involved. Since the cell extract was also unable to convert LLL-ACV to LLD-ACV, the epimerization step took place during tripeptide formation and was not a separate activity. Van Liempt et al. (1989) later obtained from a penicillin-producing strain of Aspergillus nidulans an enzyme fraction which catalyzed ACV formation and activated its constituent amino acids as their adenylates, supporting the evidence that ACVS was a single multifunctional complex.

The tripeptide LLD-ACV was cyclized to isopenicillin N by an enzyme present in lysed protoplasts of C. acremonium (Fawcett et al., 1976). The enzyme has since been subjected to detailed study and its requirements for molecular oxygen, ferrous ions and ascorbate demonstrated. The enzyme has also been purified from a number of prokaryotic and eukaryotic organisms (Pang et al., 1984; Ramos et al., 1985; Jensen et al., 1986; Castro et al., 1988).

B. Biosynthesis of hydrophobic β-lactam antibiotics

Isopenicillin N is a branch point for the synthesis of hydrophilic and hydrophobic β-lactams (Fig. 1). Penicillin G is synthesized from isopenicillin N by exchange of the aminoadipyl moeity for phenylacetate. Loder (Cited in Demain, 1983) observed cell-free conversion of isopenicillin N to benzyl penicillin when phenylacetate, CoA and isopenicillin were incubated with crude extracts of P. chrysogenum. The

enzyme catalyzing this reaction was identified as an acyltransferase. 6-APA, a hydrolytic product of isopenicillin N, accumulates in cultures deficient in phenylacetate (Demain, 1959; Batchelor et al., 1959). 6-APA can also serve as a substrate for acyltransferase in the production of penicillin G in vitro (Fawcett et al., 1975), but it is not known whether 6-APA is the true intermediate in penicillin G biosynthesis. Acyltransferase is restricted to fungi producing hydrophobic penicillins and is not found in organisms producing hydrophilic β-lactams.

C. Biosynthesis of hydrophilic β-lactams

Initially the α-amino group of the AAA component of isopenicillin N is converted to the D-configuration by an epimerase, forming penicillin N. Konomi et al. (1979) were able to demonstrate this reaction in newly prepared cell extracts of C. acremonium, but the enzyme was very labile. The IPN epimerase has been partially purified from a high-yielding mutant of C. acremonium (Lubbe et al., 1986). Partially purified epimerase from S. clavuligerus (Jensen et al., 1983) is more stable than the fungal counterpart.

In the next step, the five-membered thiazolidine ring of penicillin N is expanded to a six-membered dihydrothiazine ring by deacetoxycephalosporin C synthetase (DAOCS). The enzyme is a dioxygenase with a requirement for molecular oxygen, ferrous ions, ascorbate and α-ketoglutarate (Kupka et al., 1983a). In C. acremonium, DAOCS is bifunctional and serves not only to expand the thiazolidine ring but also as a hydroxylase converting deacetoxycephalosporin C to deacetylcephalosporin C (Kupka et al., 1983b). The enzyme from C.

acremonium has been partially purified (Dotzlaf and Yeh, 1987).

In contrast to the situation in fungi, the ring expansion and hydroxylation activities are separate in streptomycetes and both proteins have been isolated from S. clavuligerus (Jensen et al., 1985). The terminal step in cephalosporin C biosynthesis is conversion of deacetylcephalosporin C to cephalosporin C, and is catalyzed by the enzyme acetyl CoA;deacetylcephalosporin C acetyltransferase (Fujisawa et al., 1973; Fujisawa and Kanzaki, 1975). Mutants lacking this activity accumulate deacetylcephalosporin C (Fujisawa et al., 1973 and 1975). Actinomycetes can convert both cephalosporin C and deacetylcephalosporin C to cephamycins by the alternative sequences of reactions shown in Fig. 1 (O'Sullivan and Abraham, 1980).

II. Regulation of β-lactam biosynthesis

Analysis of the relatively extensive information generated from physiological studies has led to the conclusion that conditions favouring rapid growth and high biomass accumulation generally do not support optimum antibiotic production. While rapidly metabolised carbon sources for this reason are unsuitable, poorly metabolised carbon sources, although they may support excellent specific production, also fail to yield high titres (Aharonowitz and Demain, 1978). Thus, there is a critical biomass requirement for obtaining both high specific productivity and high titres. Understanding this and other factors controlling optimum antibiotic production has made it possible to devise suitable fermentation conditions for high levels of productivity. As with producers of all other antibiotics, β-lactam producers require an

adequate supply of the three major nutrients carbon, nitrogen and phosphorus. Altering the source or concentration of these nutrients in the culture medium has a controlling effect on antibiotic production. The term repression will be used consistently in this thesis to denote transcriptional or post-transcriptional repression of a gene expression whereas the term depression will be used to denote a reduction in the specific activity of an enzyme.

A. Carbon catabolite regulation

The effect of the carbon source on antibiotic production varies from one organism to another. Streptomyces clavuligerus has a limited ability to assimilate carbohydrates and can use only maltose or starch, but not glucose (Aharonowitz and Demain, 1978). It also grows with glycerol or succinate. Glycerol, which supports rapid and abundant biomass accumulation, is the most depressive carbon source. At a concentration of 1% in the medium, it reduces both the specific and volumetric cephamycin production. Lowering the concentration to 0.4% provides the optimal level for volumetric antibiotic production, emphasizing that adequate biomass is necessary to maximize the titre. This was confirmed when S. clavuligerus was grown on organic acids, which supported high specific production but failed to yield high volumetric titres. Starch, which was less rapidly metabolised, supported good volumetric titres and specific productivity. Glycerol was suppressive when combined with starch during the early phase of the fermentation (Lebrihi et al., 1988). Depression of antibiotic synthases, notably DAOCS, and not inhibition was identified as the

reason for reduced antibiotic production. On the other hand, phosphorylated intermediates of glycolysis such as glucose 6-phosphate and fructose 6-phosphate strongly inhibited DAOCS activity. Similar results were observed in Nocardia lactamdurans when glucose was used as a carbon source (Cortes et al., 1986). Glucose reduced the synthesis of ACV and depressed DAOCS but had no effect on IPNS and epimerase. Glycolytic pathway intermediates inhibited IPNS, DAOCS and epimerase activities. cAMP did not seem to have any role in glucose control of antibiotic production.

Glucose is readily metabolized in fungi and decreases β-lactam synthesis in P. chrysogenum. The onset of penicillin biosynthesis is progressively delayed with increasing glucose suplementation and in every case the onset coincides with glucose exhaustion. The specific activities of ACVS and IPNS were decreased even though the terminal step catalyzed by the acyltransferase was not depressed (Revilla et al., 1981). Cephalosporin C synthesis was also depressed by glucose in C. acremonium. Even though increasing glucose concentration stimulated growth and volumetric production, specific production declined gradually as the concentration of glucose was increased from 20 to 55 g/l (Zanca and Martin, 1983). That the decreased cephalosporin production was due to depression of one or more enzymes converting penicillin N to cephalosporin was established in resting cell experiments, and was supported by the accumulation of penicillin N in glucose-supplemented cultures. DAOCS was subsequently identified as the target of glucose depression (Heim et al., 1984). DAOCS synthesis did not begin until glucose was exhausted from the medium, and the amount of DAOCS fell when

glucose was added to cultures actively synthesizing the enzyme. No inhibition of any of the β-lactam synthases by glucose was observed in the fungi. Although the mechanism of glucose depression is not known, a relationship between glucose utilization and penicillin biosynthesis was confirmed with the discovery of a mutant derepressed for β-galactosidase and penicillin biosynthesis (Barredo et al., 1988). The mutant was defective in glucose kinase activity and glucose uptake was greatly reduced; thus it is not possible to say whether penicillin biosynthesis was directly or indirectly affected.

B. Nitrogen catabolite regulation

β-Lactam-producing actinomycetes metabolize nitrogen from such sources as ammonium, amino acids and urea. In S. clavuligerus ammonium decreased β-lactam production, whether used alone or in combination with a preferred nitrogen source, such as asparagine (Aharonowitz and Demain, 1979). The slower growth on ammonium indicated that growth rate was not a factor in decreasing antibiotic production. The ammonium effect was observed in cultures supplied with ammonium 24 h after inoculation or earlier, suggesting that it mediated an event in the trophophase that regulated antibiotic production. Brana et al. (1985) identified IPNS and to a lesser extent DAOCS but not epimerase as primary targets of ammonium depression, and suggested that IPNS catalyzed the rate limiting step in β-lactam biosynthesis. Later ACVS was identified as an additional target for depression (Zhang et al., 1988). The depressions observed were 75% for ACVS, 70% for IPNS and 50% for DAOCS. Controlling formation of the tripeptide would avoid wastage of amino acids and

energy which could be channelled into primary metabolism and growth. Therefore, ACVS rather than IPNS was suggested to be the true rate limiting enzyme in β-lactam biosynthesis.

In N. lactamdurans, ammonium not only reduces antibiotic production but also depresses IPNS, epimerase and DAOCS (Castro et al., 1985); this indicates ammonium coordinately regulates the β-lactam biosynthetic pathway. It is not known whether other enzymes converting deacetylcephalosporin C to cephamycin C are also coordinately regulated in this species. However, coordinate regulation does not occur in all β-lactam producers; epimerase is only slightly depressed in S. clavuligerus whereas in C. acremonium DAOCS synthesis is more sensitive than other enzymes to ammonium regulation (Shen et al., 1984). None of the antibiotic synthases was inhibited by high concentrations of ammonium in N. lactamdurans, indicating that ammonium regulates antibiotic production in this organism primarily by depression (Castro et al., 1985).

Ammonium depression was also observed in β-lactam-producing fungi. While asparagine and arginine were the best nitrogen sources for β-lactam biosynthesis in C. acremonium, ammonium decreased titres when added at concentrations higher than 100 mM (Shen et al., 1984). The optimum concentration of asparagine for antibiotic production was 1.2%. Accumulation of ammonium was observed in cultures supplemented with above-optimum asparagine concentrations and this ammonium probably decreased antibiotic production. β-Lactam biosynthesis was stimulated in cultures containing tribasic magnesium phosphate which complexes with free ammonium. Concomitant increase in DAOCS activity suggested that

synthesis of this enzyme was a target for ammonium depression. Removal of ammonium from the medium not only increased cephalosporin production but also improved penicillin N synthesis, suggesting that some earlier steps were also regulated by ammonium. ACVS was later identified as an additional target of ammonium depression (Zhang et al., 1987) and was also found to be 50% inhibited by a very high (250 mM) concentration of ammonium. IPNS was not strongly depressed by ammonium in any of these studies.

Since secondary metabolism depends on the supply of precursors by primary metabolism, Brana et al. reasoned that the ammonium effect on secondary metabolism was probably indirectly exerted by a primary metabolic process. Evidence for a depressive effect of ammonium on glutamine synthetase and β-lactam synthesis (Brana et al., 1986), and the recovery from depression that followed ammonium exhaustion from the medium, suggested that glutamine synthetase might have a role in controlling β-lactam biosynthesis. Moreover, the intracellular pools of glutamine, alanine and ammonium were influenced by the type of nitrogen source employed. However, there was no correlation between any of these pool sizes and IPNS activity. Glutamine synthetase levels were not closely correlated with IPNS and DAOCS activities, and ammonium decreased β-lactam biosynthesis in mutant strains with lesions in the GS, GOGAT pathway of ammonium assimilation (Brana et al., 1986). These results suggested that there is no direct regulatory linkage between the pathways of ammonium assimilation and antibiotic production. The mechanism by which ammonium does control antibiotic production is not yet known.

C. Phosphate regulation

High phosphate concentrations in a defined medium buffer the pH against drastic changes and thereby support good growth of S. clavuligerus, but antibiotic production is decreased (Aharonowitz and Demain, 1977). Lubbe et al. (1985) investigated this effect in detail and found that prior growth at high phosphate concentrations decreased resting cell production of β-lactam antibiotics. DAOCS but not IPNS or epimerase was identified as the main target of phosphate depression. Lebrihi et al. (1987) subsequently corroborated these results. ACVS was identified as an additional target for phosphate depression by Jhang et al. (1989) who also found IPNS to be depressed, but to a lesser extent. Not only were enzymes depressed by high phosphate, but the activities of DAOCS, IPNS (Lubbe et al., 1985) and ACVS (Jhang et al. 1989) were also inhibited. However, the inhibition was shown to be due to deprivation of ferrous ions and could be reversed by adding ferrous ions to enzyme incubation mixtures (Lubbe et al., 1984). Ferrous ion supplementation of the fermentation medium did not reverse the depression of antibiotic production, indicating that depression was the actual mechanism through which phosphate controlled β-lactam production. Phosphate did not have any significant effect on β-lactam production in N. lactamdurans (Cortes et al. 1986), although it inhibited DAOCS and the inhibition could not be reversed by ferrous ions. In C. acremonium on the other hand, phosphate depressed synthesis and inhibited the activities of ACVS, IPNS and epimerase (Zhang et al., 1988), paralleling the results obtained in S. clavuligerus.

III. Precursor biosynthesis and regulation

A. Cysteine

Isotope incorporation studies performed by Arnstein and Grant (1954) provided conclusive evidence that cysteine was incorporated intact into penicillin. Addition of cysteine to P. chrysogenum fermentation medium failed to stimulate penicillin production, indicating that cysteine was not a limiting precursor (Demain, 1956).

B. Valine

Whereas isotopically labeled L-valine was incorporated into penicillin (Arnstein and Grant, 1954), D-valine was a poor precursor and inhibited penicillin production. Additional evidence that L-valine is the actual precursor, even though present in the D-configuration in the penicillin molecule, was obtained by Arnstein and Margreiter (1958). α-Methyl-DL-valine inhibited penicillin formation and this was reversed by addition of L-valine (Demain, 1956). The possibility that valine is a limiting precursor in P. chrysogenum was suggested when the high yielding strain, Q176, showed a higher level of acetohydroxyacid synthetase, the first enzyme in valine biosynthesis, than the wild type (Goulden and Chattaway, 1969). The enzyme from the mutant was also less sensitive to feed back inhibition by valine and had lost one of two valine binding sites. The remaining site was noncompetitive with respect to pyruvate. End-product control by repression of acetohydroxyacid synthetase was not observed, indicating that inhibition of acetohydroxyacid synthetase was the main control mechanism determining

valine biosynthesis.

In S. lipmanii, strains with defective control of isoleucine-valine biosynthesis were superior producers of cephamycin C (Godfrey, 1973), suggesting that feedback inhibition might be responsible for limiting the rate of valine formation. Thus valine could be a rate limiting precursor in at least one prokaryote and one eukaryote.

C. α-Aminoadipate

While AAA is an intermediate in lysine biosynthesis in fungi, it is a product of lysine catabolism in actinomycetes. The consequent difference in the role of lysine in β-lactam biosynthesis in the two groups of organisms has been extensively investigated.

1. Lysine biosynthesis in fungi

Since AAA is a biosynthetic intermediate in both lysine and β-lactam biosynthesis pathways (Fig. 2), the many factors governing lysine biosynthesis would be expected to influence β-lactam biosynthesis. When lysine was added to a growth medium for P. chrysogenum, antibiotic production was decreased (Demain, 1957). This was consistent with an earlier observation by Bonner (1947) that lysine auxotrophs were defective in penicillin biosynthesis. The depression of penicillin production by lysine was reversed by addition of AAA to the culture medium (Somerson, 1961), and it was suspected that lysine depression of penicillin production was due to a feed-back control mechanism. Additional experiments by Masurekar and Demain (1972) showed that lysine inhibited an enzyme leading to penicillin biosynthesis, postulated to be

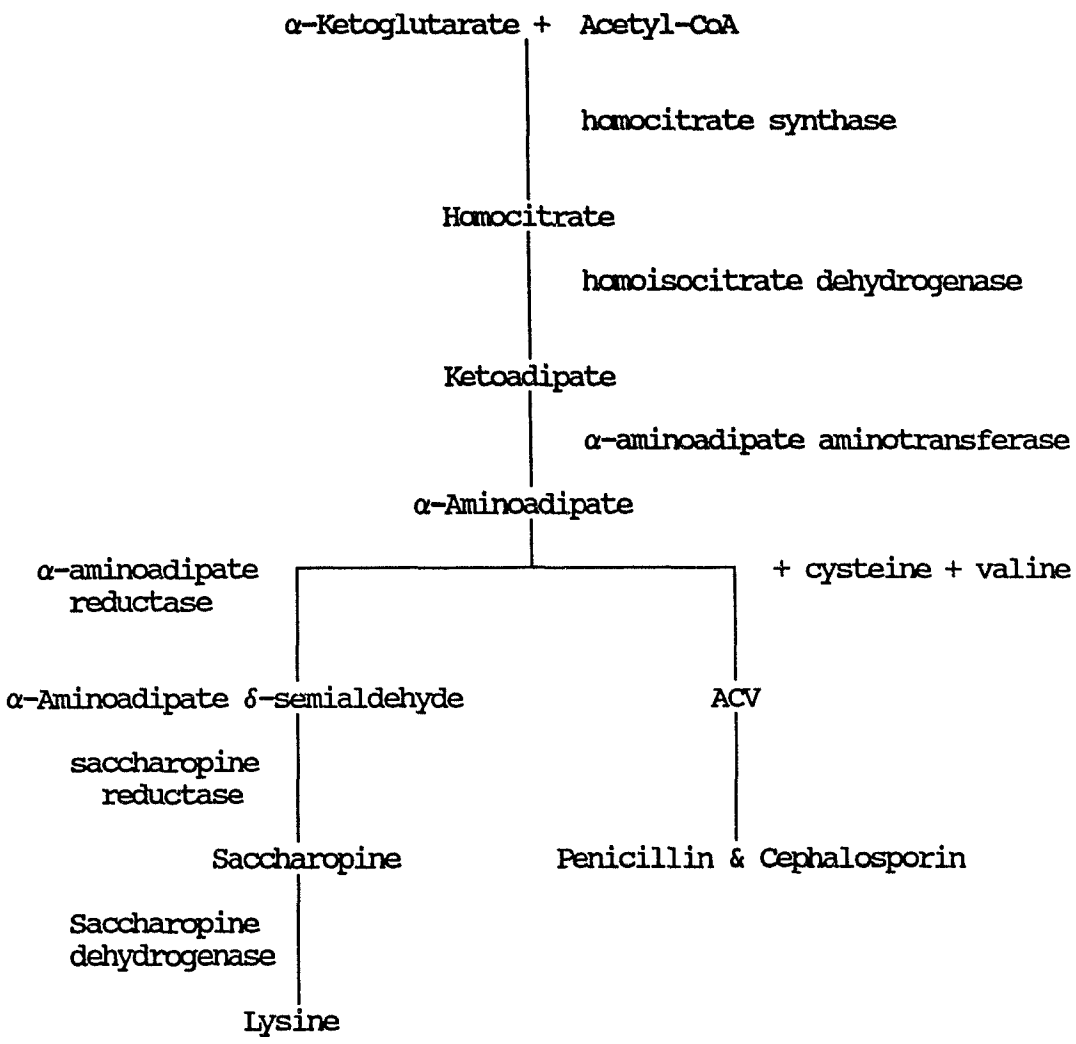


Fig. 2. Pathway for the biosynthesis of lysine in fungi (modified from Vining et al., 1990).

an early enzyme in lysine biosynthesis. Mutants deregulated in lysine biosynthesis secreted lysine and were impaired in penicillin biosynthesis, leading Masurekar and Demain (1974a) to propose that AAA is a branch point precursor for both lysine and penicillin. Subsequently an *in vivo* study identified homocitrate synthase, the first enzyme in lysine biosynthesis, as the target of lysine regulation (Demain and Masurekar, 1974). Addition of homocitrate to the fermentation medium reversed lysine inhibition of penicillin biosynthesis (Friedrich and Demain, 1977).

Luengo et al. (1980) investigated the lysine effect in detail and provided evidence for inhibition of homocitrate synthase activity *in vitro*. They also observed depression of homocitrate synthase by lysine, contradicting earlier findings (Demain and Masurekar, 1974; Masurekar and Demain, 1974b). When high- and low-producing strains of *P. chrysogenum* were examined carefully, the high-producing strain was found to have a homocitrate synthase less sensitive to feedback inhibition and depression by lysine than the low producer. Homocitrate synthase activity peaked at 48 h, which coincided with peak antibiotic production. Further analysis of the two strains indicated that even though lysine inhibited penicillin production in both to a similar extent, the onset of penicillin production occurred at high lysine concentrations in the high producer whereas in the low producer lysine had to be depleted before penicillin production could start. Luengo et al. (1979) suggested that the high producer was less sensitive to lysine regulation.

Jaklitsch et al. (1986) did not observe any inhibition of

homocitrate synthase in P. chrysogenum in the physiological range (0.1-1.0 mM) normally expected in vivo. Though the results did not conflict with those obtained by Luengo et al. (1980), Jaklitsch et al. (1986) suggested that inhibition of homocitrate synthase was not an important regulatory mechanism because the high concentrations (50 mM) necessary for inhibition of homocitrate synthase in vitro are not likely to occur in vivo. Measurements of pool sizes in cells supported this conclusion. Further detailed investigation of lysine biosynthesis indicated regulation by a general amino acid control mechanism in P. chrysogenum (Jaklitsch et al., 1987). Derepression of amino acid biosynthesis took place when the cells were starved for one of several amino acids. No depression of homocitrate synthase was observed in this investigation. Honlinger et al. (1988) investigated differences in high and low penicillin-producing strains of P. chrysogenum in the light of the new information about regulation of lysine biosynthesis. In the high producer, AAA reductase (Fig.1) was included in general amino acid control as well as the previously reported saccharopine reductase and dehydrogenase. Since AAA reductase uses AAA to generate lysine, changes in its level during general amino acid control were expected to influence the availability of AAA for penicillin biosynthesis. When general amino acid control was evoked by using a histidine analogue (amitrole), one high producer increased penicillin production while another produced less. Though there was a correlation between AAA pool sizes and penicillin production, AAA reductase activity did not match the changes in AAA pool sizes.

2. Importance of AAA pool sizes in fungi

Loder and Abraham (1971) identified the tripeptide, LLD-ACV, as an intermediate in cephalosporin-producing C. acremonium. Depression of penicillin production by lysine and its reversal by AAA addition (Demain, 1957; Somerson et al., 1961) raised a question about the role of AAA as a precursor. Because of the exchange of AAA for phenylacetate in penicillin production, AAA released during the terminal step of penicillin biosynthesis could be recycled to supply AAA to the tripeptide precursor. Friedrich and Demain (1978) demonstrated that relatively low concentrations of AAA were required for optimum penicillin production. Thus 0.01 mM AAA added externally, supported 50% of the maximum antibiotic production. However, to maximize antibiotic production required raising the external supply by a factor of ten. Based on this and other observations Friedrich and Demain (1978) concluded that AAA was recycled during penicillin production and that 10 molecules of penicillin were produced per molecule of AAA consumed. Even though AAA is absent from the penicillin molecule, its absolute requirement in penicillin production together with the fact that it serves as a common precursor for both penicillin and lysine suggested that AAA pool levels might influence penicillin production. When four strains of P. chrysogenum with different productivity levels were tested, the intracellular concentration of AAA was significantly higher for cells grown under conditions supporting penicillin production than in those propagated on a medium that did not support penicillin production (Jaklitsch et al., 1986). In the supportive medium, penicillin production in the four strains was proportional to the

intracellular AAA pool concentrations. The authors concluded that the low producing strains were in part limited by the availability of AAA. This was confirmed by Honlinger et al. (1988) who found a positive correlation between penicillin production and AAA pool sizes during their studies on general amino acid control.

The relationship between AAA pool size and penicillin biosynthesis was further investigated by Honlinger and Kubicek (1989) to test whether the correlation between AAA pool sizes and penicillin production was due to actual AAA limitation or to some other rate limiting enzyme in the penicillin biosynthetic pathway of low producers. AAA pool sizes correlated well with production of ACV and isopenicillin N in strains of differing productivity and under a variety of culture conditions. These results indicated that the low producing strains were limited by the availability of AAA. In C. acremonium, addition of small amounts of lysine stimulated antibiotic production (Mehta et al., 1979). This was explained as a sparing effect of lysine on AAA availability for β-lactam production in this species. From these results it appears that AAA is a true rate-limiting precursor, and that its pool size is controlled by mechanisms not yet fully defined.

D. AAA in actinomycetes

In actinomycetes, AAA is formed from lysine. Thus the availability of AAA for β-lactam production could be regulated at two stages, namely: 1) during lysine biosynthesis and 2) when lysine is metabolized to generate AAA.

1. Lysine biosynthesis in actinomycetes

The presence of diaminopimelate (DAP) in the cell walls of streptomycetes (Cummins and Harris, 1958; Higgins and Kastner, 1971) suggested that the pathway for lysine biosynthesis might include this intermediate. A lysine auxotroph of S. lipmanii was isolated and was shown to be defective in DAP decarboxylase (Kirkpatrick et al., 1973). The mutant accumulated DAP under conditions of lysine starvation, indicating that lysine was indeed synthesised via DAP (Fig. 3). The stability, pH and temperature optima, and cofactor requirement DAP decarboxylase from S. lipmanii were similar to those of other bacterial DAP decarboxylases. The enzyme activity was sensitive to inhibition by lysine but no repression of synthesis was observed. Incorporation of radioactivity from ^{14}C-aspartic acid into DAP provided further evidence that DAP pathway functioned in actinomycetes.

As a product of lysine metabolism, AAA would be expected to vary in concentration in response to factors affecting lysine biosynthesis. The flow of carbon from aspartic acid to AAA is regulated by feedback mechanisms (Mendelovitz and Aharonowitz, 1982). Aspartic acid serves as a common precursor for the biosynthesis of lysine, methionine, threonine and isoleucine (Fig. 3). Aspartate semialdehyde (AS) is a branch point intermediate and its relative flow to each of the pathways is likely to be regulated by feedback control of key enzymes such as homoserine dehydrogenase (HSD) and dihydrodipicolinate synthetase (DDPS), the first enzymes in their respective pathways. In S. clavuligerus, aspartate kinase (AK), the first enzyme, was feedback inhibited by a mixture of lysine and threonine (Mendelovitz and Aharonowitz, 1982). Lysine alone

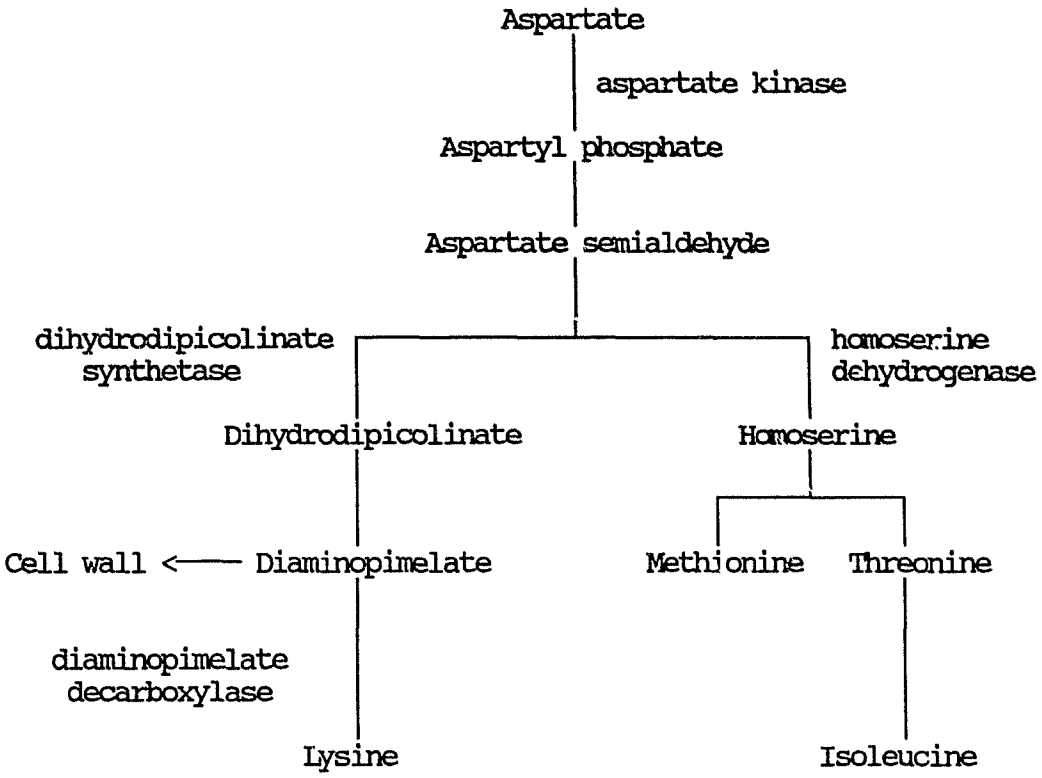


Fig. 3. Pathway for the biosynthesis of lysine in actinomycetes. Only relevant enzymes described in the text are indicated (modified from Vining et al., 1990).

stimulated AK activity while threonine alone had no effect. The pattern of stimulation and inhibition was seen in cell extracts of different degrees of purification, providing evidence for the presence of only one AK in S. clavuligerus. Addition of isoleucine and methionine to the culture medium depressed AK activity by 50% and 70% respectively while addition of lysine stimulated AK activity by 20%. The depressive effect of isoleucine was partially reversed by addition of lysine.

The flow of carbon from aspartate can potentially be regulated by the channelling of AS through either of the available pathways (Fig. 3). DDPS and HSD had similar affinities for AS with K_m values of 0.3 and 0.28 respectively. However, the specific activity of DDPS was higher than that of HSD, indicating that more AS would normally be converted to DDP than to homoserine. DDPS, the first enzyme specific for lysine biosynthesis, was significantly inhibited by high concentrations of DL-meso-DAP and AAA. No other amino acid had any effect at a concentration of 10 mM. The specific activity of DDPS during exponential growth of S. clavuligerus did not alter significantly. Addition of lysine and other members of the aspartate family of amino acids did not have any effect. DL-meso-DAP, leucine and alanine caused 20% reduction in the specific activity of DDPS when added alone whereas lysine + threonine stimulated DDPS synthesis by 25%. These results suggested that DDPS was not tightly regulated in S. clavuligerus.

HSD activity (the first enzyme specific for threonine and methionine biosynthesis) on the other hand was closely controlled by feedback mechanisms. At 1 mM, threonine inhibited HSD activity by 75% whereas homoserine and methionine inhibited HSD activity by 50% and 20%

respectively. Isoleucine strongly depressed HSD (45% depression) whereas threonine had a mildly (15%) depressive effect. The relatively loose control of DDPS compared to HSD, favouring the flow of carbon into DAP, probably reflects the importance of DAP in cell wall biosynthesis. Although DAP decarboxylase of S. clavuligerus was inhibited by lysine at a high concentration (50 mM), no depression was observed. In S. lipmanii, DAP decarboxylase was inhibited by lysine (66% inhibition) at the relatively low concentration of 10 mM (Kirkpatrick et al., 1973), but it is not certain that such a concentration can be achieved in vivo. Perhaps the β-lactam producers have evolved this loose regulatory mechanism to facilitate the channelling of aspartate via lysine to AAA and then into β-lactam antibiotics.

That β-lactam antibiotic production can be limited by the availability of lysine due to feed-back inhibition by a combination of lysine and threonine, was demonstrated by Mendelovitz and Aharonowitz (1983). Adding lysine and DL-meso-DAP to cultures stimulated antibiotic production in S. clavuligerus. Mutants of S. clavuligerus with deregulated AK were isolated by selecting for mutants resistant to the lysine analogue, 4-(2-aminoethyl)-L-cysteine (AEC). As expected, 70% of these mutants produced significantly more cephamycin C suggesting that feedback regulation of AK was a limiting factor in β-lactam biosynthesis. Analysis of the intracellular amino acids in wild type and AEC resistant mutants showed significant differences (Aharonowitz et al., 1984). In mutant strains, DAP represented approximately 15% of the total amino acid pool whereas in wild-type strains it accounted for only 0.5%. On average, the AEC-resistant mutants with high intracellular DAP

produced 2-5 times more cephamycin C than the wild type strain. The lysine pool, although higher in the mutants, increased less than did the DAP pool; the authors suggested that this was probably due to feedback inhibition of DAP decarboxylase by lysine (Mendelovitz and Aharonowitz, 1982), but the high lysine concentration needed for such feedback inhibition would not be achieved if this amino acid is able to induce a lysine catabolic pathway resulting in its degradation. In Pseudomonas, overproduction of lysine in AK deregulated mutants was shown to induce lysine oxygenase, an enzyme of the lysine catabolic pathway (Hermann et al., 1972). It may be that DAP decarboxylase is the rate limiting enzyme in lysine biosynthesis because of the importance of DAP in cell wall biosynthesis.

2. Conversion of lysine to AAA in actinomycetes

2.1. Origin

As noted earlier the origin of AAA in actinomycetes is different from its origin in fungi. Evidence that radioactivity from DL-(1-^{14}C)lysine and DL-α-amino(1-^{14}C)adipate was incorporated at comparable specific activity into the cephamycin C by S. clavuligerus (Whitney et al., 1972) implicated a pathway from lysine via AAA. Additional evidence was provided by identification of the DAP pathway for lysine biosynthesis in S. lipmanii (Kirkpatrick et al., 1973) ruling out the formation of AAA during lysine biosynthesis. Evidence for the involvement of an aminotransferase in AAA biosynthesis (Kirkpatrick et al., 1973) was subsequently confirmed by Kern et al. (1980) in N.

lactamdurans. The enzyme, lysine ε-aminotransferase, catalyzed transfer of the epsilon amino group from lysine to α-ketoglutarate to generate L-1-piperideine-6-carboxylate. The substrate and amino group acceptor specificity of the enzyme from the actinomycete was identical to the one reported for Flavobacterium (Soda et al., 1968; Soda and Misono, 1968). Lysine ε-aminotransferase activity has also been demonstrated in S. clavuligerus (Shapiro, personal communication). In the second step piperideine-6-carboxylate is converted to AAA, possibly by a nicotinamide adenine dinucleotide-linked dehydrogenase analogous to one demonstrated in Pseudomonas by Calvert and Rodwell (1966), but the reaction has not been characterized in actinomycetes.

2.2. Importance of AAA pool size

In earlier studies AAA was identified as a rate-limiting precursor in β-lactam biosynthesis (Inamine and Birnbaum, 1973). Enhanced β-lactam production was obtained by addition of AAA to the fermentation at 0.3-0.5% for N. lactamdurans and 0.025-0.05% for S. clavuligerus. The timing of AAA addition was critical only in N. lactamdurans, where supplementation at 24-48 h supported optimum production. Similar stimulation was observed when AAA was replaced by 0.1-0.2% lysine (Hallada et al., 1975). Delaying the addition of lysine until 24 h after inoculation in general gave better antibiotic titres. Mendelovitz and Aharonowitz (1982) reported that lysine, meso-DAP and AAA stimulated β-lactam production in S. clavuligerus and that the timing of addition was sometimes critical. DL-meso-DAP, at 10 mM concentration enhanced production when added 24 h after inoculation. Addition of 10-20 mM

lysine improved antibiotic production irrespective of the time of addition, but at 5 mM lysine was without effect. AAA stimulated antibiotic production at all concentrations tested when added after 24 or 48 h of growth. When a number of strains of N. lactamdurans with different cephamycin C productivities were tested for lysine ε-aminotransferase activity, the specific activity of the enzyme correlated with antibiotic titre (Inamine et al., 1980. Cited in Martin and Aharonowitz, 1983). The intracellular pool of AAA increased in a similar way, suggesting that AAA was rate limiting for β-lactam biosynthesis.

2.3. Function of AAA in actinomycetes

Whether AAA has a role in actinomycetes other than in β-lactam biosynthesis is not known, but the following observations suggest that it does not: 1) In N. lactamdurans, lysine ε-aminotransferase activity peaked during early exponential growth and then rapidly declined (Kern et al., 1980). Production of cephamycin C began only in late exponential growth. This suggested to the authors that a pool of AAA accumulated and was later drawn upon for β-lactam biosynthesis, 2) Similar results were obtained in S. clavuligerus (Shapiro, pers. comm.). Though the cytoplasmic AAA pool level did not change significantly during growth phase, the AAA concentration in the culture medium gradually increased and later declined dramatically. The decline in AAA levels coincided with peak antibiotic production, 3) Romero et al. (1988) isolated mutants of S. clavuligerus with lesions in lysine ε-aminotransferase; these mutants did not produce any β-lactam

antibiotics, but showed normal growth kinetics and were able utilize a number of amino acids, including lysine, as nitrogen sources. It can be concluded that the pathway generating AAA is not necessary for growth; its only known function is to provide a key precursor in β-lactam biosynthesis. The question of whether lysine is catabolized by other pathways during growth on lysine as nitrogen source can be answered in the affirmative from the results reported by Romero et al., (1988). The presence of a separate pathway argues in favour of AAA being produced exclusively for β-lactam biosynthesis thus representing part of a secondary metabolic pathway.

2.4. Lysine catabolism in aerobic bacteria

Lysine catabolism is notable for its biochemical diversity (Fig. 4). In the genus Pseudomonas, there are four inducible pathways. Two of these lead to a common intermediate δ-aminovalerate. One pathway is initiated by decarboxylation of lysine to cadaverine (Fothergill and Guest, 1977; Stewart, 1970), and the second is a monooxygenase-catalyzed conversion of lysine to δ-aminovaleramide (Reitz and Rodwell, 1970; Vandecasteele and Hermann, 1972). In the remaining pathways, lysine is either transaminated to 1-piperideine-6-carboxylate (Fothergill and Guest, 1977) or converted to pipecolate (Miller and Rodwell, 1971b), both of which are ultimately catabolized to α-aminoadipate. In Flavobacterium lutescens, lysine catabolism proceeds through 1-piperideine-6-carboxylate to AAA (Soda et al., 1968; Soda and Misono,1968). When the present work was begun, the route used for lysine catabolism in actinomycetes was not known (Madduri et al., 1989).

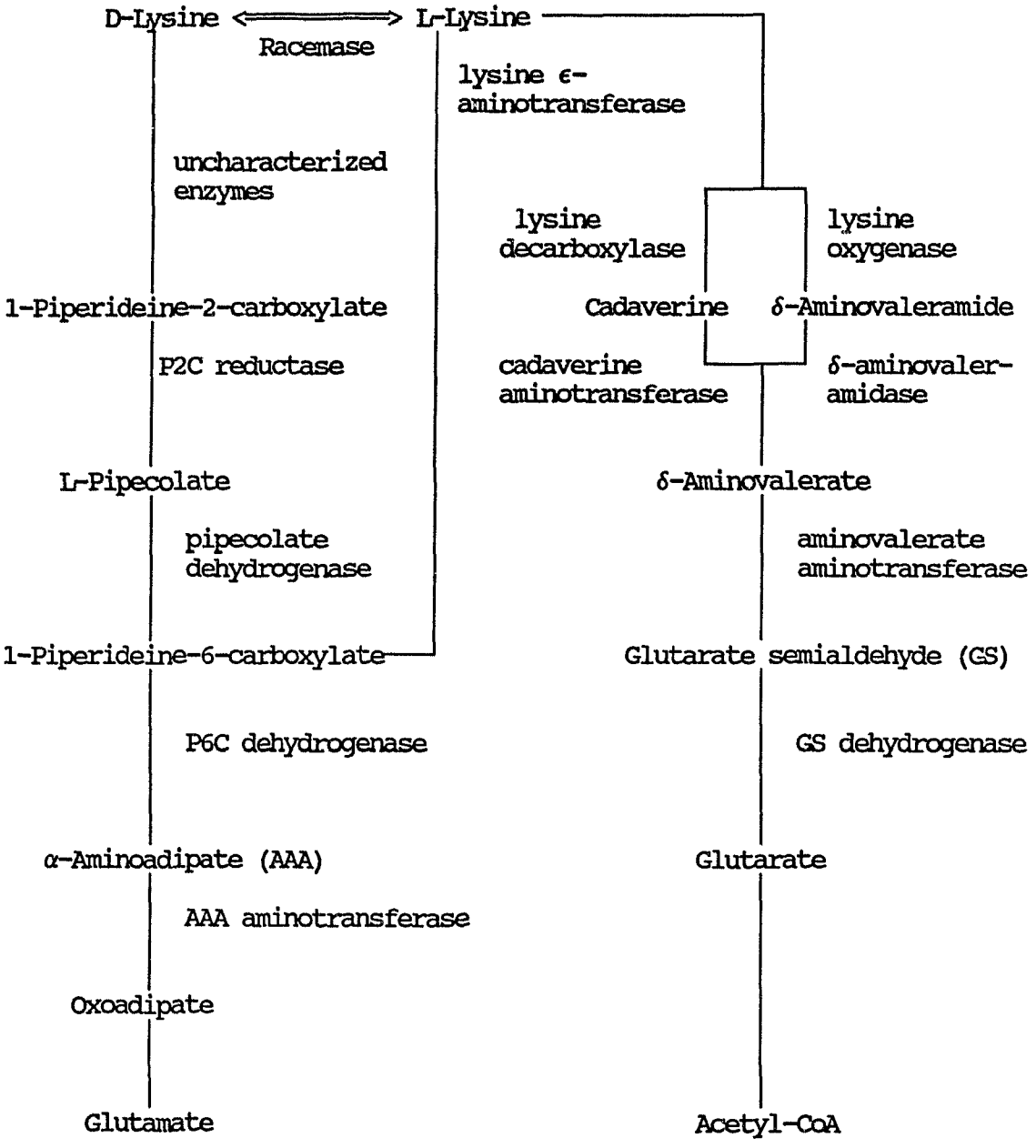


Fig. 4. Pathways for lysine catabolism in aerobic bacteria (modified from Fothergill and Guest, 1977).

Romano and Nickerson (1958) reported that lysine supported modest growth of S. fradiae when it was supplied as the sole source of carbon and nitrogen, but provided no information on the pathway involved. Subsequently, the α-aminoadipyl side chain of β-lactam antibiotics was found to be derived from lysine, and the first enzyme catalyzing this conversion was identified as lysine ε-aminotransferase (Kern et al., 1980).

2.5. Regulation

Enzymes of amino acid catabolic pathways are usually regulated by induction, carbon catabolite repression and nitrogen catabolite repression. δ-Aminovaleramidase, an enzyme in the δ-aminovaleramide pathway for lysine catabolism in Pseudomonas putida , is induced by lysine (Reitz and Rodwell, 1970). Lysine transport is induced in P. putida when lysine was the sole source of carbon and nitrogen, and uptake is followed closely by an increase in the amounts of lysine catabolic enzymes, such as lysine oxygenase, δ-aminovaleramidase and pipecolate oxygenase (Miller and Rodwell, 1971a). A survey of enzymes belonging to various amino acid catabolic pathways indicated that they had high K_m values, a safety mechanism to avoid degrading amino acids destined for protein biosynthesis (Miller and Rodwell, 1971b). Chang and Adams (1971) found that enzymes of the L- and D-lysine catabolic pathways were induced by the L- and D-isomers, respectively. Some apparent cross induction of the D-lysine pathway enzymes by L-lysine was probably due to a weak racemase activity. That lysine was the actual inducer of lysine oxygenase and δ-aminovaleramidase was shown by

comparing the induction patterns in a wild-type and a mutant strain blocked in lysine oxygenase (Vandecasteele and Hermann, 1972). The first three enzymes in the cadaverine pathway for lysine catabolism, namely lysine decarboxylase, cadaverine aminotransferase and 1-piperideine dehydrogenase, and the first enzyme of the AAA pathway, lysine ϵ-aminotransferase, were all induced by lysine in P. aeruginosa (Fothergill and Guest, 1977).

No repression of lysine catabolic pathway enzymes was observed when several carbon sources were tested in P. putida (Vandecasteele and Hermann, 1972). Similarly no end-product repression by δ-aminovalerate, glutarate and ammonium was noticed. Contrary results were obtained for histidine dissimilation in Bacillus subtilis: at least three enzymes in the histidine catabolic pathway (Hut) were repressed by preferred carbon sources such as glucose and glycerol (Chasin and Magasanik, 1968). Subsequent investigation led to the identification of a positive control mechanism mediated by cAMP and catabolite gene activator protein (CAP) (reviewed in Magasanik, 1978). In Klebsiella, strong catabolite repression of the Hut pathway by glucose was relieved under conditions of nitrogen limitation (Prival and Magasanik, 1971). Glutamine synthetase is also regulated by nitrogen availability and is regulated in parallel with histidase by a complex mechanism encoded by ntr genes. This system includes: glnD which encodes uridyl transferase/uridyl removing enzyme; glnB, encoding protein P_{II}; glnG, encoding protein NR_I; glnL, encoding protein NR_{II} which is an NR_I kinase/phosphatase. The products of ntr genes control the genes for nitrogen assimilation by a cascade mechanism reviewed by Magasanik (1982) and Magasanik and

Neidhardt (1987). In Streptomyces coelicolor, histidine catabolic enzymes are induced by histidine and to a lesser extent by urocanate, the product of histidase activity (Kendrick and Wheelis, 1982). Urocanate has been identified as the primary inducer of urocanase and formiminoglutamate iminohydrolase, two later enzymes of the catabolic pathway in Streptomyces griseus (Kroening and Kendrick, 1989). The first enzyme, histidase, is formed constitutively but remains in an inactive form under noninducing conditions; its activation is regulated by a cascade mechanism that requires participation of urocanase, inactivation factor (IF), activating factor (AF) and a phosphatase. In the proposed mechanism, urocanate a positively regulates a phosphatase which activates AF; active AF in turn converts inactive histidase (H_I) to active histidase (H_A). Under noninducing conditions IF converts H_A to H_I (Kroening and Kendrick, 1989). Histidine dissimilation in S. coelicolor, unlike other eubacterial counterparts, is not under carbon catabolite repression or nitrogen regulation (Kendrick and Wheelis, 1982).

IV. β-lactam biosynthesis genes

In recent years, β-lactam biosynthesis genes have been cloned from several organisms. The pcbC gene for IPNS has been cloned from S. clavuligerus (Leskiw et al., 1988), S. lipmanii (Weigel et al., 1988), P. chrysogenum (Barredo et al., 1989; Carr et al., 1986; Smith et al., 1990), A. chrysogenum (Skatrud et al., 1985; Smith et al., 1990a), and A. nidulans (Ramon et al., 1987; Smith et al., 1990a). The cefE gene which encodes DAOCS, a late pathway enzyme, has been cloned from S.

clavuligerus (Kovacevic et al., 1989) and A. chrysogenum (Samson et al., 1987). The acvA (also called pcbAB) gene encoding ACVS was recently identified in DNA cloned from S. clavuligerus, P. chrysogenum and A. nidulans (Smith et al., 1990b). That the genes governing β-lactam biosynthesis in Streptomyces cattleya form a discrete cluster was indicated when Chen et al. (1988) cloned from this species a 29.3-kb genomic DNA fragment that directed the synthesis of cephamycin C when transferred into S. lividans, an organism that does not normally produce β-lactam antibiotics. Kovacevic et al. (1989) provided further evidence of gene clustering when they isolated the cefE gene from S. clavuligerus by using pcbC from S. lipmanii to probe a genomic library for adjacent genes. Recently a DNA fragment from S. clavuligerus containing cefD, encoding IPN epimerase, and cefE was cloned and shown to direct the synthesis of a polycistronic mRNA of about 10 kb (Kovacevic et al., 1990), indicating that genes of the β-lactam biosynthesis pathway are organised in operons (Fig. 5). The cloning from P. chrysogenum and A. nidulans of acvA and and a region (penDE) encoding activities for IPN amidolyase and acetyl CoA:aminopenicillanic acid acyltransferase, which convert isopenicillin N to penicillin G, demonstrated that these genes are near pcbC (Diez et al., 1989; Smith et al., 1990a; Smith et al., 1990b) and implies that in at least in two filamentous fungi, β-lactam biosynthesis genes are clustered (Smith et al., 1990b).

V. Genetic improvement and β-lactam production

When a high-yielding strain of P. chrysogenum was subjected to Southern blot analysis, it was found to have multiple (8-16) copies of

Fig. 5. Organization of β-lactam biosynthesis genes in Streptomyces clavuligerus (excluding present work). IPNS - isopenicillin N synthetase, ACVS - ACV synthetase, DAOCS - deacetoxycephalosporin C synthetase, Ep - IPN epimerase, Oc-H - O-carbomoyldeacetylcephalosporin C hydroxylase. Solid bars represent the approximate location of β-lactam biosynthesis genes. DAOCS and Ep are encoded in a single transcript and hence are represented as a single solid bar. Figure not drawn to scale (modified from Smith et al., 1990b). B - BamHI, Bg - BglII and E - EcoRI.

pcbC. The steady state mRNA levels were between 32 and 64-fold higher than those of the wild type strain (Smith et al., 1989). This indicated that during strain improvement, not only had the copy number increased but also the transcriptional efficiency of some or all the copies of the pcbC gene had improved. The IPNS activity of the high yielding strain correlated with the amount of IPNS mRNA. Both IPNS-specific mRNA and enzyme activities were very high during the early stages of growth and a half maximal level of enzyme activity was attained at 14 h. The mRNA and enzyme activities observed were higher throughout the fermentation than were those of wild-type strain. Penicillin production correlated with IPNS activity and peaked after 40 h. In the low producer production stopped early when the IPNS activity declined, whereas in the high producer penicillin continued to accumulate with the continued IPNS activity during later stages of the fermentation. From this evidence, IPNS was the rate-limiting enzyme in this species; increasing the gene copy number overcame this limitation.

This was not the situation in a β-lactam producing strain of C. acremonium where increasing the copy number of pcbC (via recombinant DNA) did not improve antibiotic production (Skatrud et al., 1986). The authors suggested that IPNS was not rate-limiting in this species. When cefEF cloned on a plasmid was introduced by transformation, the plasmid stably integrated and the introduced extra DNA was stably maintained without selection pressure (Skatrud et al., 1989). The recombinant strain had twice as much DAOCS as the parent strain and produced 23-39% more cephalosporin C. The increase in DAOCS activity also reduced the amount of penicillin N excreted into the medium. The recombinant

strains produced more cephalosporin C in pilot-scale fermentations and the validity of this approach for improving the yield of antibiotics is now established. A key factor is selection of the rate-limiting step in a process for yield improvement.

MATERIALS AND METHODS

I. Bacteria and Plasmids

The strains and plasmids used in this study are listed in Table 1.

II. Chemicals and Biochemicals

Yeast extract, bacto-peptone, bacto-tryptone, casamino acid, malt extract, nutrient broth. tryptic soya broth were purchased from Difco Laboratories, Detroit, Michigan. 3-[N-Morpholino]propanesulfonic acid (MOPS), N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), trichloroacetic acid, ficoll, polyvinylpyrrolidone, triton X-100, bovine albumin (fraction 5), adenosine 5'-triphosphate, o-aminobenzaldehyde, pyridoxal-5-phosphate, α-ketoglutaric acid, ribonuclease A, deoxyribonuclease, pronase, lysozyme (grade 1) ampicillin (sodium salt), thiostrepton and all amino acids were purchased from Sigma Chemical Co., St. Louis, Mo. High-pressure liquid chromatography grade acetonitrile was from Fisher Scientific Co., Fairlawn, N.J. Silica Gel G thin-layer chromatography plates were from E. Merck AG, Darmstadt, Federal republic of Germany. Tris[hydroxylmethyl]aminomethane (Tris), random primed DNA labelling kit were purchased from Boehringer Mannheim, Dorval, Que. All restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase were purchased from Bethesda Research Laboratories, Burlington, Ont. [α-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from Du Pont Canada Inc., Mississauga, Ont. Nitrocellulose, ultra pure DNA grade agarose and sodium dodecyl sulfate were from Bio-Rad Laboratories Ltd., Mississauga, Ont., and Hybond N membrane was purchased from Amersham Canada Ltd.,

Table 1. Bacteria and Plasmids used.

Strain	Genotype/phenotype	Source/reference[a]
β-Lactam producers		
S. clavuligerus		
NRRL3585	Wild type	ARS
DCC1 to DCC10	Lut$^-$	This study
S. lipmanii	Wild type	ARS
NRRL3584		
S. griseus	Wild type	ARS
NRRL3851		
N. lactamdurans	Wild type	ARS
NRRL3802		
β-Lactam nonproducers		
S. lividans		
TK24	SLP1$^-$, SLP2$^-$, *str*	Hopwood et al., 1983
DCL1 to DCL4	SLP1$^-$, SLP2$^-$, *str*, Lut$^-$	This study
S. venezuelae		
ISP5230 (10712)	Wild type	E. Wellington
VS206	*hisA6*, *adeA10*, *strA6*	C. Stuttard
DCV1 to DCV4	*hisA6*, *adeA10*, *strA6*, Cut$^-$	A. Manning
VS1	*lysA1*	C. Stuttard
DCV5 to DCV8	*lysA1*, Lut$^-$	This study

Table 1. (continued)

S. phaeochromogenes NRRLB2119	Wild type	ARS
S. griseofuscus NRRLB5429	Wild type	R.H. Baltz
S. viridochromogenes CUB416	Wild type	K.F. Chater
S. glaucescens GLAO	Wild type	R. Hütter
S. parvulus ISP5048	Wild type	K.F. Chater
S. rimosus NRRL2234	Wild type	K.F. Chater
E. coli LE392	F^-, hsdR514, supE44, supF58, lacY1 or (lacIZY)6, galK2, galT22, metB1, trpR55, lambda$^-$	Promega
E. coli DH5α	F^-, φ80dlacZ M15 (lacZYA-argF) 169 recA1, endA1, hsdR17, supE44, lambda$^-$, thi-1, gyrA, relA1	BRL
E. coli ESS	β-lactam supersensitive	Hu et al., 1984
Plasmids		
pLJ702	tsr, mel$^+$	Katz et al., 1983
pIZ18R	amp, lacZ	Mead and Kemper, 1988.
pBL1	pUC119 containing 9.6-kb S. clavuligerus DNA	Leskiw et al., 1988

a ARS, U.S. Agricultural Research station, Peoria, Il.; BRL, Bethesda Research Laboratories, Burlington, Ont.; Promega, Madison, Wis.;

Oakville, Ont. Lambda GEM-11 cloning kit was from Promega, Madison, Wis. Polyethylene glycol 1000 was from Koch light, Haverhill, Suffolk, United Kingdom. Other chemicals were of reagent grade.

III. Media

Agar media

1) Complex MYM medium (Stuttard, 1982):

Maltose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
Agar	20.0 g
Distilled water	1000 ml

2) Minimal medium (Hopwood et al., 1985)

Maltose	10.0 g
Dipotassium hydrogen phosphate	0.5 g
Magnesium sulfate heptahydrate	0.2 g
Ferrous sulfate heptahydrate	9.0 mg
Nitrogen source*	0.5 g
Agar	15.0 g
Distilled water	1000 ml

* Nitrogen sources used were L-lysine.HCl for the isolation of lysine non-utilizing (Lut^-) mutants; cadaverine, δ-aminovalerate, α-aminoadipate (AAA) and asparagine for testing the phenotype of Lut^- and Cut^- mutants; AAA for testing the utilization of this amino acid by various streptomycetes.

3) Defined medium for bioassays (modified from the defined medium of Aharonowitz and Demain, 1977):

Glycerol	10.0 g
Proline	2.0 g
Dipotassium hydrogen phosphate	1.0 g
Magnesium sulfate	0.6 g
Trace element solution*	1.0 ml
Agar	15.0 g
Distilled water	1000 ml

* Ferrous sulfate heptahydrate	100 mg
Manganese chloride tetrahydrate	100 mg
Zinc sulfate heptahydrate	100 mg
Calcium chloride	100 mg
Distilled water	100 ml

3) R5 medium for protoplast regeneration (Hopwood et al., 1985)

Sucrose	103.0 g
Potassium sulfate	0.25g
Magnesium chloride hexahydrate	10.12 g
Glucose	10.0 g
Casamino acids	0.1 g
Trace element solution*	2.0 ml
Yeast extract	5.0 g
TES buffer	5.73 g
Agar	22 g
Distilled water	to 1000 ml

Just before use the medium was melted and supplemented (per 100 ml) with the following:

Dihydrogen potassium phosphate (0.5%)	1.0 ml
Calcium chloride dihydrate (5 M)	0.4 ml
Proline (20%)	1.5 ml
Sodium hydroxide (1 N)	0.7 ml

* Composition of trace element solution (per litre):

Zinc chloride	40 mg
Ferric chloride hexahydrate	200 mg
Cuprous chloride dihydrate	10 mg
Manganese chloride tetrahydrate	10 mg
$Na_2B_4O_7.10H_2O$	10 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	10 mg

4) Regeneration medium for S. venezuelae (Aidoo et al., 1990)

The composition was the same as R5 medium except that sucrose was

replaced with 0.3 M sodium chloride as an osmotic stabilizer.

5) Medium for the sporulation of S. lividans

Same as R5 medium except that sucrose was omitted and glucose was replaced with maltose.

6) LB agar (modified from L agar of Hopwood et al., 1985):

Bacto-tryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1000 ml

7) Soft nutrient agar (Hopwood et al., 1985):

Nutrient broth powder	8.0 g
Agar	3.0 g
Distilled water	1000 ml

8) Top agarose (Maniatis et al., 1982):

Bacto tryptone	10.0 g
Sodium chloride	5.0 g
Agarose	8.0 g

The medium was autoclaved, allowed to cool to 60°C and 10 ml of 1 M magnesium sulfate was added.

Liquid media

1) YEME medium (Hopwood et al., 1985):

Yeast extract	3.0 g
Malt extract	3.0 g
Bacto peptone	5.0 g
Glucose	10.0 g
Sucrose	340 g
Distilled water	to 1000 g

After the medium had been autoclaved, magnesium chloride hexahydrate

(2.5 M) was added at 2 ml/litre, and for protoplasting 25 ml/litre of 20% glycine was also added.

2) Trypticase soya broth:

Tryptone soya broth powder	30.0 g
Glycerol	10.0 g
Distilled water	1000 ml

3) Luria broth (LB) (modified from L broth of Hopwood et al., 1985):

Bacto-tryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml

The medium was adjusted to pH 7.5.

4) TB medium:

Bacto-tryptone	10.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml

5) Defined medium for studying lysine catabolism (glucose-isoleucine medium of Chatterjee et al.[1983] modified by substituting lysine for isoleucine):

Glucose	30.0 g
Magnesium sulfate heptahydrate	0.2 g
Potassium dihydrogen phosphate	4.5 g
Dipotassium hydrogen phosphate	10.5 g
Lysine	5.4 g
Sodium chloride	90 mg
Calcium chloride	90 mg
Ferrous sulfate heptahydrate	9.0 mg
Zinc sulfate heptahydrate	4.0 mg
Cupric sulfate pentahydrate	0.18 mg
Boric acid	26 µg
Ammonium molybdate tetrahydrate	17 µg
Manganese sulfate tetrahydrate	27 µg
Distilled water	1000 ml

6) Defined medium for physiological studies (Shapiro, unpublished):

Carbon source	1.0%
0.5% Calcium chloride solution	1.0% (v/v)
Dipotassium hydrogen phosphate	0.12%
MOPS buffer	1.05%
Trace mineral solution*	1.0% (v/v)
Nitrogen source	30.0 mM

* Magnesium sulfate heptahydrate	5.0 g
Ferrous sulfate heptahydrate	0.32 g
Zinc sulfate heptahydrate	0.3 g
Manganese sulfate tetrahydrate	0.16 g
Cuprous sulfate pentahydrate	0.12 g
Distilled water	100 ml

IV. Culture conditions

A. Preparation of spore stocks

Streptomyces strains were allowed to sporulate on appropriate media in petridishes and the spores were dispersed in 10 ml of sterile water. The spore suspension was filtered through cotton wool to remove mycelial fragments, then pelleted by centrifugation. The spore pellet was resuspended in 20% glycerol and stored at -20°C.

B. Single plaque lambda eluate

Using the narrow end of a sterile pasteur pipette a single plaque was transferred to 1 ml of phage buffer containing a few drops of chloroform. The phages were allowed to elute at room temperature for at least 2 h and subsequently stored at 4°C.

C. Vegetative inoculum

A vegetative inoculum of S. lividans for studies of lysine catabolism was grown for 48 h at 30°C on a shaker rotating at 250 rpm

from spores (100 µl) inoculated into YEME medium. The mycelium was then harvested by centrifugation, washed with physiological saline and transferred to the defined medium. In physiological studies on the regulation of lysine catabolic pathway enzymes, mycelium was initially grown in trypticase soya broth for 48 h at 30°C; the washed mycelium was then transferred to defined medium containing 15 mM asparagine and 1% starch and allowed to grow for 24 h. The culture adapted to defined medium now served as inoculum for defined medium containing different carbon and nitrogen sources.

D. Culture conditions for plasmid isolation

For small scale preparation of Streptomyces plasmids 100 µl of spore suspension was used to inoculate into 25 ml of YEME medium contained in a 250-ml Erlenmeyer flask and shaken at 250 rpm for 48 h at 30°C. For large-scale plasmid isolation, mycelium was propagated as above and transferred to 500 ml of YEME medium contained in two 1000-ml Erlenmeyer flasks. The culture was grown as before for 48 h at 30°C.

E. Growth of mycelium for protoplast preparation

A spore suspension (100 µl) was transferred to 25 ml of YEME medium contained in a 250-ml Erlenmeyer flask and shaken at 30°C. After 48 h the mycelium was harvested and washed twice with 10.3% sucrose. If not used immediately it was kept frozen at -20°C until needed.

F. Growth of E. coli

A single colony of E. coli LE392 from LB agar was transferred to

25 ml of TB medium containing 0.25 ml of 1 M magnesium sulfate and 0.25 ml of 20% (w/v) maltose. The culture was shaken overnight at 37°C and stored at 4°C. This culture was used in preparing phage lambda DNA and for plating recombinant lambda PFU containing S. clavuligerus genomic libraries.

To prepare competent cells of E. coli a culture of E. coli DH5α, initiated by inoculating 10 ml of LB medium with one colony of E. coli from an LB plate, was shaken overnight at 37°C. The next day 25 ml of LB was inoculated with 0.25 ml of tha culture and shaken at 37°C until the OD_{550} reached 0.5 to 0.6. The resulting culture was used to prepare competent cells.

G. Growth measurement

A sample of cell suspension (250 μl) was vortexed with 250 μl of 1M sodium hydroxide and incubated at 37°C for 24-48 h to solubulize the cells. The resulting suspension was assayed for protein content by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

V. β-Lactam bioassay

Cultures of S. clavuligerus were plated on defined fermentation medium. When single colonies became visible, they were excised on agar plugs (7 x 5 mm) and incubated in moist conditions at 30°C for 48 h. They were then transferred to trays (22.5 x 22.5 cm), each containing 100 ml of 3% tryptone soya broth seeded with the β-lactam-supersensitive strain, E. coli ESS. The trays were incubated at 37°C overnight and

examined for zones of growth inhibition.

VI. Isolation and characterization of lysine nonutilizing mutants (Lut$^-$)

Mutants unable to catabolize lysine (Lut$^-$) were isolated from streptomycetes after treatment with 5 mg of N-methyl-N'-nitro-N-nitrosoguanidine per ml in 0.05 M tris-maleic acid buffer, pH 8.0. To obtain the desired 5% survival of colony forming units (CFU), spores of S. clavuligerus, S. lividans or S. venezuelae were treated for 20, 120 and 100 min, respectively. For the detection of Lut$^-$ mutants, replica platings on aspargine and lysine containing defined minimal media were compared. For the phenotypic characterization of Lut$^-$ mutants, defined minimal media without asparagine but containing intermediates of lysine catabolism, such as cadaverine, δ-aminovalerate and α-aminoadipate, were used.

VII. Isolation of lysine catabolic products

Samples (5 ml) of an S. lividans culture grown in defined medium containing lysine as the sole nitrogen source were collected at 24-h intervals and were centrifuged at 900 X g. The mycelium was washed twice with physiological saline, resuspended in 5 ml of 70% ethanol and heated in boiling water for 10 min to extract amino acids. The resulting suspension was clarified by centrifugation at 10,000 X g for 10 min. The mycelium extract was freeze-dried and the culture filtrate was dried either at room temperature or under a jet of air. The dried samples were resuspended in 100 μl of distilled water.

VIII. Thin-layer chromatography and amino acid analyses

The amino acid extract and reference compounds were chromatographed on thin (250 μm) layers of silica gel G with a solvent system containing methanol, ammonium hydroxide and acetic acid (7:3:0.1). The plates were dried at room temperature; amines and amino acids were visualized by dipping the chromatogram in 0.25% ninhydrin in acetone, then heating it at 100°C for several minutes.

Amino acid analyses were obtained with a module 119CL amino acid analyzer (Beckman Instruments Inc., Fullerton, Calif.) by using a column of W-3H cation-exchange resin (Beckman) and sodium citrate (0.2 N Na^+ [pH 3.53], 0.4 N Na^+ [pH 4.12], and 1.0 N Na^+ [pH 6.40]) as sequential eluting buffers.

IX. High-pressure liquid chromatography

Reference amino acids (2 mg) and mycelial extracts (10 to 20 μl) were derivatized for detection by being mixed with 10 mg of picryl sulfonic acid (2,4,6-trinitrobenzene sulfonic acid) in 0.5 ml of 2% sodium bicarbonate as described by Vitt et al. (1983). The solution was kept at room temperature in the dark for 2 h. Derivatized amino acids were applied to a Beckman Ultrasphere ODS (5 μm) column (0.46 by 25 cm) equilibrated with 35% acetonitrile in citric acid (0.098 M)-acetic acid (0.43 M)-disodium hydrogen phosphate (0.016 M) at pH 2.5. The column was developed isocratically with the solvent mixture at a flow rate of 1 ml/min using a model 330 high-pressure liquid chromatograph (Beckman). The eluate was monitored at 350 nm with a model 450 variable wavelength detector (Waters Associates, Inc., Milford, Mass.).

X. Lysine ε-aminotransferase and cadaverine aminotransferase assay

Mycelium harvested by centrifugation was washed with 0.85% sodium chloride, suspended in 0.2-M potassium phosphate buffer, pH 7.5, and disrupted by sonic oscillation (Branson Sonifier, four to six 10-s pulses with a 1-min interval between each pulse). Cell debris was removed by centrifugation for 15-20 min at 4°C and 10,000 X g. The supernatant fluid was used as the cell extract.

The assay for lysine ε-aminotransferase was based on that of Kern et al. (1980). The incubation mixture contained 1 ml of cell extract, 40 μmol of α-ketoglutarate, and 0.15 μmol of pyridoxal phosphate in a final volume of 2.0 ml. Cadaverine aminotransferase was assayed by the method of Fothergill and Guest (1977). The incubation mixture consisted of 1 ml of cell extract, 300 μmol of cadaverine, 25 μmol of α-ketoglutarate, 0.25 μmol of pyridoxal phosphate, and 1 ml of sodium carbonate buffer, pH 10.25. To stop both enzyme reactions, 0.8 ml of the reaction mixture was mixed with 0.4 ml of 5% trichloroacetic acid in absolute ethanol. Precipitated proteins were removed by centrifugation at 11,600 X g. The amounts of 1-piperideine-6-carboxylate and 1-piperideine in 1 ml of deproteinized reaction mixture were measured by adding 1.5 ml of 4 mM o-aminobenzaldehyde in 0.2-M phosphate buffer, pH 7.5. The mixtures were heated at 37°C for 1 h, and the yellow-orange products were measured at 465 nm. To estimate the amounts of 1-piperideine-6-carboxylate and 1-piperideine formed, an extinction coefficient of 2,800 litres/mol/cm was used (Fothergill and Guest, 1977). One enzyme unit is defined as that amount of enzyme catalyzing the appearance of 1 nmol of product in 1 min. Protein in the cell

extract was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The values given in Tables 6 to 12 represent average values from duplicate or triplicate experiments.

XI. Isolation of total DNA

A. Large-scale isolation

Mycelium (1 g wet weight) was suspended in 5 ml of TE buffer (10-mM tris-HCl, pH 8.0, and 1-mM sodium EDTA, pH 8.0) containing 10 mg of lysozyme and vortexed and incubated at 30°C until a drop of the suspension was completely cleared by addition of one drop of 10% SDS. To this suspension 1.2 ml of 0.5-M EDTA and 0.13 ml of 0.2 mg/ml pronase solution were added, mixed in gently and incubated at 30°C. After 5 min, 0.7 ml of 10% SDS was added, mixed in gently and incubated at 37°C until the solution cleared and appeared to be viscous. Into the viscous solution, 6 ml of phenol solution (500 ml phenol, 0.5 g hydroxyquinoline and 65 ml of TE buffer containing 0.1-M sodium chloride, mixed thoroughly to form a single phase) was mixed thoroughly by gently shaking for 10 min. The creamy white suspension was shaken with 6 ml of chloroform for 5 min, then centrifuged for 10 min at 6,600 x g. The aqueous phase was transferred to another screw-cap bottle and reextracted with phenol-chloroform. The aqueous phase was mixed with 40 µg/g of RNase (prepared by dissolving 10mg/ml of RNase in distilled water and incubating at 90°C for 10 min to inactivate contaminating DNase) and incubated at 37°C for 1 h. To the RNase-treated solution, 0.1 vol of unbuffered 3 M sodium acetate and 2 vol of absolute ethanol

were added; the DNA that precipitated when the solutions were mixed was collected by spooling and transferred to a fresh screw-cap tube. The DNA pellet was washed with 70% ethanol, dried and finally dissolved in TE buffer and refrigerated.

B. Small-scale isolation

Mycelium (50 mg) was resuspended in 500 µl of lysozyme solution (2 mg/ml of lysozyme and 50 µg/l of RNase in 0.3-M sucrose, 25 mM tris, pH 8.0, and 25-mM EDTA, pH 8.0) and incubated at 37°C until the suspension became translucent. To the suspension 250 µl of 2% SDS was added and the mixture was vortexed until the viscosity decreased. Neutral phenol-chloroform was prepared by mixing 5 ml of phenol containing 5 mg of 8-hydroxyquinoline with 5 ml of chloroform-isoamyl alcohol (24:1); the resulting mixture was equilibrated first with 0.5 vol of 1-M tris, pH 8.0, then with 0.5 vol of 0.1-M tris, pH 8.0. The resulting neutral phenol-chloroform (250 µl) was added to the DNA sample, vortexed and centrifuged for 5 min. The aqueous phase was transferred to a new centrifuge tube and treated with neutral phenol-chloroform repeatedly until very little or no residue collected at the interphase. To the aqueous phase, 0.1 vol of unbuffered 3-M sodium acetate and 1 vol isopropanol were added and incubated at room temperature for 5 min. The precipitated DNA was collected by centrifugation and the pellet was washed with 70% ethanol, then dissolved in TE buffer.

XII. Isolation of plasmid

A. large-scale isolation of Streptomyces plasmids

Strains containing plasmids were grown in 500 ml of YEME medium containing 5 μg/ml thiostrepton. After 48 h the mycelium was collected by centrifugation and resuspended in 45 ml of lysis buffer (0.3-M sucrose, 25-mM tris.HCl, pH 8.0, and 25-mM EDTA, pH 8.0) containing 100 mg of lysozyme. The cell suspension was incubated at 37°C for 30 min. Alkaline SDS (25 ml of a 2% solution of SDS in 0.3 N sodium hydroxide) was then added and mixed in by syringing 8-10 times with a 20-ml plastic syringe (without a needle). The mixture was incubated at 70°C for 20 min. After the mixture had cooled to room temperature 8 ml of acid phenol-chloroform (5 ml of redistilled phenol mixed with 5 mg of 8-hydroxyquinoline and 5 ml of chloroform-isoamyl alcohol [24:1]) was added and the phases were mixed thoroughly by shaking. The creamy white emulsion was centrifuged at 6,600 x g for 15 min and the aqueous phase was transferred to a screw-cap bottle containing 0.1 vol of unbuffered 3-M sodium acetate and 1 vol of isopropanol. After 5 min at room temperature, the precipitated DNA was recovered by centrifugation at 6,600 x g for 15 min. The pellet was dissolved in 5-10 ml of TE and transferred to a screw-cap tube. To the DNA solution, 0.1 vol of sodium acetate and 0.5 vol of neutral phenol-chloroform were added and the mixture was vortexed for 1 min. The creamy white suspension was centrifuged at 6,600 x g for 10 min and the aqueous phase was transferred to a new screw cap tube to which was added 1 vol of isopropanol. The DNA was allowed to precipitate at room temperature for

5 min and was recovered by centrifugation at 10,000 rpm. The DNA pellet was dissolved in 8 ml of TE. To the DNA solution, 8.4 g of cesium chloride was added and dissolved by warming at 30°C for a few minutes. To the DNA-cesium chloride solution 0.05 ml/ml of ethidium bromide solution (10 mg/ml) was added and the resulting solution was transferred with a syringe to heat-sealable ultracentrifuge tubes. They were filled with paraffin oil and freed of air bubbles before they were heat sealed. The tubes were centrifuged at 36,000 rpm for 60 h in a Beckman Ti50 rotor. After centrifugation, the layer of plasmid DNA was detected under long-wave UV and recovered using a 1-ml syringe fitted with an 18G needle. The recovered fraction was extracted 4-5 times with water-saturated butanol to remove ethidium bromide. To the clear solution, 2 vol of distilled water and 6 vol of absolute ethanol were added. The DNA that precipitated after 15 min at 4°C was recovered by centrifugation at 6,600 x g for 15 min. The pellet was washed once with 70% ethanol, dried, dissolved in TE buffer, and stored at 4°C.

B. Small-scale isolation of Streptomyces plasmids

Plasmid-containing strains were grown in 25 ml of YEME medium containing 5 μg/ml of thiostrepton. Mycelium from the cultures was used to isolate plasmid DNA by the steps outlined in the large-scale isolation procedure, except that the cesium chloride-density gradient separation was omitted. The reagents were scaled down proportionally.

C. Small-scale isolation of E. coli plasmid (rapid boiling method)

E. coli strains harboring plasmids were grown overnight at 37°C in

LB medium containing 50 μg/ml ampicillin. A 1.5-ml portion of the culture was centrifuged for 2 min at 11,600 X g. The supernatant fluid was discarded and the cell pellet was resuspended in 400 μl of lysis buffer (250 μg/ml of lysozyme in a solution containing 8% sucrose, 0.5% Triton X-100, 50-mM EDTA, pH 8.0, and 10-mM tris.HCl, pH 8.0). The cell suspension was heated in a boiling water bath for 45 s and immediately centrifuged at 11,600 X g for 10 min. The pellet was removed using a toothpick, and to the supernatant fluid 40 μl of 3-M sodium acetate and 420 μl of isopropanol were added. After 15 min at -20°C, precipitated DNA was recovered by centrifugation for 15 min, washed with 70% ethanol and dissolved in 50 μl of TE buffer.

XIII. Isolation of phage lambda DNA

A single-plaque eluate (5-10 μl) was added to 300 μl of E. coli LE392 grown in TB medium and incubated at 37°C for 20 min to allow phage to adsorb. Top agarose (7 ml) was added to the phage-bacteria mixture, mixed in by vortexing and plated on LB agar contained in a 15-cm diameter Petri plate. After overnight incubation at 37°C, the plate was flooded with 10 ml of phage buffer (20-mM tris.HCl, pH 7.4, 100-mM sodium chloride, 10-mM magnesium sulfate and 0.1% gelatin) and incubated at room temperature with constant shaking for 2 h. The phage eluate was decanted, centrifuged and incubated with RNase (1 μg/ml) and DNase (1 μg/ml) at 37°C for 30 min. The lysate was centrifuged at 45,000 X g for 2 h to pellet the phage, after which the phage DNA was isolated as follows.

The pellet was suspended in RNase solution (50 μg/ml in MF buffer).

MT buffer was prepared by combining 1 ml of 1-M tris.HCl, pH 7.5, 0.1 ml of 1-M magnesium chloride and 100 ml of distilled water. After incubation on a shaker for 20 min at 37°C with gentle shaking, the RNase treated suspension was transferred to a 1.5-ml plastic microfuge tube and 80 μl of SDS mix (1 vol of 2-M tris.HCl, pH 9.6, 2 vol of 0.5-M EDTA, pH 7.4, and 1 vol of 10% SDS mixed shortly before use) was added. After 30 min at 70°C, 100 μl of 8-M potassium acetate was added and the mixture was immediatly stored on ice for 15 min. Precipitated material was removed by centrifugation and the supernatant solution was transferred to a fresh microfuge tube. Proteins were removed by repeated extraction with an equal volume of neutral phenol-chloroform. To the aqueous phase an equal volume of isopropanol was added; after 5 min at room temperature, the precipitated DNA was recovered by centrifugation. The DNA pellet was washed with 70% ethanol and dissolved in 100 μl of TE buffer.

XIV. Preparation of S. lividans protoplasts

The procedure required P buffer, which has the following composition:

Sucrose	103 g
Potassium sulfate	0.25 g
Magnesium chloride hexahydrate	2.02 g
Trace element solution*	2.00 ml
Distilled water	to 800 ml

The solution was dispensed in 40 ml aliquots and autoclaved. Before use, the following solutions were added:

0.5% Dipotassium hydrogen phosphate	0.50 ml
3.68% Calcium chloride	5.00 ml
5.73% TES, pH 7.2)	5.00 ml

* The composition of trace element solution is given in the media section for R5 medium.

Mycelium obtained after growth of 25-ml cultures for 48 h in YEME medium was resuspended in 4 ml of P buffer containing 1 mg/ml lysozyme and incubated for 30 min at 30°C. Every 10 min the mycelium-lysozyme suspension was mixed by pipetting up and down 3-4 times. After 30 min, 5 ml of P buffer was added and the suspension was mixed by pipetting up and down 4-5 times. The suspension was filtered through cotton wool and protoplasts were sedimented at low speed in a table-top centrifuge for 10 min. The supernatant fluid was discarded and protoplasts were resuspended by gently tapping the side of the tube.

XV. Transformation of Streptomyces protoplasts

The procedure required transformation and P buffers. The composition of P buffer is given in the section on protoplasting. To prepare transformation buffer (Hopwood et al., 1985), The following solutions were mixed.

10.3% Sucrose	25.00 ml
Trace element solution*	0.20 ml
2.5 % potassium sulfate	1.00 ml
Distilled water	75.00 ml

* The composition of trace element solution is given in the media section for R5 medium.

At the time of use the following solutions were added to 3.1 ml of the above mixture:

5 M calcium chloride	67 µl
Tris-maleic acid buffer*	167 µl

To 3 ml of the resulting solution was added 1 ml of previously sterilized PEG1000. The final mixture (transformation buffer) was used in transformation.

* Tris-maleic acid buffer was prepared by adjusting the pH of 1-M tris buffer to 8.0 with maleic acid.

A. Large-scale transformation

To construct genomic libraries of Streptomyces species, protoplasts obtained from one 25-ml culture were mixed with 1 μg of plasmid DNA by tapping the tube 4-5 times. Immediately, 0.5 ml of transformation buffer was added and mixed in by pipetting 4-5 times. P buffer (5 ml) was added and the transformed protoplasts were sedimented in a bench top centrifuge at low speed. The pellet was resuspended in 1 ml of P buffer and the suspension was plated on R5 medium (100 μl/Petri dish). The plated protoplasts were incubated for 18-20 h at 30°C and were overlayed with 2.5 ml soft nutrient agar containing thiostrepton to give a final concentration of 50 μg thiostrepton/ml of R5 medium. The plates were incubated at 37°C until sporulation occurred.

B. Small-scale transformation

A protoplast suspension (50 μl) dispensed in sterile microfuge tubes was supplemented with 5 μl of DNA and mixed by tapping 5-6 times. Immediately, 200 μl of transformation buffer was mixed in by pipetting 4-5 times. The transformed protoplasts were spread on R5 agar in two Petri dishes and incubated for 18-20 h. The regenerated protoplasts were overlayed with soft nutrient agar containing thiostrepton (final

concentration 50 μl/ml of R5 medium) and incubated at 30°C until sporulation occurred.

XVI. Transformation of E. coli

A. Preparation of competent cells

The E. coli culture described in the section on culture conditions was stored on ice for 10 min, after which it was centrifuged at 4,200 x g for 5 min at 4°C. The culture supernatant was discarded and 12.5 ml of an ice-cold mixture of 50-mM calcium chloride and 10-mM tris.HCl, pH 8.0, was added and the bacterial pellet was resuspended. The cell suspension was stored on ice for 15 min, then centrifuged at 4,200 x g for 5 min at 4°C. The bacterial pellet was suspended in 1.6 ml of 50-mM calcium chloride and 10-mM tris.HCl, pH 8.0. Portions (200 μl) were dispensed in prechilled microfuge tubes and used immediately or stored at 4°C for 12-24 h.

B. Transformation

To 200 μl of competent cells, DNA (dissolved in not more than 50 μl of TE buffer) was added. After mixing, the cell suspension was stored on ice for 30 min. The cells were then heat shocked for 2 min at 42°C and stored on ice for 1 min. The transformed cells were supplemented with 800 μl of LB medium, incubated on a roller drum for 1 h at 37°C, then plated on LB agar containing ampicillin at a final concentration of 50 μg/ml and X-gal (to detect transformants with insertional inactivation of the lacZ gene) at a final concentration of

40 μg/ml. The plates were incubated at 37°C overnight.

XVII. Genomic libraries

A. In an S. lividans Lut⁻ mutant

Genomic DNA from S. clavuligerus was partially digested by incubation with Sst1 (1 U/10 μg of DNA) for 40 min using the reaction buffer suggested by the supplier. Plasmid pIJ702 was completely digested with Sst1 (1U/μg of DNA). The partially digested genomic DNA (10 μg) and 2 μg of completely digested plasmid DNA were mixed and co-extracted with an equal volume of neutral phenol-chloroform. The DNA was precipitated with 0.1 vol of 3-M sodium acetate and 2 vol of absolute ethanol, and washed with 70% ethanol. The DNA was dissolved in 266 μl of distilled water and mixed with the following solution (total volume

300 μl):

DNA solution	266 μl
5 x ligation buffer*	30 μl
T_4 DNA ligase	4 μl

* 0.25 M tris.HCl, pH 7.6, 50-mM magnesium chloride, 5-mM ATP, 5-mM DTT, 25%

(w/v) polyethylene glycol-8,000.

The ligation reaction mixture was incubated at 16°C for 24 h. The ligated DNA was used to transform protoplasts from two 25-ml cultures of an S. lividans Lut⁻ strain by the large-scale method. The transformed protoplasts were spread on R5 agar in 40 Petri dishes. The transformed protoplasts were incubated at 30°C for 17 h and were overlayed with soft

nutrient agar containing thiostrepton (to a final concentration of 50 μg of thiostrepton/ml of R5). They were then incubated for one week at 30°C until sporulation occurred. Colonies were replicated on minimal medium (MM) with asparagine and on MM with lysine as nitrogen sources to isolate transformants containing cloned lysine catabolic pathway genes.

B. In phage lambda GEM-11

The strategy adopted to make genomic libraries was based on the half-site cloning system (Zabrovsky and Allikmets, 1986). The ends of partially digested genomic DNA fragments were half filled (see below) and ligated to lambda phage arms in which the single-strand ends had been half filled after digestion to completion with the restriction enzyme, Xho1. Using this strategy it was possible to avoid false linkages obtained by random ligation of target DNA fragments from two different regions of the genome. Size fractionation of target DNA is unnecessary as only fragments 9-23 kb in length ligated to lambda arms can be packaged.

Conditions for partial digestion of S. clavuligerus genomic DNA were established by treating a fixed amount of DNA with varying amounts of Sau3A restriction enzyme. Small fractions of the digested DNA were resolved on a 0.4% agarose gel and the fractions which contained DNA fragments predominantly in 9-23 kb range were identified. Approximately 5 μg of DNA obtained by pooling three such fractions (from restriction digests with 0.0037 U, 0.0075 U and 0.015 U/μg of DNA) were used to ligate to lambda arms.

Sau3A-digested genomic DNA fragments (with single stranded GATC

ends) were incubated with a reaction mixture containing nucleotides complementary to the two internal bases of the sticky ends (i.e A + G). The two outer bases remained free and are complementary to bases similarly generated by half filling fragment ends after complete digestion of lambda GEM-11 with the restriction enzyme Xho1. The following reaction conditions used for half filling Sau3A ends were those suggested by the supplier (Promega, Madison, Wis.).

Partially digested genomic DNA (5 μg)	20 μl
10X fill-in buffer*	5 μl
Klenow enzyme (6 U/μl)	2 μl
Distilled water	23 μl
Total volume	50 μl

10X fill-in buffer:

Tris.HCl, p^H 7.5	400 mM
Magnesium chloride	100 mM
DTT	100 mM
dATP + dGTP	10 mM
BSA	500 μg/ml

The partially filled in Sau3A fragments were ligated to the lambda GEM-11 half-site arms using the following conditions:

Partially filled-in insert DNA (1.4 μg)	2 μl
Lambda GEM-11 half-filled arms (0.5 μg)	1 μl
10 X ligation buffer*	0.6 μl
ATP (10 mM)	0.3 μl
BSA (1 mg/ml)	0.6 μl
Distilled water	0.5 μl
T4 ligase (1 U/μl)	1.0 μl
Total volume	6.0 μl

* Ligation buffer:

Tris.acetate, pH 7.4	20.0 mM
Magnesium chloride	7.5 mM
EDTA	0.1 mM
DTT	1.0 mM

The ligated DNA was packaged into lambda heads using the Packagene packaging system as recommended by the supplier (Promega). A packaging extract (stored in liquid nitrogen) was allowed to thaw on ice. The entire ligation mixture (6 µl) was added to the packaging system and mixed by tapping the tube. The packaging reaction mixture was incubated at 23°C for 2 h, then mixed with 444 µl of phage buffer to obtain a final volume of 500 µl. The packaged DNA constituted a genomic library in lambda phage and was stored at 4°C.

XVIII. Transfer of DNA from plaques to nitrocellulose filters

Packaged DNA (30-50 µl) was mixed with 300 µl of a culture of E. coli LE392, grown overnight in TB medium, and incubated at 37°C for 20 min. To this 7 ml of top agarose was added, mixed in by vortexing and spread on a 15-cm diameter Petri plate containing LB agar. The culture was incubated overnight at 37°C to obtain 5,000-10,000 plaques/plate. A 14-cm diameter nitrocellulose filter was placed on the surface of LB agar, marked to allow later location of plaques, and allowed to stand for 0.5-1.0 min. The nitrocellulose filter was removed and placed on a Whatman 3 MM filter paper saturated with denaturing solution (1.5-M sodium chloride, 0.5-M sodium hydroxide) for 1 min with the DNA side up. The filter was transferred to another Whatman 3 MM filter paper saturated with neutralizing solution (1.5-M sodium chloride, 0.5-M Tris.Hcl, pH 8.0) and allowed to stand for 5 min. The filter was removed and transferred to a baking dish containing 2xSSC (3-M sodium chloride, 0.3-M sodium citrate, pH 7.0) and gently washed to remove

bacterial debris and agarose overlay. The filter was air dried for 30-60 min and baked in an oven at 80°C for 2 h between two layers of Whatman 3 MM filter papers.

XIX. Labelling by random primer extension

The DNA fragment to be labelled was either purified from agarose gel using GENE CLEAN (Promega) or obtained by linearizing plasmid DNA with a restriction enzyme.

A. Gene clean

A gel slice containing the DNA fragments of interest was excised from an agarose electrophoresis gel and the approximate volume of the gel slice was determined. The gel slice, mixed with 2.5 vol of saturated sodium iodide solution, was incubated in a water bath at 50°C for 5 min to dissolve the agarose. Following this incubation 5 μl of GLASS MILK (silica matrix) was added to the liquified gel slice in saturated sodium iodide solution, mixed by vortexing and stored on ice for 5 min for the DNA released from gel slice to adsorb to the silica matrix. The silica matrix was sedimented by centrifugation in a microfuge for 10 s. The supernatant was discarded and the pellet was washed 3 times (by resuspending) with ice cold washing solution (supplied by the manufacturer and containing Tris, EDTA, sodium chloride and ethanol). DNA bound to the silica matrix was eluted by incubating the pellet with 5 μl of TE buffer at 50°C for 3 min. The silica matrix was sedimented by centrifugation in a microfuge for 30 s and supernatant fluid containing DNA was transferred to a new microfuge tube. After a

second elution to recover additional DNA, the two fractions were pooled for labelling.

B. Labelling

Labelling reactions were performed with a Boehringer Mannheim kit (Dorval, Que.) which contained (total volume 20 μl).

1 μl each of dATP, dGTP, dTTP (0.5 mmol/l in tris buffer)	3 μl
10x reaction buffer (containing random hexanucleotide mixture)	2 μl
50 μCi (α^{32}P) dCTP, 3000 Ci/mmol	5 μl
Klenow enzyme (2 U/μl)	1 μl
50-100 ng DNA	in 9 μl

The DNA was incubated in a boiling water bath for 10 min and cooled on ice. The components of the reaction mixture were added to the DNA solution and incubated at room temperature for 2 h. To estimate ^{32}P incorporation, 1 μl of the reaction mixture was diluted to 100 μl with water and the DNA was precipitated from 50 μl of the diluted sample with 5 μl of tRNA (10 mg/ml), 150 μl water and 2 ml of 5% TCA 0.1 M sodium pyrophosphate solution. The precipitation was allowed to proceed on ice for 30 min after which the precipitated DNA was filtered through a glass microfibre filter (Whatman, Clifton, NJ). The filter was rinsed once with a solution containing 5% TCA, 0.1-M sodium pyrophosphate, twice with 1% TCA and finally once with absolute ethanol. The filter containing precipitated DNA represented incorporated radioactivity. The percent incorporation was estimated by comparing TCA-precipitated counts with total counts before precipitation. Labelled DNA was separated from unincorporated nucleotides by precipitation with 0.5 vol of ammonium acetate, 2 vol of absolute ethanol and 1 μl of tRNA (10 mg/ml).

XX. 5' End labelling

The synthetic oligonucleotide probe used in isolating cefEF gene was labelled at its 5' end by using T4 polynucleotide kinase. The labelling reaction was of the following composition (total volume 50 µl):

Oligonucleotide (150-200 ng)	3 µl
200 µci (τ^{32}P) ATP, 4500 Ci/mmol	20 µl
10 x T_4 polynucleotide kinase buffer*	5 µl
T4 polynucleotide kinase (10 U/µl)	2 µl
Distilled water	20 µl

* Polynucleotide kinase buffer:

Tris.HCl, pH 7.4	0.5 M
Magnesium chloride	0.1 M
DTT	50 mM

The labelling reaction was incubated 2-3 h at 37°C after which amount of radioactivity incorporated into the DNA was estimated by the procedure outlined for measuring random primer extension. The labelled DNA was used without further purification.

XXI. Screening lambda plaques by hybridization

A. Using long probes

The purified DNA fragments were labelled by random primer extension using a Boehringer Mannheim kit (Dorval, Canada). The blots containing immobilized DNA were prewashed at 42°C for 2 h using prewashing solution (50-mM tris.HCl [pH 8.0], 1-M sodium chloride, 1-mM EDTA, and 0.1% SDS) to remove bacterial debris. The filters were then incubated for 2 h at 70°C in a prehybridization solution containing 5x SSC, 5x Denhardt's solution, 0.1% SDS, and salmon sperm DNA (100 µg/ml). This was followed

by overnight incubation at 70°C with hybridization solution (prehybridization solution supplemented with 10^7 cpm of ^{32}P-labelled DNA). The filters were washed 3-4 times at room temperature (5 min each wash) in 500 ml of 2x SSC and 0.1% SDS. The filters were then washed twice at 70°C for a total of 2 h, in 250 ml of 1x SSC and 0.1% SDS. Finally the filters were washed once more at 70°C for 1 h in 250 ml of 0.2x SSC and 0.1% SDS. The filters were air dried and autoradiographed.

B. Using an oligonucleotide probe

Blots of immobilized DNA, after prewashing, were incubated for 2-3 h at 65°C in a prehybridization solution containing 2x SSC, 20 mM-sodium pyrophosphate buffer, 5x Denhardt's solution, 0.1% SDS, and salmon sperm DNA (100 μg/ml). This was followed by overnight incubation at 65°C in hybridization solution (prehybridization solution supplemented with $2x10^7$ cpm of end-labelled DNA). The blots were washed twice in 2x SSC containing 0.1% SDS for 5 min at room temperature, then for 5 min in 2x SSC containing 0.1% SDS at 65°C, and finally in 1x SSC containing 0.1% SDS for 5 min at 65°C. The filters were air dried and autoradiographed.

C. Autoradiography

The washed filters were placed on a Whatman filter paper and secured in place with scotch tape. Pieces of masking tape with radioactive spots, were placed on three corners and the entire set up was wrapped in Saran wrap. The filters were then exposed to Kodak X-omat AR film at -70°C with an intensifying screen on top of the X-ray film. The X-ray films were treated first with developer for 5 min, then

with fixer for 5 min and finally washed with running water for 10-15 min.

D. Selection of clones from the library

The X-ray film was aligned with the LB agar plate containing the plaques and plaques in the region corresponding to positive signals were marked. Agar plugs were removed and phages were eluted with phage buffer. The phage eluate was diluted and plated on an 9-cm diameter Petri dish containing LB agar to obtain 50-100 discrete plaques/plate. The plaques were subjected to one more round of screening and a single, well-separated positive plaque was picked for subsequent analyses.

XXII. Restriction analysis of recombinant DNA

Restriction maps of recombinant DNA from lambda clones were obtained by two methods:

1) By single and double digests with various restriction enzymes: The lambda DNA was digested with restriction enzymes singly and in pairwise combinations. The DNA fragments were resolved on a 0.7% agarose gel and the sizes of individual fragments were estimated from their mobilities in relation to fragment size markers generated by digesting wild-type lambda DNA to completion with HindIII. The restriction sites were then ordered on the basis of the restriction fragment lengths.

2) By partial digestion with Kpn1 enzyme (Kohara et al., 1987): As there were many sites for Kpn1 in the lambda clones it was not possible to map them by method 1. Therefore, the lambda DNA was

partially digested with varying amounts of Kpn1 and a small fraction of the partially digested DNA was resolved on 0.4% agarose gel. The fraction which contained predominantly large fragments (>9 kb) was chosen and resolved on a 20 cm-long 0.4% agarose gel for 40 h at 25 volts in tris-phosphate-EDTA buffer (80-mM tris-phosphate, pH 8.0, 8-mM EDTA). Wild-type lambda DNA partially digested with Ava1 was used to obtain fragment size standards.

After electrophoresis the gel was blotted on a Hybond-N nylon membrane (Amersham, Oakville, Ont.). The filter was probed with a ^{32}P-labelled 4.3-kb lambda fragment (containing a cos end) obtained by digesting lambda DNA with HindIII. The filter was washed twice with 2x SSC containing 0.1% SDS for 10 min each followed by one wash with 1x SSC containing 0.1% SDS for 15 min at 70°C. The filter was wrapped in Saran wrap and autoradiographed. The sizes of bands on the X-ray film were estimated from the mobilities of the Ava1 size markers. The Kpn1 sites on insert portions of lambda clones were ordered after deducting the size of the lambda arm (approx. 9 kb) containing the 4.3-kb HindIII fragment. The sizes of individual Kpn1 fragments were confirmed by single digestion with Kpn1.

XXIII. Southern blot hybridization

DNA was digested with a chosen restriction enzyme and the fragments were separated on an agarose gel either in tris-acetate buffer(0.04-M tris-acetate, 0.002-M EDTA) or in tris-phosphate buffer (for mapping by partial digestion); The agarose gel was stained with 0.5 μg/ml ethidium bromide in water for 30 min. The stained gel was placed

on a UV transilluminator (Photodyne) and photographed. The separated fragments were transferred to Hybond-N nylon membrane.

A. Transfer of DNA from agarose gels to nylon membrane

The gel was trimmed and treated with 0.25-M hydrochloric acid for 15 min. It was then rinsed with water and treated twice with denaturing solution (0.5-N sodium hydroxide, 1.5-M sodium chloride) for 15 min each. The denaturing solution was removed and excess liquid was blotted off. Alkali transfer buffer (ATB), (0.25-M sodium hydroxide, 1.5-M sodium chloride) was added to the gel and left at room temperature for 10-15 min. Alkali transfer buffer was poured into a shallow baking dish to half fill the dish. A glass plate was placed on a central support and covered with a piece of Whatman 3 MM filter paper large enough to be in contact with ATB on all sides. Another piece of Whatman paper slightly smaller than the glass plate support was placed on the top. The gel was placed on the paper on the glass support and covered with a piece of Hybond-N nylon membrane, 2 mm larger than the gel dimensions. Two pieces of Whatman paper slightly larger (2 mm) than the gel and the same size as the membrane were soaked in ATB and placed on the nylon membrane. A stack of paper towels each slightly smaller than the gel was placed on the top of the Whatman paper to a height of 2-3 cm. This was followed by another stack of paper towels (each larger than the first stack) to a final total thickness of approximately 5 cm. A glass plate was placed on top of the paper towels and the entire unit was enclosed in Saran wrap. A weight (0.5 - 1 kg) was placed on the top glass plate. The next day the membrane was removed, air dried and DNA

was cross linked to it by exposing the DNA side of the membrane to UV for 1 min on a transilluminator.

B. Probing

The membrane with bound DNA was prehybridized for 1-2 h at 70°C in a solution containing 5x SSC, 5X Denhardt's solution, 0.5% SDS, and salmon sperm DNA (100 μg/ml). This was followed by overnight incubation at 70°C in hybridization solution (prehybridization solution supplemented with ^{32}P-labelled DNA). The membrane was washed twice at 70°C for 10 min each in 2x SSC containing 0.1%, followed by one wash in 1x SSC, 0.1% SDS for 15 min at 70°C and finally in 0.1x SSC, 0.1% SDS for 10 min at 70°C. When related sequences were probed, the membrane was first washed at 70°C in 2x SSC and the stringency was gradually increased by reducing the salt concentration to 0.5x SSC. The membrane was wrapped in Saran wrap immediately after washing and autoradiographed.

XXIV. Dot blot analysis

To 200 ng of DNA in 4.5 μl of water, 0.5 μl of 1-N sodium hydroxide was added and allowed to stand at room temperature for 15-20 min to denature the DNA. 2.1 μl of 20x SSC was added to neutralize the denatured DNA solution and the mixture was added to a nitrocellulose or nylon membrane with a pipette. The membrane was then washed briefly in 2x SSC and air dried. Nitrocellulose membranes were baked in an oven at 80°C for 2 h to fix DNA to the membrane, whereas nylon membranes were exposed to UV for 1 min to crosslink the DNA to the membrane. The

membranes were prehybridized, hybridized and washed according to the procedure outlined for Southern blotting.

XXV. Subcloning fragments from lambda into a shuttle plasmid

Sst1 fragments excised from recombinant lambda DNA were cloned into an E. coli-Streptomyces shuttle vector in two steps. First, the Sst1 fragment was cloned into an Sst1 site in the polylinker region of E. coli plasmid pIZ18R. The resulting plasmid was linearized with Pst1 and inserted into Streptomyces plasmid pIJ702 at its Pst1 site to obtain a shuttle vector containing the DNA fragment from S. clavuligerus; each vector carried amp for ampicillin selection in E. coli, and tsr for thiostrepton selection in Streptomyces.

RESULTS

I. Lysine catabolism in streptomycetes

Although AAA was known to be formed from lysine in streptomycetes (Kern et al., 1980), it's potential role in lysine catabolism had not been investigated when the work described in this thesis was begun. This section deals with lysine catabolism in streptomycetes with particular reference to the distribution of alternative pathways in β-lactam producing and nonproducing species.

A. Isolation and identification of metabolic products

Lysine catabolism was investigated in the β-lactam nonproducer, S. lividans, to determine whether the pathway included AAA. This organism could not grow if lysine was provided as the sole source of nitrogen and carbon but did use lysine as a nitrogen source if supplied with either maltose or glucose. Therefore, S. lividans was grown in a defined medium with lysine serving only as the sole nitrogen source; the mycelium and culture filtrate were examined for accumulation of intermediates produced during lysine metabolism.

Thin-layer chromatography of 70% ethanolic extracts from cells grown for 48, 72, 96 and 120 h showed four main ninhydrin-positive compounds, the R_F values of which matched those of cadaverine, glutamate, δ-aminovalerate and lysine (Table. 2). As the culture aged, the increased intensity of the zone at R_f 0.52 on thin layer chromatograms indicated that δ-aminovalerate accumulated preferentially. Cell extracts from cultures grown for 48 and 120 h were derivatized with

Table 2. Thin-layer chromatographic mobility (R_f value) of reference compounds and metabolites in cell extracts from S. lividans supplied with lysine as the sole source of nitrogen*

Compound	Reference	Cell extract
Cadaverine	0.18	0.16
Lysine	0.26	0.27
δ-Aminovalerate	0.52	0.53
Glutamate	0.60	0.62

* Samples were chromatographed on silica gel plates using the solvent system methanol-30% ammonium hydroxide-acetic acid (7:3:0.1). Amino acids were visualized by treatment with ninhydrin.

picryl sulfonic acid and analysed by HPLC. The results confirmed that glutamate and δ-aminovalerate were present (elution times of 5.43 min and 14.3 min, respectively). The area of the peak for δ-aminovalerate from cell extracts of a 120-h culture was larger than that obtained from a 48-h culture, confirming the observations from thin-layer chromatography. The HPLC procedure did not detect cadaverine. When amino acids from cell extracts from 48-h cultures were analysed qualitatively by ion-exchange chromatography and post-column reaction with nihydrin, glutamate, δ-aminovalerate and lysine were found at concentrations of 1.06, 0.28, and 1.25 μmol/ml, respectively. However, this procedure also did not measure cadaverine.

Examination of filtrates from 48, 72, 96 and 120 h cultures by thin-layer chromatography showed cadaverine and lysine as the only ninhydrin-positive compounds present. Moreover, accumulation of cadaverine in the culture correlated with disappearance of lysine. α-Aminoadipate could not be detected in any of the above analyses, although it was readily detected in cell extracts and culture filtrates of S. clavuligerus (S. Shapiro and R.L. White, unpublished).

The above observations, suggest that S. lividans supplied with lysine as the sole nitrogen source catabolizes the amino acid via cadaverine and δ-aminovalerate.

B. Isolation and characterization of Lut⁻ mutants

To explore further the results from biochemical analyses of the lysine catabolic pathway in S. lividans, mutants unable to grow on lysine as the sole source of nitrogen were isolated from S. lividans, S.

venezuelae and S. clavuligerus after treatment with NTG. S. venezuelae was included because knowledge of its genetic systems, such as conjugation and generalised transduction, could be used to obtain a fine structure map of the lysine utilization (lut) genes. Mutants of S. lividans and S. clavuligerus were classified into three groups based on their ability to grow when supplied with various intermediates [Table 3]. One group grew on cadaverine and δ-aminovalerate, indicating that these mutants are probably blocked in the first step of lysine catabolism via the cadaverine pathway. A second group was unable to use lysine and cadaverine but grew on δ-aminovalerate. The mutants in this group are probably blocked in the second step of lysine catabolism (cadaverine aminotransferase) but could also contain multiple lesions in the early steps. Mutants in the third group were unable to catabolize lysine, cadaverine or δ-aminovalerate. These mutants are either regulatory mutants or have multiple lesions in the earlier steps of lysine catabolism.

All the S. lividans Lut$^-$ mutants, including the third group, were able to grow on AAA. Bioassays of the S. clavuligerus Lut$^-$ mutants showed that all were able to produce β-lactam antibiotics, suggesting that the pathway to α-aminoadipate was functioning. Efforts to isolate Lut$^-$ mutants from S. venezuelae VS1 (lysA1) using NTG as a mutagen gave only one type, distinguished by the inability to use lysine or any of the intermediates of lysine catabolism as a nitrogen source. Failure to isolate any but this one type, which may be nitrogen assimilation mutants, raised the question of whether S. venezuelae has more than one pathway for lysine catabolism.

Table 3. Growth of different groups of lysine non-utilizing (Lut$^-$) mutants of S. lividans and S. clavuligerus[1]

Lut$^-$ group	Nitrogen source[2]		
	Lysine	Cadaverine	δ-Aminovalerate
I	-	+	+
II	-	-	+
III	-	-	-
Parent	+	+	+

1 +, growth; -, no growth after 4-5 days of incubation at 30°C. All S. lividans strains, but no S. clavuligerus strains could grow on α-aminoadipate.

2 Supplied to minimal medium [6] without asparagine, at a concentrations of 0.05% [w/v].

c. Characterization of Lut$^-$ mutants of S. venezuelae

In contrast to S. lividans and S. clavuligerus, S. venezuelae strain VS1, a lysine auxotroph, was able to grow on D-lysine as well as intermediates of D-lysine catabolism such as pipecolate and α-aminoadipate. To test whether the inability to isolate different types of S. venezuelae Lut$^-$ mutants was due to the existence of a pathway for lysine catabolism via pipecolate in addition to one via cadaverine, strain VS1 was grown on minimal agar with D-lysine or pipecolate as the nitrogen source, with and without lysine auxotrophic supplementation. In the lysine supplemented culture, growth was visible within 24 h on both D-lysine and pipecolate. When the required auxotrophic supplementation was omitted, growth on minimal agar with D-lysine as the nitrogen source did not commence until 4-5 days after replica plating. Pipecolate as the nitrogen source gave only a trace of growth even after 4-5 days. Because VS1 without lysine supplementation showed no growth whatsoever on minimal medium with asparagine as the nitrogen source, the growth observed on D-lysine was not due to leakiness of the mutant but rather to an activity which converted D-lysine to the L-isomer. However, the much faster growth of S. venezuelae VS1 on D-lysine or pipecolate as nitrogen source when L-lysine was also supplied suggested that a separate catabolic pathway via D-lysine existed.

To further test whether S. venezuelae has more than one pathway for lysine catabolism, mutants unable to assimilate cadaverine (Cut$^-$) isolated from S. venezuelae VS206 (hisA6, adeA10, strA6) by A. Manning, were characterised (Table 4). The Cut$^-$ mutants could be divided into two types. One type was similar to the Lut$^-$ mutants isolated from VS1

Table 4. Growth of Lut⁻ and Cut⁻ mutants of S. venezuelae on various nitrogen sources[1].

Group	Nitrogen source[2]						
	Asn	L-Lys	Cad	δ-Ava	D-Lys	Pip	AAA
Lut⁻ mutants							
Parent VS1	+	+	+	+	+	+	+
Lut⁻	-	-	-	-	-	-	-
Cut⁻ mutants							
Parent VS206	+	+	+	+	+	+	+
Type 1	+	+	-	+	+	+	+
Type 2	-	-	-	-	-	-	-

[1] +, growth; -, no growth after 24-36 h of incubation at 30°C.

[2] All strains were grown on minimal medium (Hopwood et al., 1985) or on this medium with asparagine replaced by the indicated nitrogen source at a concentration of 0.05% (w/v). Asn- aspargine, Lys- lysine, Cad- cadaverine, δ-Ava- δ-aminovalerate, Pip- pipecolate, AAA- α-aminoadipate.

in that they were unable to use L- or D-lysine or their catabolic pathway intermediates. The other type was blocked specifically in cadaverine utilization but was still able to use lysine, indicating that an alternative pathway for lysine catabolism was present. This type could metabolize D-lysine and intermediates in the D-lysine pathway. The results suggested that lysine might be used by an alternative pathway initiated by epimerization of L-lysine and subsequent catabolism of D-lysine via α-keto-ε-aminocaproic acid and pipecolate. However, they did not exclude other potential routes, of which the most plausible is the conversion of L-lysine to δ-aminovaleramide (Fig. 4).

D. Lysine and cadaverine aminotransferases

To assess whether inability to make β-lactam antibiotics correlated with the absence of LAT, a number of β-lactam-producing and nonproducing streptomycetes were analysed for LAT and cadaverine aminotransferase (CAT) activities. Cadaverine aminotransferase, an enzyme catalyzing the second step in the pathway for lysine assimilation via cadaverine, was readily detected in all cell extracts (Table 5). That the activity was due to aminotransferase and not to an amine oxidase was indicated by the marked decrease in enzyme activity when α-ketoglutarate (the putative amino group acceptor) was omitted from incubation mixtures. Activity was optimum at pH 10.5; in this respect CAT from streptomycetes was similar to the enzyme in Pseudomonas (Fothergill and Guest, 1977).

Lysine ε-aminotransferase could not be detected in extracts of S. lividans cells but was present in extracts of S. clavuligerus. To test

whether disruption of S. lividans cells released inhibitors or caused loss of enzyme activity, cell extracts prepared from S. lividans and S. clavuligerus were mixed. Enzyme activity was present in the mixture; the amounts indicated that no inhibition or rapid degradation had occurred, and that the enzyme was completely absent from the S. lividans extract. Of ten actinomycetes tested, lysine ε-aminotransferase was detected only in the three β-lactam producers (Table 5). This contrasted with the presence of cadaverine aminotransferase activity in every strain.

E. Catabolism of α-aminoadipate by different species of Streptomyces

The accumulation of AAA in cultures of S. clavuligerus grown in a defined medium (S. Shapiro and R.L. White, unpublished results) suggested that β-lactam producers do not catabolize AAA. Therefore, several β-lactam producing and nonproducing actinomycetes were tested for their ability to catabolize AAA in the absence of other nitrogen sources. The growth responses (Table 5) suggested that ability to make efficient use of α-aminoadipate is correlated with inability to make β-lactams. However, the converse is probably not true: some species not known to produce β-lactams showed relative (or total) inability to use AAA for growth

The overall conclusion from this investigation of lysine catabolism in streptomycetes was that there is a general pathway present in all species. This converts lysine via cadaverine and δ-aminovalerate to ammonia and central pathway intermediates (Madduri et al., 1989). In S. venezuelae a second pathway catabolizing lysine via the D-isomer is also present. β-Lactam producers have an additional pathway via AAA

Table 5. Specific activity of two early enzymes of lysine catabolism in actinomycete species, and growth of the species on α-aminoadipate.

Species	Cadaverine aminotransferase (units/mg protein)	Lysine ε-aminotransferase (units/mg protein)	Growth*
β-Lactam producers			
S. clavuligerus NRRL3585	2.38	1.16	-
S. griseus NRRL3851	1.92	0.85	+
N. lactamdurans NRRL3802	0.64	0.21	+
β-Lactam nonproducers			
S. lividans TK24	2.10	0	++
S. phaeochromogenes B2119	2.48	0	+
S. viridochromogenes CUB416	1.67	0	-
S. glaucescens GLAO	1.16	0	++
S. venezuelae ISP5230	1.56	0	++
S. parvulus ISP5048	0.64	0	tr
S. rimosus NRRL2234	1.1	0	tr

* α-aminoadipate (0.05%, w/v) was added to minimal agar made without asparagine; ++, growth as on asparagine; +, poor growth; tr, faint growth; -, no growth after 4-5 days at 30°C.

that provides a precursor for the biosynthesis of β-lactam secondary metabolites (Fig. 6) (Madduri et al., 1989).

II. Regulation

In the previous section biochemical and genetic approaches were described which were used to define the role of the pathway generating AAA in β-lactam-producing streptomycetes. This section describes physiological studies providing support for the hypothesis that the formation of AAA is a secondary metabolic activity. The regulation of enzymes in the cadaverine and AAA pathways was investigated 1) to identify differences in metabolic control of the two pathways, and 2) to determine if there were similarities in the regulation of LAT and other β-lactam biosynthesis enzymes.

A. Induction

Enzymes of amino acid dissimilatory pathways, like other catabolic enzymes, are often subject to induction by their substrates (Chang and Adams, 1971; Vandecasteele and Hermann, 1972; Fothergill and Guest, 1977). Secondary metabolic pathway enzymes, on the other hand, are not inducible and respond to an entirely different set of regulatory factors. When S. clavuligerus was grown in a defined medium, lysine ε-aminotransferase specific activity was not altered by lysine addition, irrespective of the carbon source employed. In contrast, CAT acivity was higher in cultures supplemented with lysine than in control cultures without this amino acid (Table 6). The effect of lysine supplementation was observed in cultures with various carbon sources. The ratio of

Fig. 6. Catabolic pathways for lysine in Streptomyces lividans and Streptomyces clavuligerus. Enzyme activities are: 1, lysine decarboxylase; 2, cadaverine aminotransferase; 3, 1-piperideine dehydrogenase; 4, lysine ϵ-aminotransferase; 5, 1-piperideine-6-carboxylate dehydrogenase. The pathway on the right is essential for the growth of Streptomyces on lysine; the pathway on the left is present only in β-lactam-producing Streptomyces. Another pathway, for the catabolism of α-aminoadipate (dashed line), exists in S. lividans but not in S. clavuligerus. Bracketed intermediate is hypothetical (Madduri et al., 1989).

Fig. 6

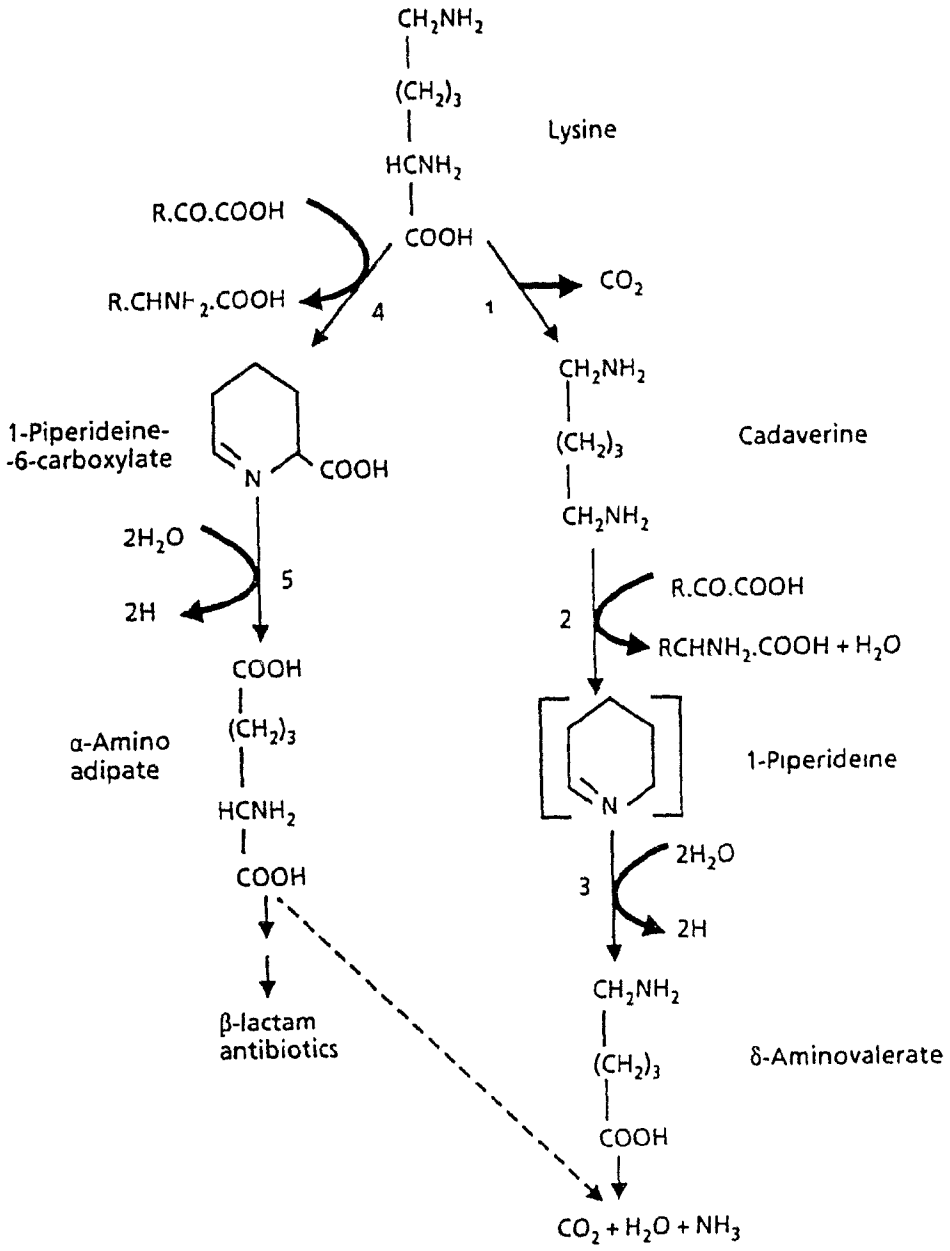

CH2NH2
(CH2)3
HCNH2
COOH
Lysine
R.CO.COOH
R.CHNH2.COOH
4
1
CO2
1-Piperideine-
-6-carboxylate
N
COOH
CH2NH2
(CH2)3
CH2NH2
Cadaverine
2H2O
5
2H
R.CO.COOH
2
RCHNH2.COOH + H2O
COOH
(CH2)3
α-Amino
adipate
HCNH2
COOH
1-Piperideine
N
2H2O
3
2H
CH2NH2
(CH2)3
COOH
δ-Aminovalerate
β-lactam
antibiotics
CO2 + H2O + NH3

Table 6. Effect of lysine supplementation on the specific activity of aminotransferases of S. clavuligerus cultures grown with different carbon sources*

Carbon source	Lysine ε-aminotransferase units/mg protein			Cadaverine aminotransferase units/mg protein		
	- Lysine	+ Lysine	Ratio(+/-)	- Lysine	+ Lysine	Ratio(+/-)
Glycerol	0.3	0.3	1.0	0.2	0.8	4.0
Maltose	1.5	1.5	1.0	0.2	1.6	8.0
Starch	1.1	0.7	0.6	0.3	1.0	3.0

* Cultures were grown in the defined medium for physiological studies containing 1% (w/v) carbon. Asparagine was supplied as the nitrogen source at 15 mM concentration and, where indicated, lysine at 15 mM concentration was added as an inducer.

Cultures were assayed for aminotransferases after growth for 48 h.

enzyme activity in cultures with lysine to that in cultures without lysine was highest (8.0) when maltose was the carbon source. The effect of lysine on CAT activity was seen throughout growth in a starch-asparagine medium supplemented with lysine, whereas LAT activity remained unchanged (Table 7). To test whether lysine is the primary inducer of CAT, S. clavuligerus was grown in starch-asparagine medium containing cadaverine or δ-aminovalerate. CAT activity was highest in 48-h cultures supplemented with cadaverine. δ-Aminovalerate, on the other hand, had no effect (Table 8). The results suggested that cadaverine, not lysine, is the primary inducer of this enzyme, and that the effect of lysine was probably due to its catabolism to cadaverine.

B. Carbon source regulation

Rapidly metabolized carbon sources may depress some catabolic and also some secondary metabolic enzymes. When S. clavuligerus is grown in a defined medium containing glycerol as the carbon source, β-lactam antibiotic synthesis is appreciably lower than in starch-supplemented cultures (Aharonowitz and Demain, 1978). DAOCS has been identified as a target for glycerol depression (Lebrihi et al., 1988). Glycerol strongly depressed LAT activity whereas maltose was the most favourable carbon source (Table 6). When enzyme activities were compared during growth of S. clavuligerus in starch and glycerol-supplemented cultures, glycerol proved to be more depressive for LAT activity than was starch at the 24 and 48-h sampling times (Table 9). At 72 h, the enzyme activity was very low in both, probably reflecting depletion of carbon sources in the medium. The ratio of enzyme activity in glycerol and starch grown

Table 7. Specific activity of aminotransferases of S. clavuligerus cultures at different stages of growth with and without lysine supplementation*

Age of culture (h)	Lysine ε-aminotransferase units/mg protein		Cadaverine aminotransferase units/mg protein	
	- Lysine	+ Lysine	- Lysine	+ Lysine
24	2.2	2.2	0.9	1.7
48	0.6	0.6	0.7	1.6
72	0.2	0.2	0.8	1.3

* Cultures were grown in the defined medium for physiological studies with starch as the carbon source at 1% (w/v). Asparagine was supplied as a nitrogen source at 15 mM concentration and where indicated lysine was supplied at 15 mM concentration as an inducer.

Table 8. Effect on cadaverine aminotransferase activity in S. clavuligerus of supplementing media with lysine catabolic pathway intermediates.

Medium composition*	Cadaverine aminotransferase units/mg protein
Starch + asparagine	1.0
Starch + asparagine + δ-aminovalerate	0.3
Starch + asparagine + cadaverine	9.5
Starch + asparagine + lysine	6.2

* Cultures were grown in the defined medium for physiological studies with starch at 1% (w/v) as the carbon source; the other components listed were provided as nitrogen sources, each at 15 mM. Cultures grown for 48 h were assayed for CAT activity.

Table 9. Effect of carbon source on intracellular lysine ε-aminotransferase activity during the growth of *S. clavuligerus**

Age of culture (h)	Carbon source	Growth mg protein/ ml culture	Lysine ε-aminotransferase	
			Total (units)	Specific activity (units/mg protein)
24	Glycerol	0.24	2.0	1.1
	Starch	0.18	3.3	2.7
48	Glycerol	0.80	4.2	0.8
	Starch	0.74	9.3	1.7
72	Glycerol	1.30	1.5	0.3
	Starch	0.70	3.3	0.6

* Cultures were grown in the defined medium for physiological studies with 1% (w/v) carbon and 15 mM asparagine as the nitrogen source.

cultures was approximately 1:3 after 24 and 48 h of growth. Increasing the glycerol concentration from 0.2% to 1.0% did not alter the activity of LAT significantly. Thus, the source of carbon but not the concentration seems to determine LAT activity in S. clavuligerus.

In a survey to identify carbon sources that affected CAT activity, glycerol, starch and to a lesser extent maltose at 15 mM concentration were found to be depressive (Table 10). Histidine and succinate, which are relatively poor carbon sources, were the least depressive when supplied at 15 mM concentration. In this experiment 15 mM ammonium sulfate was supplied as the nitrogen source and 15 mM lysine was present as an inducer.

C. Phosphate effect

The effect of high phosphate concentrations on secondary metabolism has been investigated in considerable detail. High phosphate concentrations depress β-lactam biosynthesis (Aharonowitz and Demain, 1977) and the synthases involved (Lubbe et al., 1985). In contrast, increasing the phosphate concentration from 6 mM to 100 mM in the defined starch-asparagine medium increased LAT activity in a 48-h culture (Fig. 7). The amount of biomass accumulated peaked with 20 mM and then declined with increasing phosphate concentration. The effect of phosphate concentration on LAT was also seen when starch was replaced by glycerol as the carbon source. The specific activity of LAT in glycerol supplemented cultures was 0.4 nmol/min/mg protein in 6 mM phosphate and 1.4 nmol/min/mg protein in 100 mM phosphate.

The total and specific activities of LAT in S. clavuligerus

Table 10. Effect of different carbon and nitrogen sources on cadaverine aminotransferase activity in S. clavuligerus[1]

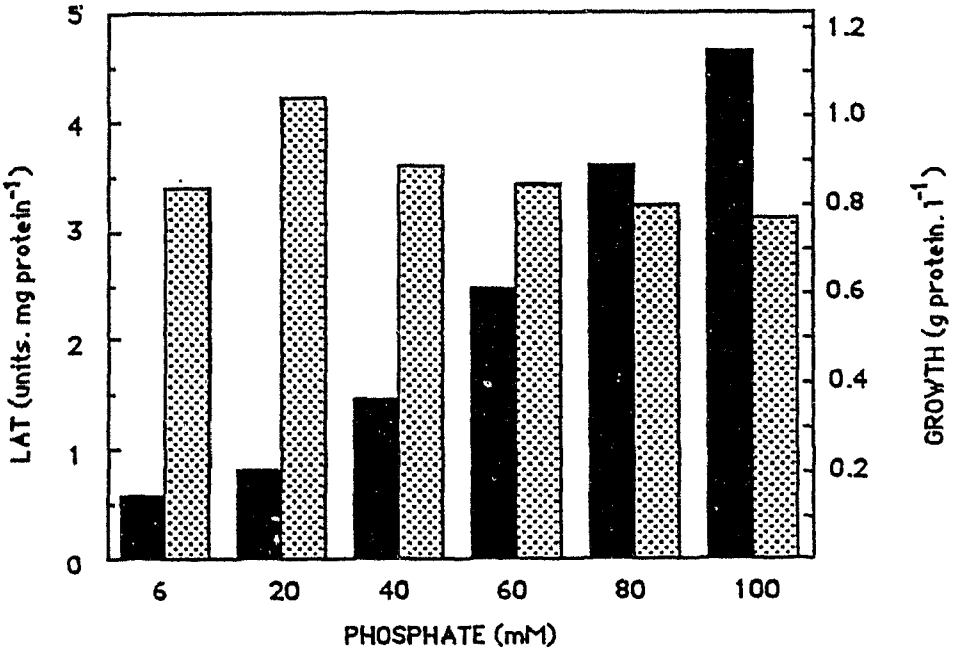
Medium composition	Cadaverine aminotransferase[2] units/mg protein
Glycerol + lysine	1.9
Glutamate + lysine	0.7
Glycerol + lysine + ammonium	0.7
Glycerol + lysine + glutamate	0.2
Glutamate + lysine + ammonium	0.9
Starch + lysine + ammonium	1.0
Maltose + lysine + ammonium	1.3
Histidine + lysine + ammonium	4.0
Succinate + lysine + ammonium	7.0
Lysine + ammonium	3.0
Lysine	4.7

[1] Cultures were grown in the defined medium for physiological studies. All of the carbon and nitrogen sources listed were provided at 15 mM concentration except for starch where the concentration was 0.5% (w/v).

[2] Cultures were assayed for CAT activity after growth for 24 h.

Fig. 7. Effect of increasing phosphate concentration on biomass production and lysine ε-aminotransferase specific activity in a 48-h culture of S. clavuligerus. Solid bars indicate lysine ε-aminotransferase activity and the hatched bars indicate growth.

Fig. 7.


LAT (units. mg protein^{-1})
GROWTH (g protein. l^{-1})
PHOSPHATE (mM)
0
1
2
3
4
5
0.2
0.4
0.6
0.8
1.0
1.2
6
20
40
60
80
100

mycelium grown in 100 mM phosphate were consistently higher than in mycelium grown in 20 mM phosphate, regardless of the stage of growth (Table 11). The difference in specific activities were the highest at 72 h (5.25 fold compared to 1.69 fold at 24 h), as were the differences for total enzyme activities. To test whether an activator present in 100-mM phosphate cultures or an inhibitor in 20 mM cultures was responsible for the observed differences, enzyme mixing experiments were performed. The enzyme activities observed were proportional to the amounts of each cell extract and indicated that no activators or inhibitors were present. Boiling one component before mixing the cell extracts supported this conclusion.

The specific activity of CAT in starch-asparagine-lysine medium was 1.1 nmol/min/mg protein in 6 mM phosphate and 1.3 nmol/min/mg protein in 100 mM phosphate; this indicated that CAT was not influenced by the concentration of phosphate supplied.

D. Nitrogen source regulation

In a glycerol-lysine-ammonium containing medium, the depressive effects of various nitrogen sources on CAT activity (Table 10) were partially relieved when ammonium was omitted. Supplementing glycerol-lysine cultures with glutamate drastically reduced CAT activity, and omitting glutamate increased CAT activity to a larger extent than did removing ammonium. However, omitting ammonium from glutamate-lysine medium had little effect on CAT activity, indicating that glutamate is the primary depressor. The presence of both glycerol and glutamate in medium supplemented with lysine not only depressed CAT activity but also

Table 11. Effect of phosphate concentration on lysine ϵ-aminotransferase activity at different stages in the growth of S. clavuligerus cultures*

Age of culture (h)	Phosphate concentration (mM)	Growth mg protein/ ml culture	Lysine ϵ-aminotransferase units/mg protein	
			Total	Specific
24	20	0.41	11.4	3.5
	100	0.54	17.8	5.9
48	20	1.06	4.8	0.8
	100	0.76	24.5	4.7
72	20	1.17	2.0	0.4
	100	0.82	10.2	2.1

* Cultures were grown in the defined medium for physiological studies with 1% (w/v) starch as carbon source and 15 mM asparagine as nitrogen source.

(based on visual observation) restricted the growth of S. clavuligerus.

The influence of various nitrogen sources on both LAT and CAT activities was tested in media containing succinate as a nondepressive carbon source and lysine as an inducer (Table 12). While different nitrogen sources did not have any significant effect on LAT after 24 h growth of S. clavuligerus, they did effect the specific activity of CAT. Compared to the control succinate-lysine medium the specific activity of CAT was reduced in succinate-ammonium and succinate-glutamate. However, the effect of these nitrogen sources on CAT activity in succinate medium was not as pronounced as in glycerol-containing medium.

Table 12. Effect of several nitrogen sources on the specific activity of lysine and cadaverine aminotransferases in cultures of S. clavuligerus*

Medium composition*	LAT units/mg prot.	CAT units/mg prot.
Lysine	4.4	3.7
Succinate + lysine	5.8	3.3
Succinate + lysine + asparagine	8.3	4.0
Succinate + lysine + ammonium	6.0	2.5
Succinte + lysine + glutamate	5.7	2.3

* All of the components listed including succinate were supplied at 15 mM concentration to cultures grown in defined medium for physiological studies. Cultures grown were assayed for aminotransferases after 24 h.

III. Cloning the lysine ε-aminotransferase gene

To clone the lysine ε-aminotransferase gene (*lat*) two strategies were explored. The first was based on suppression of the Lut⁻ phenotype in cadaverine-pathway mutants by introducing on the high-copy number plasmid, pIJ702, cloned genes for converting lysine to AAA. Even though *S. lividans* does not have the pathway generating AAA, biochemical investigations described earlier had shown that AAA is an excellent nitrogen source for this species. Introducing the genes for AAA pathway from *S. clavuligerus* into a Lut⁻ mutant of *S. lividans* on a high-copy number plasmid should then enable mutants blocked in the cadaverine pathway to obtain an adequate supply of nitrogen (by providing glutamate as nitrogen source as a result of LAT activity). This strategy yielded putative cadaverine pathway genes but failed to yield cloned DNA containing the *lat* gene. The second strategy was based on "chromosome walking" from a cloned β-lactam biosynthesis gene encoding IPNS. This was successful in yielding the *lat* gene. Together the results served to confirm the evidence obtained earlier from biochemical characterization that lysine is metabolized by two independent pathways in β-lactam producing streptomycetes. The experiments used in the two strategies and their outcomes are described in this section.

A. Cloning by suppression of a Lut⁻ mutation

The experimental strategy is represented schematically in Fig. 8. A type-1 mutant of *S. lividans* blocked in the first step of lysine catabolism was chosen as a host for cloning of genomic DNA fragments from *S. clavuligerus*. The low reversion frequency (6×10^{-6}/cfu) of

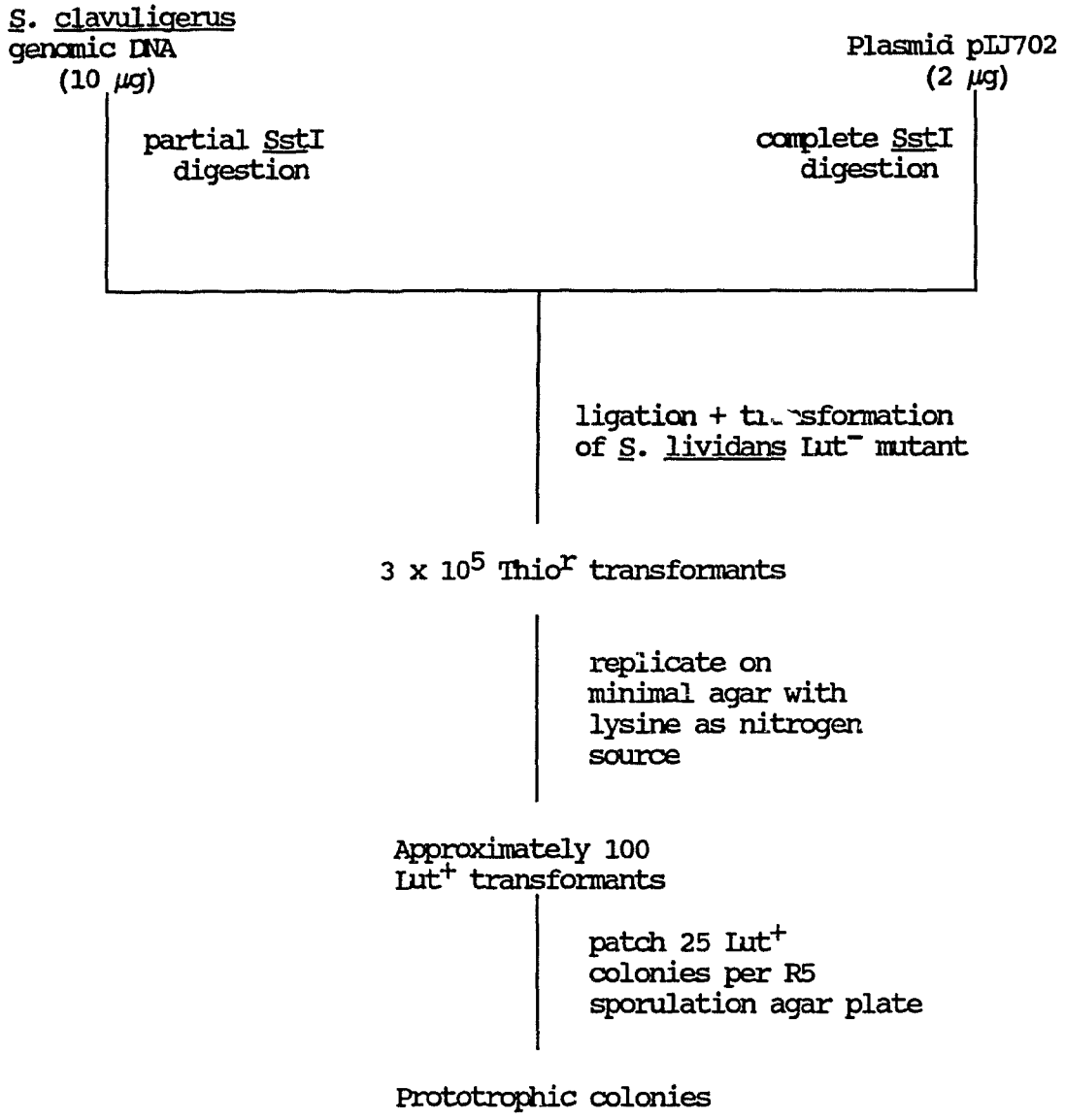


Fig. 8. Schematic representation of the cloning of S. clavuligerus DNA suppressing the Lut⁻ phenotype in S. lividans strain DCL1.

this mutant and availability of procedures for cloning in S. lividans made the use of this strain attractive. Moreover, the mutant stably retained its Lut$^-$ phenotype during protoplasting and transformation with the plasmid pIJ702.

Approximately 3 x 10^5 transformants were obtained using the strategy outlined in Fig. 8. Replication of the transformants on minimal medium supplemented with lysine as the sole nitrogen source gave approximately 100 Lut$^+$ transformants. The transformants were analyzed by a simple sib selection procedure to find any that harbored a recombinant plasmid carrying the lat gene. Lut$^+$ transformants were patched on R5 sporulation agar (25 per plate) and allowed to sporulate. The spores were scraped off and transferred to liquid YEME medium containing 5 μg/ml of thiostrepton and grown at 30^{o}C. After 48 h, mycelium was collected and analysed for LAT activity. None was observed, indicating that either none of Lut$^+$ transformants present on the four plates contained a recombinant plasmid with lat, or if a lat containing transformant was present, the amount of LAT it produced was below the detection limits of the assay.

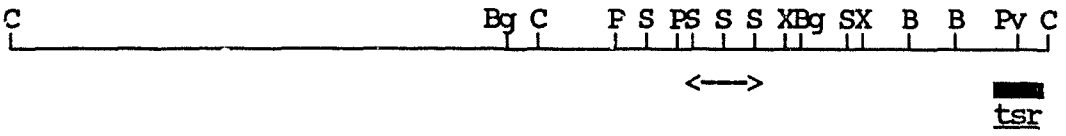
When 50 of the Lut$^+$ transformants were screened by gel electrophoresis of their plasmid DNA for the presence of recombinant plasmids, only three were found to contain plasmids larger than the vector. These were grown in YEME medium and assayed for LAT activity. None was detected. The results overall suggested that the Lut$^-$ phenotype could not readily be suppressed by the AAA pathway in S. lividans, and therefore that it might not be possible to metabolically link this pathway to the cadaverine route for lysine catabolism. When

the three plasmids that suppressed the Lut$^-$ phenotype were isolated and each was separately reintroduced into the Lut$^-$ mutant, all transformants were Lut$^+$, indicating that the recombinant plasmids contained a gene(s) governing some step(s) in the cadaverine pathway. One of these plasmids, pDQ401, was analysed in more detail to study the organisation and location of cadaverine pathway genes.

B. Plasmid pDQ401

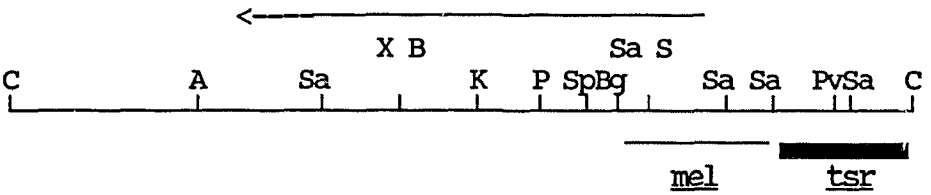
Since SstI-digested S. clavuligerus DNA had been cloned into the single SstI site of pIJ702 to produce pDQ401, it was expected that complete digestion of pDQ401 with SstI would reveal the size of the insert in the recombinant plasmid. However, electrophoresis of the digest on an agarose gel did not show a 5.8-kb fragment corresponding to linearized pIJ702. Altogether, five fragments of approximately 14.2, 1.8, 0.56, 0.45 and 0.40 kb were seen, indicating that pIJ702 had suffered a large deletion in its DNA or had undergone extensive rearrangement to produce pDQ401. A restriction map of pDQ401 was constructed by digesting the plasmid singly or with several restriction enzymes in pairwise combinations (Fig. 9). The restriction maps of pIJ702 and pDQ401 were then compared. The results showed that pDQ401 apparently lacked SstI sites at both ends of the insert; and many sites in the pIJ702 component of pDQ401 DNA were apparently missing. Because of the lack of correspondence between the two maps the exact size and location of the insert in pDQ401 could not be determined. The discrepancies between pIJ702 and pDQ401 maps are presently inexplicable, but may be resolved by hybridization tests.

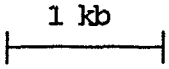
a.



<—> Orientation of the two SstI fragments is not known

b.





————> DNA segment apparently deleted in pDQ401

————————> End of the deletion not known

Fig. 9. a) Restriction map of pDQ401 (approximately 18.5 kb); b) restriction map of pLJ702 (5.8 kb) showing the approximate location of the pLJ702 sequence apparently deleted in pDQ401. A - ApaI, B - BamHI, Bg - BglII, C -ClaI, K - KpnI, P - PstI, Pv - PvuII, Sa - SalI, Sp - SphI, S - SstI, and X - XhoI. Only one of several PvuII sites present in pDQ401 is shown.

C. Complementation of different types of Lut⁻ mutations in S. lividans and S. venezuelae

Recombinant plasmid pDQ401 was introduced into Lut⁻ mutants of S. lividans and S. venezuelae to test whether genes on the plasmid complemented different types of Lut⁻ mutations and thereby to find out if the lut genes are clustered in streptomycetes.

Strains transformed with plasmids pDQ401 and pLJ702 were examined for growth with lysine as the sole nitrogen source. DCL1, a type-1 mutant of S. lividans transformed with pDQ401 showed growth comparable to the growth of the parent strain, TK24. Since a type-1 mutant is probably blocked in the first step of lysine catabolism, complementation of a mutation in this strain indicated that pDQ401 contained gene(s) catalysing the first step in lysine assimilation (Table 13). Two type-2 mutants, DCL2 and DCL3, also grew well on lysine as the sole nitrogen source after transformation with pDQ401. Since the type-2 mutants of S. lividans were blocked either in the second step or both early steps of lysine catabolism, complementation of the type-1 and type-2 mutations suggested that pDQ401 contained more than one lut gene for early steps in lysine catabolism via cadaverine. Type-3 mutants were poor growers and their Lut⁻ phenotype was very unstable through protoplast regeneration. Hence the effect of pDQ401 on these mutants could not be assessed.

pDQ401 did not complement representative strains (DCV2 and DCV5) belonging to the two types (Lut⁻ and Cut⁻, respectively) of lysine catabolic pathway mutations of S. venezuelae. To test whether absence of complementation was due to instability of the plasmid in this host,

Table 13. Growth of S. lividans Lut⁻ mutants on lysine as the sole nitrogen source after transformation with pIJ702 and recombinant plasmid pDQ401[a]

Mutant type	Growth on lysine with plasmid*	
	pIJ702	pDQ401
Type 1	-	+
Type 2	-	+
Wild type	+	+

* +, normal growth; -, no growth; after 4-5 days of incubation at 30°C.

[a] Lysine was added to the minimal medium (Hopwood et al., 1985) at 0.05% (w/v).

plasmid DNA was isolated from transformants of S. venezuelae strains DCV2 and DCV5 and analyzed on an agarose gel after digestion with restriction enzymes. The plasmid obtained lacked a large segment of pDQ401 DNA, indicating that the presence of lut genes on a high copy number plasmid might be lethal to the host. Despite the failure of pDQ401 to complement S. venezuelae, the results obtained with S. lividans suggested that the plasmid did contain gene(s) catalyzing one or more steps in lysine catabolism via cadaverine.

b. Southern hybridization of the genomic DNA from streptomycetes with pDQ401

The genomic DNA of various streptomycetes, including S. clavuligerus, was hybridized with pDQ401 DNA: to identify the size of Sst1 fragments in pDQ401 before the deletion had occurred; and to test for sequence similarities between the cloned S. clavuligerus DNA and the genomic DNA from other streptomycetes.

The complete insert in pDQ401 could not be retrieved for labelling because the Sst1 sites used for cloning were either absent, or uncleavable. Hence, Southern hybridization analysis was performed in two steps. Fragments of SstI-cleaved genomic DNA from various streptomycetes were separated on an agarose gel and then transferred to a nylon membrane. In the first step the membrane was probed with ^{32}P labelled pDQ401 DNA and autoradiographed. In the second step, the ^{32}P-labelled pDQ401 probe DNA was removed from the membrane and the membrane was reprobed with ^{32}P labelled pIJ702. The reprobed membrane was autoradiographed. The two autoradiograms were compared to identify the

location of the sequences hybridizing to cloned S. clavuligerus DNA in pDQ401.

The S. clavuligerus insert DNA in pDQ401 contained five internal sites giving four SstI fragments of 1.8, 0.56, 0.45 and 0.40 kb (Fig. 9). These were flanked by S. clavuligerus DNA which should correspond to two more SstI fragments, but due to loss of SstI sites on both ends of the insert, the size of the flanking SstI fragments could not be determined by electrophoresis of a restriction enzyme digest. Theoretically, however, the size of flanking SstI fragments could be measured from Southern hybridization analysis of SstI digested genomic DNA of S. clavuligerus with ^{32}P-labelled pDQ401. Since pIJ702 did not hybridize to S. clavuligerus genomic DNA, any hybridization observed was due to S. clavuligerus DNA carried in the recombinant plasmid probe. The size of the internal SstI fragments was already known from the restriction map; therefore any additional hybridizing fragments in S. clavuligerus genomic DNA would represent SstI fragments flanking the DNA contained within four internal SstI fragments. Thus pDQ401 was expected to hybridize to six fragments from S. clavuligerus genomic DNA digested with SstI. However, at a washing stringency of 1x SSC at 70°C, only two hybridizing fragments, of approximately 17 and 7 kb, were present (Fig. 10) When the stringency was increased by reducing the salt concentration in washing solution to 0.2x SSC, the intensity of 17-kb hybridization signal was reduced suggesting that the overlapping region of homology was small. The results indicated that the SstI sites in the cloned DNA of pDQ401 were absent from the genomic DNA. When BglII-digested genomic DNA from S. clavuligerus was probed with ^{32}P labelled

Fig. 10. A) Ethidium bromide stained electrophoresis gel containing genomic DNA samples from β-lactam-producing (lanes 1-4) and nonproducing (lanes 5-8) streptomycetes. B) Autoradiograph of a nylon membrane blot of the gel in A after hybridization with ^{32}P labelled pDQ401. 1) S. clavuligerus, 2) S. clavuligerus, 3) S. lipmanii, 4) S. griseus, 5) S. lividans, 6) S. venezuelae, 7) S. phaeochromogenes, 8) S. griseofuscus. Arrow indicates the sequence hybridizing to pIJ702. DNA from lane 1 was digested with BglII and DNA from lanes 2 to 8 were digested with SstI. Washing stringency was 1x SSC at 70°C.

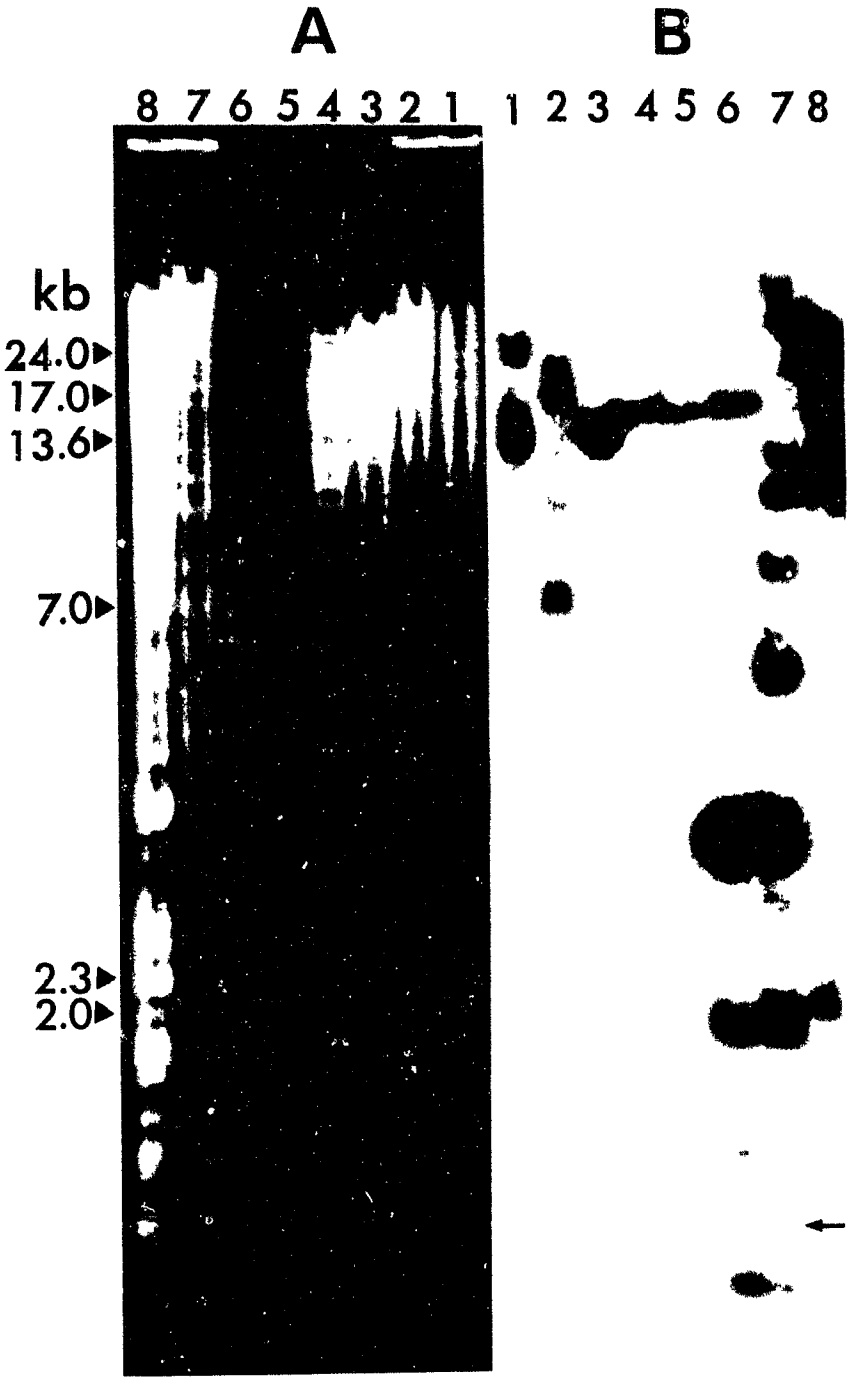


Fig. 10

pDQ401 two hybridizing bands of 24 and 13.6 kb were observed (at a washing stringency of 1X SSC at 70°C). When the stringency was increased by reducing the salt concentration in the washing solution to 0.2x SSC, the 24-kb hybridization signal was almost completely eliminated. No hybridization signals corresponding to the 1.85, 0.56, 0.45 and 0.4-kb internal SstI fragments, or to the 4.7-kb internal BglII fragment were observed. Similar results were obtained when the experiment was repeated several times. Not only could the size of the SstI fragments flanking the four internal SstI fragments not be established but the absence of hybridization signals corresponding to the four internal SstI fragments themselves necessitated a reinterpretation of the data. Either the four internal fragments were not of S. clavuligerus origin or they were produced by rearrangement of sequences present in the genome of S. clavuligerus.

At a washing stringency of 1x SSC at 70°C, pIJ702 hybridized to genomic DNA from all streptomycetes tested except S. clavuligerus (presumably via the mel gene). However, except for one fragment from S. phaeochromogenes, the hybridization signals did not match any of those obtained with pDQ401. Thus all hybridization signals obtained with pDQ401 must have been due to the S. clavuligerus DNA insert portion in pDQ401.

The cloned S. clavuligerus DNA in pDQ401 hybridized to and therefore contained sequences similar to genomic DNA from several β-lactam producing and nonproducing streptomycetes (Fig. 10). The plasmid hybridized to several SstI fragments of S. venezuelae and S. phaeochromogenes. The intensities of hybridizing bands varied

considerably and the intensity of signals was not proportional to the size of hybridizing bands. This suggested that different regions of the cloned S. clavuligerus DNA had different levels of sequence similarity to the genomic DNA of these two strains. Alternatively, some sequences in S. venezuelae and S. phaeochromogenes genomes corresponding to S. clavuligerus insert DNA were amplified.

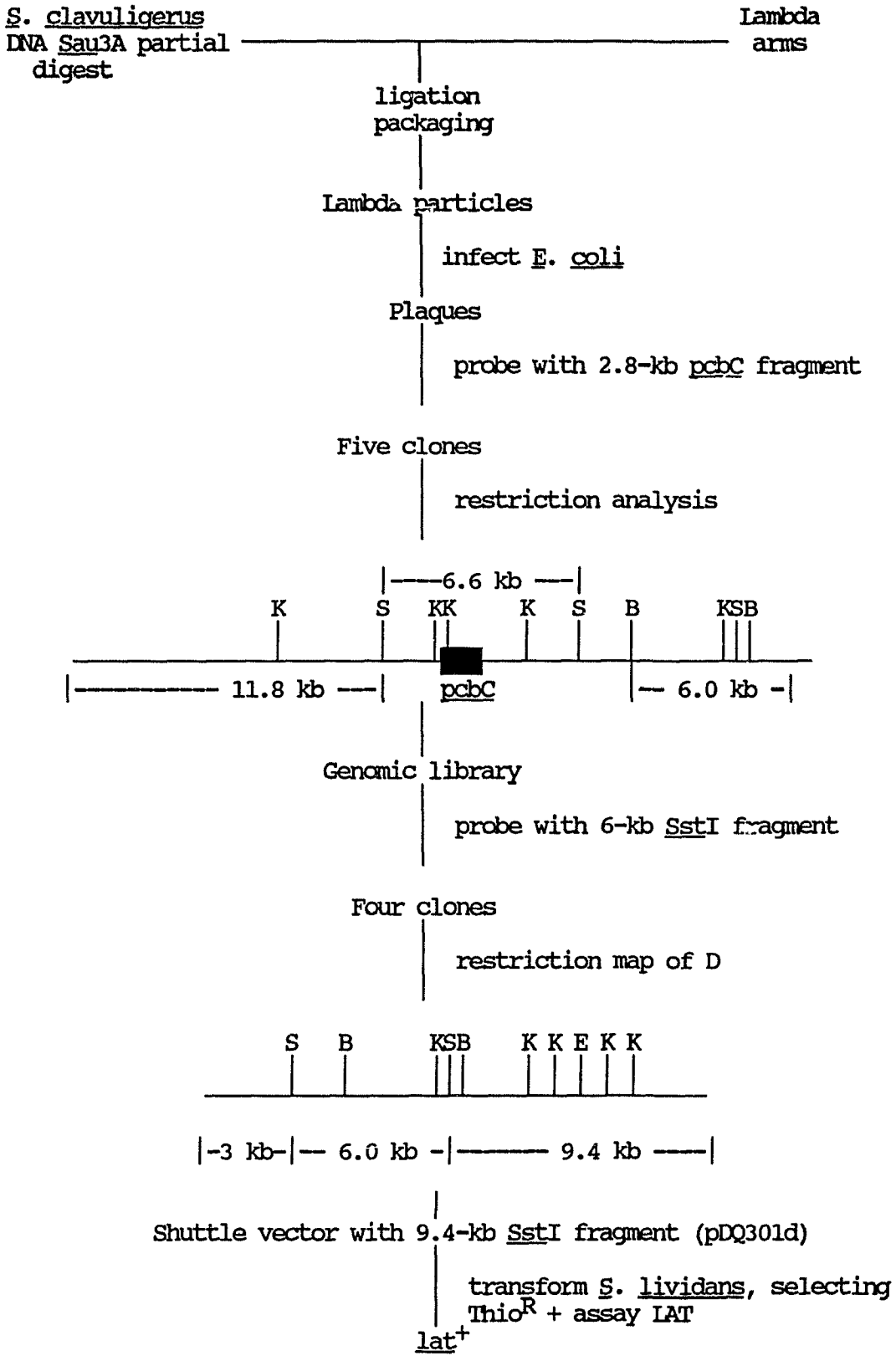
E. Cloning a putative LAT gene by chromosomal walking

The material in this section is included in a manuscript already submitted for publication in the Journal of Bacteriology. Since known β-lactam biosynthesis genes are clustered in the S. clavuligerus genome and since a 29.3-kb fragment from S. cattleya directed synthesis of cephamycin B in S . lividans in a medium without AAA supplementation (Chen et al., 1988), the genes governing AAA biosynthesis were presumed to be present in the β-lactam biosynthesis gene cluster. A promising approach was therefore to use the cloned pcbC gene encoding IPNS to isolate DNA adjacent to pcbC, and to examine the isolated DNA for the presence of the lat gene.

The isolation of lat by using the pcbC gene of S. clavuligerus cloned by Leskiw et al. (1988) on plasmid pBL1 as a probe in chromosomal walking is presented schematically in Fig. 11. Southern hybridization analysis of S. clavuligerus genomic DNA with ^{32}P-labelled pBL1 showed that the DNA insert in this plasmid was not derived from a single contiguous segment of the chromosome, and probably contained DNA from two different regions of the S. clavuligerus genome. The segment to the left of the SstI site on pBL1 (Fig. 12) was identified as the region

Fig. 11. Schematic representation of the isolation of an S. clavuligerus genomic DNA fragment containing lat. S - SstI and K - KpnI and B - BamHI.

Fig. 11.


S. clavuligerus DNA Sau3A partial digest
Lambda arms
ligation packaging
Lambda particles
infect E. coli
Plaques
probe with 2.8-kb pcbC fragment
Five clones
restriction analysis
6.6 kb
K S KK K S B KSB
11.8 kb
pcbC
6.0 kb
Genomic library
probe with 6-kb SstI fragment
Four clones
restriction map of D
S B KSB K K E K K
3 kb
6.0 kb
9.4 kb
Shuttle vector with 9.4-kb SstI fragment (pDQ301d)
transform S. lividans, selecting Thio^R + assay LAT
lat+

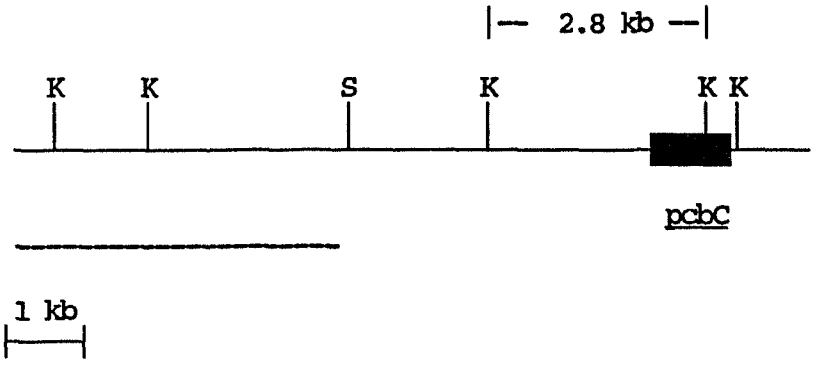


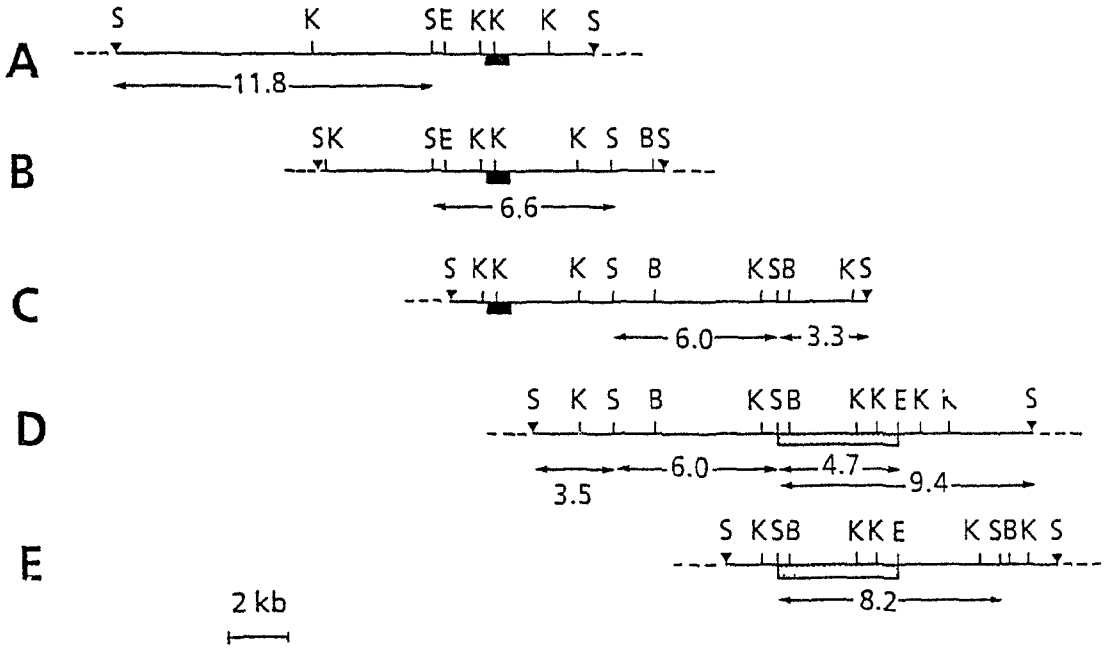
Fig. 12. Restriction map of pBL1 showing the location of the pcbC gene encoding IPNS. The approximate location of the DNA sequence extraneous to the β-lactam biosynthesis region is shown by the dashed line. The 2.8-kb KpnI fragment used as a probe in isolating lambda clones A-D is indicated above the restriction map. K - KpnI, S - SstI.

extraneous to pcbC.

To ensure that the probe consisted of a contiguous DNA sequence, the KpnI fragment of S. clavuligerus DNA (approximately 2.8-kb) containing part of the cloned pcbC gene (Leskiw et al., 1988) was labelled with ^{32}P. This probe was used to search a genomic library prepared by partially digesting S. clavuligerus DNA with Sau3A and ligating the fragments into a lambda replacement vector. Altogether, five hybridizing clones were obtained. Restriction mapping of the inserts with SstI, KpnI, EcoRI and BamHI showed that the separately cloned DNA fragments contained overlapping S. clavuligerus genomic sequences. Matching of overlaps in three of the clones (A-C) gave a unique region of approximately 28 kb (Fig. 13). Most of this region was included within three contiguous segments of 11.8, 6.6 and 6.0 kb defined by SstI sites. Plasmids containing S. clavuligerus DNA retrieved from lambda clones were unstable when introduced into S. lividans. Analysis of the plasmids recovered from S. lividans $Thio^R$ transformants indicated there were deletions in the S. clavuligerus DNA insert portions. Since Streptomyces secondary metabolic pathway genes are not normally expressed in E. coli, recombinant E. coli plasmids containing Streptomyces DNA usually are stably maintained in their bacterial host. Therefore, the 11.8, 6.6 and 6.0-kb SstI segments excised from lambda clones A, B and C, respectively, were subcloned in the polylinker Sst1 site of the E. coli vector, pIZ18R. To obtain expression of Streptomyces genes, each recombinant pIZ18R plasmid was fused with the Streptomyces vector pIJ702, giving the recombinant shuttle plasmids pDQ301a, pDQ301b and pDQ301c, respectively. Each

Fig. 13. Restriction maps of the S. clavuligerus genomic DNA inserts in recombinant lambda clones. Clones A, B and C were identified by plaque hybridization using a 2.8-kb KpnI fragment containing part of pcbC as a probe; thickened lines indicate the location of pcbC. Clones D and E were identified using a 6.0-kb SstI fragment of clone C as a probe. The SstI sites at both ends of the inserts are from the polylinker region of the lambda vector; continuous lines indicate the S. clavuligerus insert in lambda clones; dashed lines denote the lambda vector arms. The approximate location of lat is shown as a hatched region. S - SstI, K - KpnI, E - EcoRI, B - BamHI.

Fig. 13

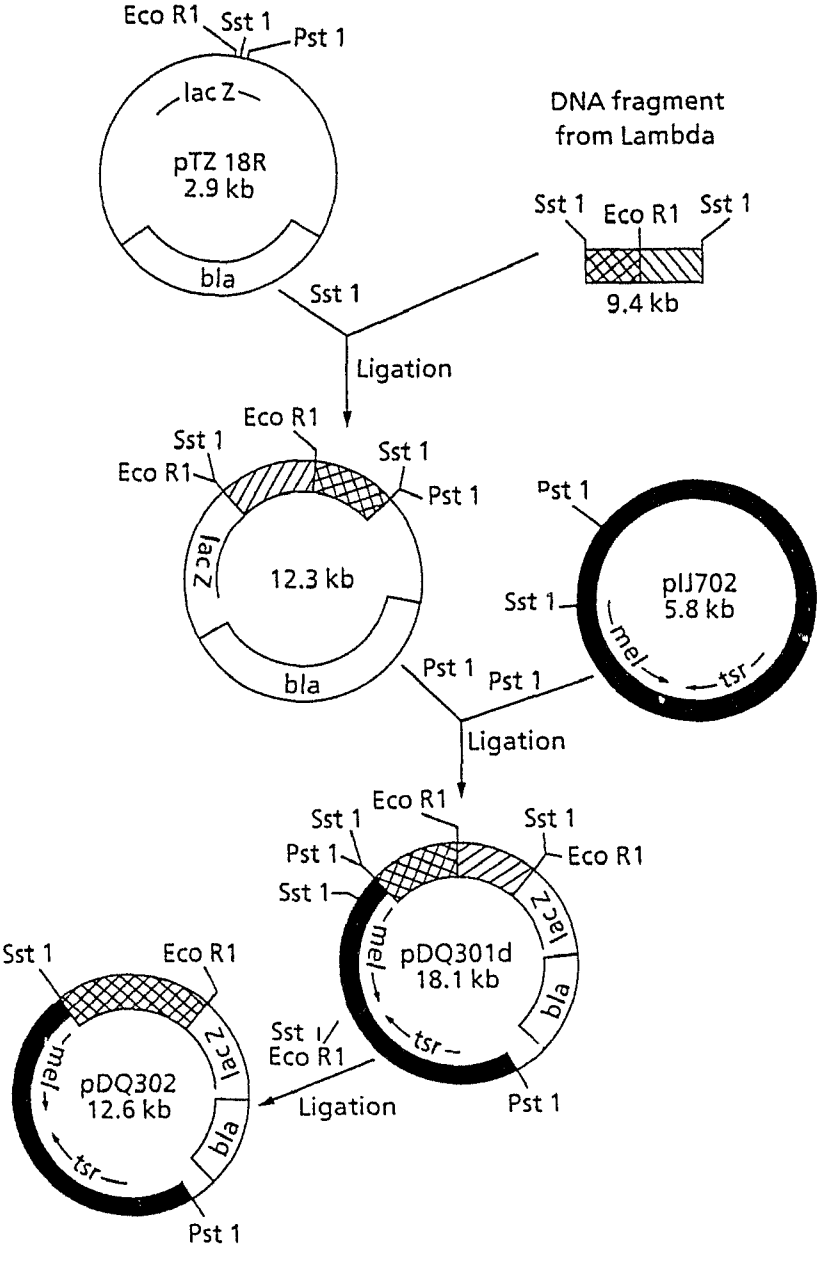

A
S
K
SE KK
K
S
11.8
B
SK
SE KK
K S BS
6.6
C
S KK
K S B
KSB
KS
6.0
3.3
D
S K S B
KSB
KK E K
S
3.5
6.0
4.7
9.4
E
S KSB
KK E
K SBK S
8.2
2 kb

shuttle plasmid was introduced into S. lividans, and transformants were selected by their resistance to thiostrepton. The $Thio^R$ transformants given by each plasmid were propagated together in a shaken culture, and cell extracts from each culture were assayed for LAT activity. Since LAT activity is normally absent from S. lividans, any transformant exhibiting it could be presumed to harbor the lat gene or possibly an activator gene (Jones, 1989) in its cloned DNA. However, no activity was detected.

When the 6.0-kb SstI fragment excised from recombinant lambda clone C was labelled with ^{32}P and used to probe the S. clavuligerus genomic library, it hybridized with four additional clones. Restriction enzyme mapping of DNA from the recombinant phage showed that one of these, clone D, contained approximately 19 kb of S. clavuligerus DNA. Within the insert, SstI sites defined three regions: the 6.0-kb probe sequence, a 3.5-kb region that overlapped part of the 6.6-kb sequence in clone A, and 9.4 kb of DNA extending the 6.0-kb sequence distal to the 3.5-kb overlap (Fig. 14). The 9.4-kb segment was recovered by digesting the recombinant phage DNA with SstI (taking advantage of an SstI site in the polylinker region of the vector as well as one in the insert DNA) and was subcloned in pTZ18R. Incorporation of the recombinant vector into a shuttle vector furnished plasmid pDQ301d (Fig. 14). When pDQ301d was introduced into S. lividans, $Thio^R$ transformants from one of two regeneration medium plates exhibited LAT activity indicating that the 9.4-kb fragment contained a gene directing the synthesis of LAT. This does not rule out the possibility that the cloned DNA might contain an activator gene activating a silent AAA pathway in S. lividans. Until

Fig. 14. Construction of plasmids pDQ301d and pDQ302. A 9.4-kb SstI fragment from lambda clone D (Fig. 1) was ligated into the SstI site in the polylinker region of E. coli plasmid pTZ18R. The recombinant E. coli plasmid was digested with PstI and ligated to the PstI-digested Streptomyces plasmid pIJ702, resulting in the E. coli-Streptomyces recombinant shuttle vector, pDQ301d. pDQ301d was digested with a mixture of SstI and EcoRI; the digested DNA was treated with T4 DNA ligase and used to transform E. coli DH5α; some of the transformants harbored pDQ302.

Fig. 14


Eco R1
Sst 1
Pst 1
lac Z
pTZ 18R
2.9 kb
bla
DNA fragment
from Lambda
Sst 1
Eco R1
Sst 1
9.4 kb
Sst 1
Ligation
Eco R1
Sst 1
Eco R1
Sst 1
Pst 1
lac Z
12.3 kb
bla
Pst 1
Sst 1
pIJ702
5.8 kb
mel
tsr
Pst 1
Pst 1
Ligation
Eco R1
Sst 1
Pst 1
Sst 1
Sst 1
Eco R1
mel
pDQ301d
18.1 kb
lac Z
bla
tsr
Pst 1
Sst 1
Eco R1
Ligation
Sst 1
Eco R1
mel
pDQ302
12.6 kb
lac Z
bla
tsr
Pst 1

further evidence is available to show that the gene encoding the LAT activity is a structural gene for LAT, the cloned gene directing LAT activity in S. lividans can be considered only as a putative lat gene. However, for the sake of brevity the term lat is used to describe the cloned gene directing the synthesis of LAT (see dicussion for more details).

When pDQ301d was digested to completion with a mixture of the restriction enzymes SstI and EcoRI, incubated with T4 DNA ligase and used to transform E. coli DH5α, plasmid pDQ302, containing a 4.7-kb EcoRI:SstI insert, was isolated from some of the transformants (Fig. 14 and 15). Introduction of pDQ302 into S. lividans yielded transformants that initially showed LAT activity. This property was stably maintained when the transformants were grown on R5 medium containing lysine.

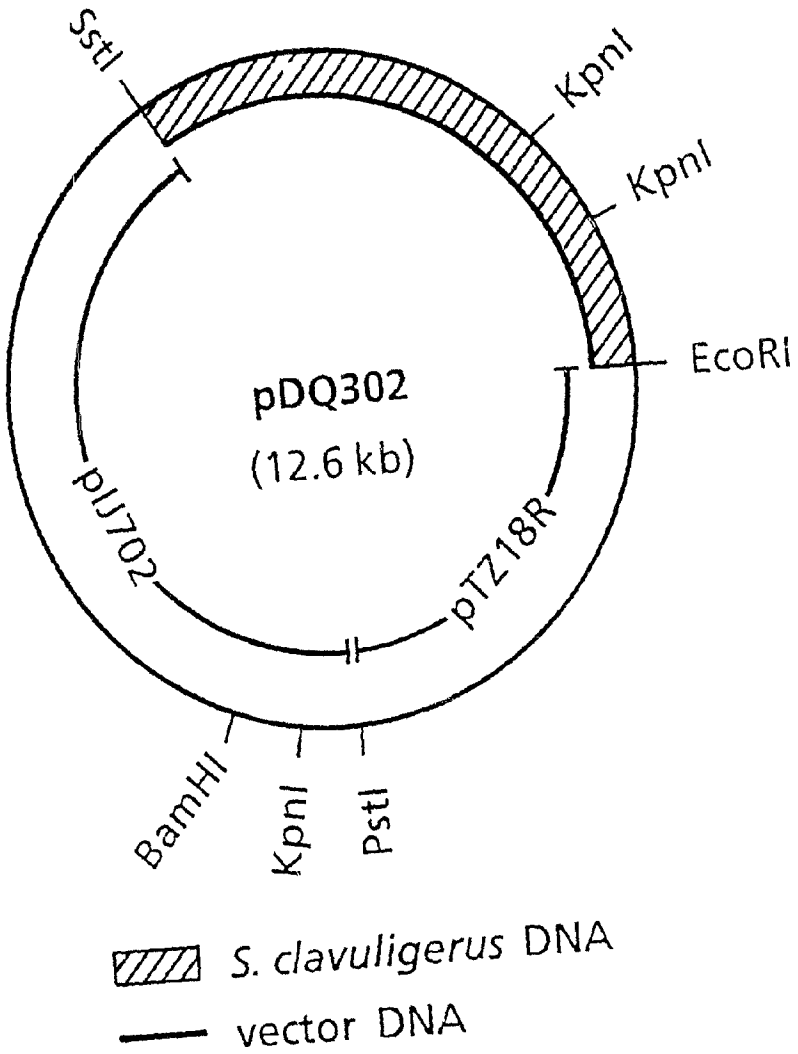
F. Restriction fragment-length polymorphism in the region flanking lat

Lambda clone E and two other clones isolated from the S. clavuligerus genomic library concurrently with clone D, contained the lat in an 8.2-kb SstI sequence which replaced the 9.4-kb sequence of clone D (Fig. 14). The presence of LAT activity was demonstrated by subcloning the 8.2-kb SstI fragment in pTZ18R and combining the recombinant plasmid with pIJ702 to give the shuttle vector pDQ301e. When pDQ301e was used to transform S. lividans, some of the transformants exhibited LAT activity.

Restriction mapping showed distinct differences in the 9.4 and 8.2-kb DNA fragments from clones D and E, respectively (Fig. 14). To

Fig. 15. Restriction map of plasmid pDQ302 containing *lat*. Only relevant restriction sites are shown.

Fig. 15


SstI
KpnI
KpnI
EcoRI
pDQ302
(12.6 kb)
pIJ702
pTZ18R
BamHI
KpnI
PstI
S. clavuligerus DNA
vector DNA

compare these fragments with the genomic region that furnished them, DNA from the clones as well as S. clavuligerus genomic DNA were digested with KpnI and the restriction fragments were separated on an agarose gel. When the fragments were transferred to a nylon membrane and probed with the ^{32}P labelled 9.4-kb SstI fragment from clone D, each sample showed hybridizing components at 3.5 and 0.8 kb (Fig. 16). Based on restriction maps of the clone D and E inserts obtained earlier (Fig. 13), these KpnI fragments encompassed much or all of the lat locus (Fig. 17). The 3.7-kb fragment which mapped to the right of the 0.8-kb fragment in the clone-E DNA insert was present as a hybridizing fragment in DNA from the S. clavuligerus genome and from clone E, but was absent from clone-D DNA. Therefore the sequence in clone E represents that of the S. clavuligerus genome. The 1.0-kb KpnI fragment mapped in clone D was present as a hybridizing fragment in DNA from the S. clavuligerus genome as well as from clone D but was absent from clone E DNA. This suggested that a DNA sequence containing two KpnI sites separated by 1.0-kb had inserted into the 8.2-kb SstI:SstI region from elsewhere in the genome. Since the 1.75-kb KpnI fragment that mapped to the right of the 0.8-kb fragment in the clone D insert was absent from both clone E and the S. clavuligerus genome, it may have been generated by insertion of an extraneous sequence into the 3.7-kb KpnI genomic fragment. This insertion would have occurred immediately to the right of the EcoRI site. The 4.4-kb hybridizing KpnI fragment in clone D contained only 2.9 kb of S. clavuligerus DNA; attached to it was 1.5 kb of lambda DNA, extending to the KpnI site in the left arm of the phage genome. Whether the 2.9-kb fragment contained a portion of the 3.7-kb genomic fragment

Fig. 16. Agarose gel electrophoresis and Southern hybridization of KpnI-digested DNA samples from S. clavuligerus (1) and from lambda clones E (2) and D (3): A) the gel treated with ethidium bromide. B) Autoradiograph of a blot of the gel in A after hybridization with a 9.4-kb SstI fragment of clone D. The washing stringency was 0.1x SSC at 70°C.

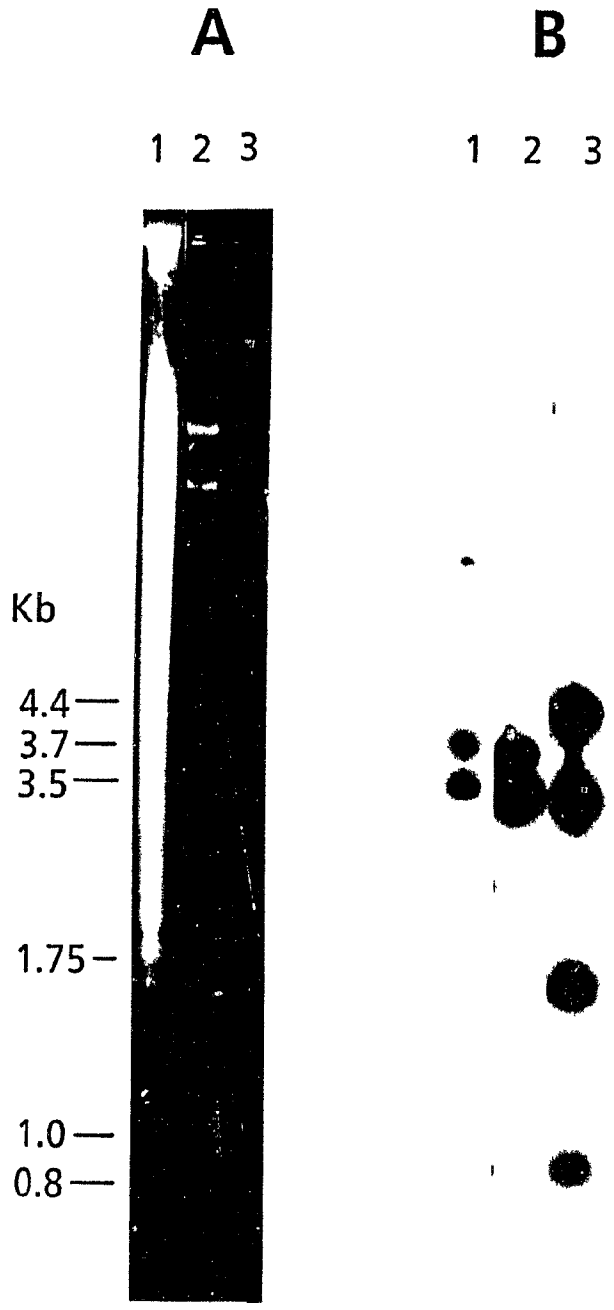
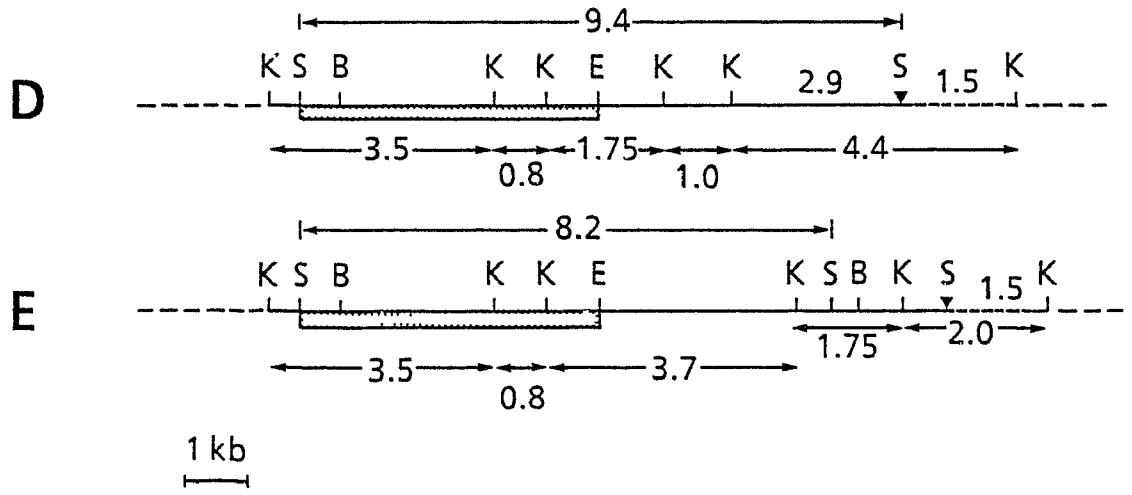


Fig. 16

Fig. 17. Restriction maps of relevant sections of clones D and E showing their differences. The SstI site at the right end of each insert was derived from the polylinker region of the lambda vector; continuous lines indicate the S. clavuligerus insert portion of lambda clones; dashed lines indicate lambda vector arms. The approximate location of lat is shown as a hatched region. S - SstI, K - KpnI, E - EcoRI, B - BamHI.

Fig. 17


D
9.4
K S B
K K E
K
K
2.9
S
1.5
K
3.5
0.8
1.75
1.0
4.4
E
8.2
K S B
K K E
K S B K S
1.5
K
1.75
2.0
3.5
0.8
3.7
1 kb

was not determined; thus the size of the putative insertion is not known. A comparison by dot-blot hybridization of the genomic DNA from E. coli and S. clavuligerus indicated that there was no sequence similarity between the ^{32}P-labelled 9.4-kb SstI fragment of clone D, used as a probe, and E .coli DNA.

When the 9.4-kb fragment was labelled with ^{32}P and used as a probe to screen the genomic library of S. clavuligerus, it hybridized to several lambda clones. Restriction digestion with KpnI indicated that two of these clones differed significantly from each other as well as from the clones containing the lat gene. This indicated that the cloned 9.4-kb fragment contained a sequence which was present elsewhere in the S. clavuligerus genome. One plausible explanation for this observation is that the 9.4-kb fragment contained an insertion sequence. It has been reported (Leskiw et al., 1990) that S. clavuligerus contains a 1.4-kb mobile element able to insert into the plasmid pIJ702. The insertion sequence hybridized to homologous sequences in S. clavuligerus but not to genomic DNA from S. lividans. An alternative explanation for the additional DNA sequence adjacent to lat is that duplication had occurred to generate the 9.4-kb fragment. The presence of amplifiable DNA sequences has been reported in several streptomycetes.

G. Instability of hosts carrying the cloned lat gene

The lat gene was not stably maintained in S. lividans during growth on R5 agar medium, even when thiostrepton was included to select for pDQ301d-containing strains. When plasmid DNA isolated from the S. lividans transformants was introduced into E. coli, extraction of the

E.coli transformants yielded four types of plasmid. Gel electrophoresis and restriction analysis showed that three of these plasmid types had deletions in the 9.4kb DNA segment derived from S. clavuligerus (Fig. 18). None of the plasmids with deletions conferred LAT activity when they were used to transform S. lividans. Since LAT activity was fully retained in S. lividans transformants containing pDQ301d when colonies were grown on R5 medium containing 30-mM lysine, the apparent plasmid instability may have been due to avid conversion of endogenous lysine to piperideine-6-carboxylate in strains carrying multiple copies of the cloned DNA conferring LAT activity. The consequent lysine starvation would be expected to select for mutants in which lat had been inactivated.

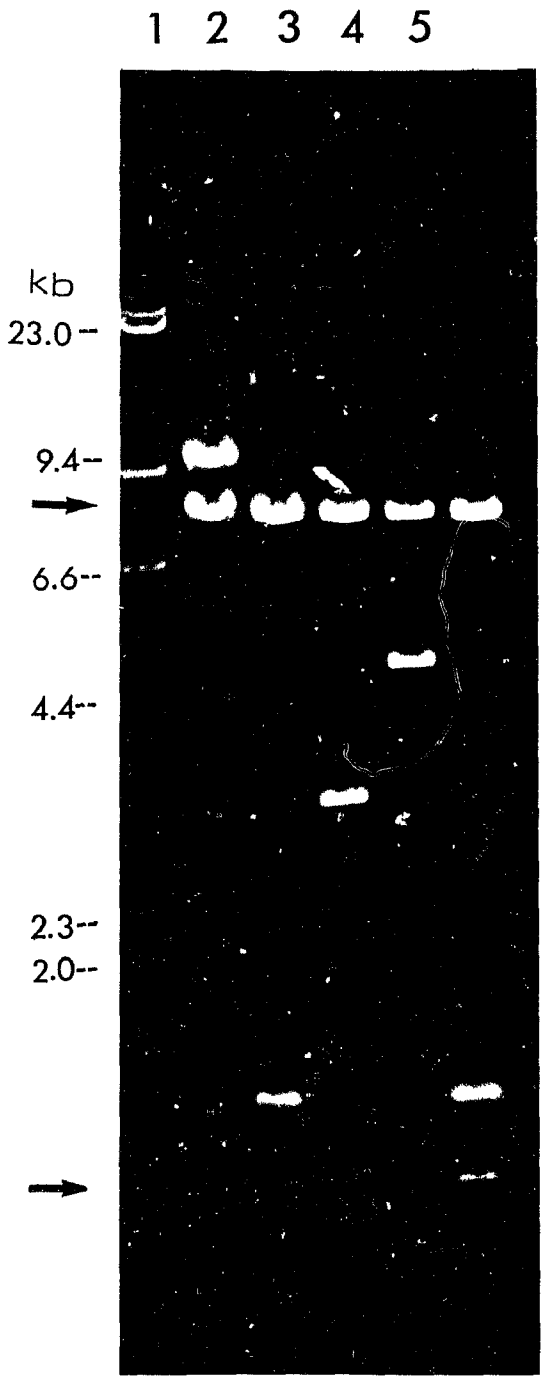
Plasmid pDQ301e was very unstable. Out of eight primary transformants tested only two showed LAT activity despite the presence of 30-mM lysine in the regeneration, sporulation and liquid media. Of the two transformants one showed very low LAT activity indicating that a large proportion of transformants on the sporulation plate had lost lat. Evidently the exogenous supply of lysine did not protect the transformants from lysine starvation. One possible explanation is that the lysine uptake system was unable to transport the amino acid rapidly enough to prevent lysine starvation. Alternatively, the presence on the cloned fragment of some other sequence, detrimental to the stability of the transformants, might have been responsible.

H. Location of lat within the β-lactam biosynthesis gene cluster

Restriction analysis of the region of S. clavuligerus DNA between

Fig. 18. Ethidium bromide-stained agarose gel containing DNA samples digested with SstI and separated by electrophoresis. The DNA samples were from the four types of pDQ301d isolated from E. coli transformants: 1) lambda DNA digested with HindIII, 2) unmodified plasmid, 3) to 5) modified plasmids with deletions.

Fig. 18



→ Vector DNA

pcbC and the SstI:EcoRI fragment encoding LAT activity indicated that lat was located 10-12 kb from the pcbC gene (Fig. 13). To position lat in relation to known β-lactam biosynthesis genes, and to elaborate in greater detail the organization of the pcb gene cluster, lambda clones D and E were digested with KpnI and the fragments were separated on an agarose gel. They were then transferred to a nylon membrane and examined for overlap with genomic DNA sequences containing cefE. To obtain the latter sequences, clones in the S. clavuligerus genomic library were probed with a 33-mer synthetic oligonucleotide, the sequence of which was based on the published N-terminal amino acid sequence of DAOCS (Kovacevic et al., 1989). Three hybridizing lambda clones (F-H) were isolated. Insert DNA was excised with SstI from each of the recombinant phages, labelled with ^{32}P and allowed to hybridize with the KpnI fragments of clones D and E. A 13.2-kb Sst1 fragment from one (clone F) of the cefE-containing clones hybridized to the 1.75 and 2.0-kb KpnI fragments from clone E (Fig. 19). These two fragments are present at the extreme right of the insert DNA in clone E (Fig. 17). Indeed, the 2.0-kb fragment contains only 0.5 kb of S. clavuligerus DNA, the rest being from the lambda arm. Restriction mapping of the DNA insert in clone F placed lat 13-14 kb to left of cefE, i.e., between pcbC and cefE (Fig. 20).

I. Hybridization of lat with Streptomyces genomic DNA

To examine whether the gene for LAT was present only in β-lactam producers, the 4.7-kb SstI:EcoRI fragment cloned in pDQ302 was used as a

Fig. 19. Agarose gel electrophoresis and Southern hybridization of a KpnI-digested DNA sample from lambda clone E: A) the gel treated with ethidium bromide. B) Autoradiograph of a blot of the gel in A after hybridization with a 13.2-kb SstI *S. clavuligerus* insert containing *cefE* from lambda clone F. The upper two bands contained mostly lambda DNA; their hybridization to the probe was due to small amounts of contaminating lambda DNA remaining after purification of the probe DNA. The washing stringency was 0.1x SSC at 70°C.

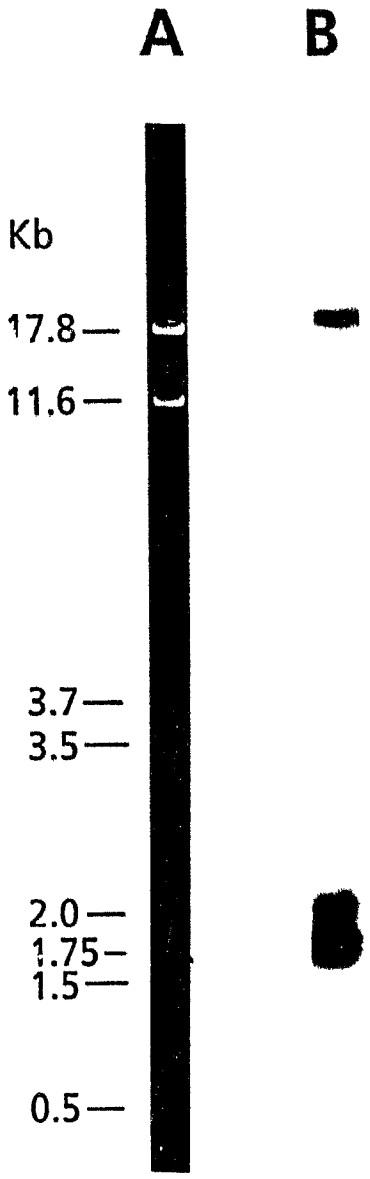
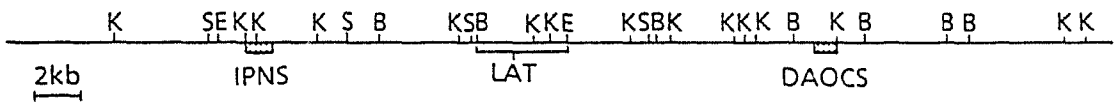


Fig. 19

Fig. 20. A restriction map of the β-lactam biosynthesis region of the *S. clavuligerus* genome, generated by aligning overlapping *S. clavuligerus* DNA inserts from lambda clones A-G. The approximate location of *lat* (as defined by the *S. clavuligerus* DNA insert in pDQ302), and more precise locations of genes encoding IPNS and DAOCS are shown. S-*Sst*I, K-*Kpn*I, E-*Eco*RI, B - *Bam*HI.

Fig. 20



source of *lat*. Samples of genomic DNA from three β-lactam-producing and four nonproducing *Streptomyces* sp. were digested to completion with *Sst*I. The DNA fragments were separated by electrophoresis on an agarose gel and transferred to a nylon membrane. When the membrane was probed with the ^{32}P-labelled 4.7-kb fragment, hybridization was observed only with DNA from the β-lactam producers such as *S. clavuligerus*, *S. lipmanii* and *S. griseus* (Fig. 21). The signal was retained when the stringency of washes was increased by decreasing the salt concentration from 2x SSC to 0.5x SSC. This washing stringency indicated that there was a significant sequence similarity between the *S. clavuligerus* putative gene encoding LAT and the gene from the other β-lactam producing *Streptomyces*.

J. Attempted complementation of Lut$^-$ mutation with pDQ302

Transformation of Lut$^-$ mutants, with pDQ302 to test whether the cloned *lat* DNA in this plasmid suppressed the Lut$^-$ phenotype of cadaverine pathway mutants gave no transformants that could grow with lysine as their sole source of nitrogen. The putative *lat* gene present on a high-copy number plasmid vector, pDQ302, did not suppress the Lut$^-$ phenotype caused by different types of cadaverine pathway mutations. Only a trace of growth was visible with lysine as the sole nitrogen source even on prolonged incubation at 30°C. During the same period no growth was visible in Lut$^-$ strains transformed with pLJ702. This supported the earlier conclusion that the Lut$^-$ phenotype cannot be suppressed by *lat*. *S. venezuelae* could not be transformed with pDQ302.

Fig. 21. Agarose gel electrophoresis and Southern hybridization of Sst1-digested genomic DNA from β-lactam-producing (lanes 2-4) and nonproducing streptomycetes: A) the gel treated with ethidium bromide. B) Autoradiograph of a blot of the gel in A probed with a 4.7-kb SstI:EcoRI fragment containing lat. 1) Lambda DNA digested with HindIII, 2) S. clavuligerus, 3) S. lipmanii, 4) S. griseus, 5) S. venezuelae, 6) S. lividans, 7) S. phaeochromogenes, 8) S. griseofuscus. The washing stringency was 0.5x SSC at 70°C.

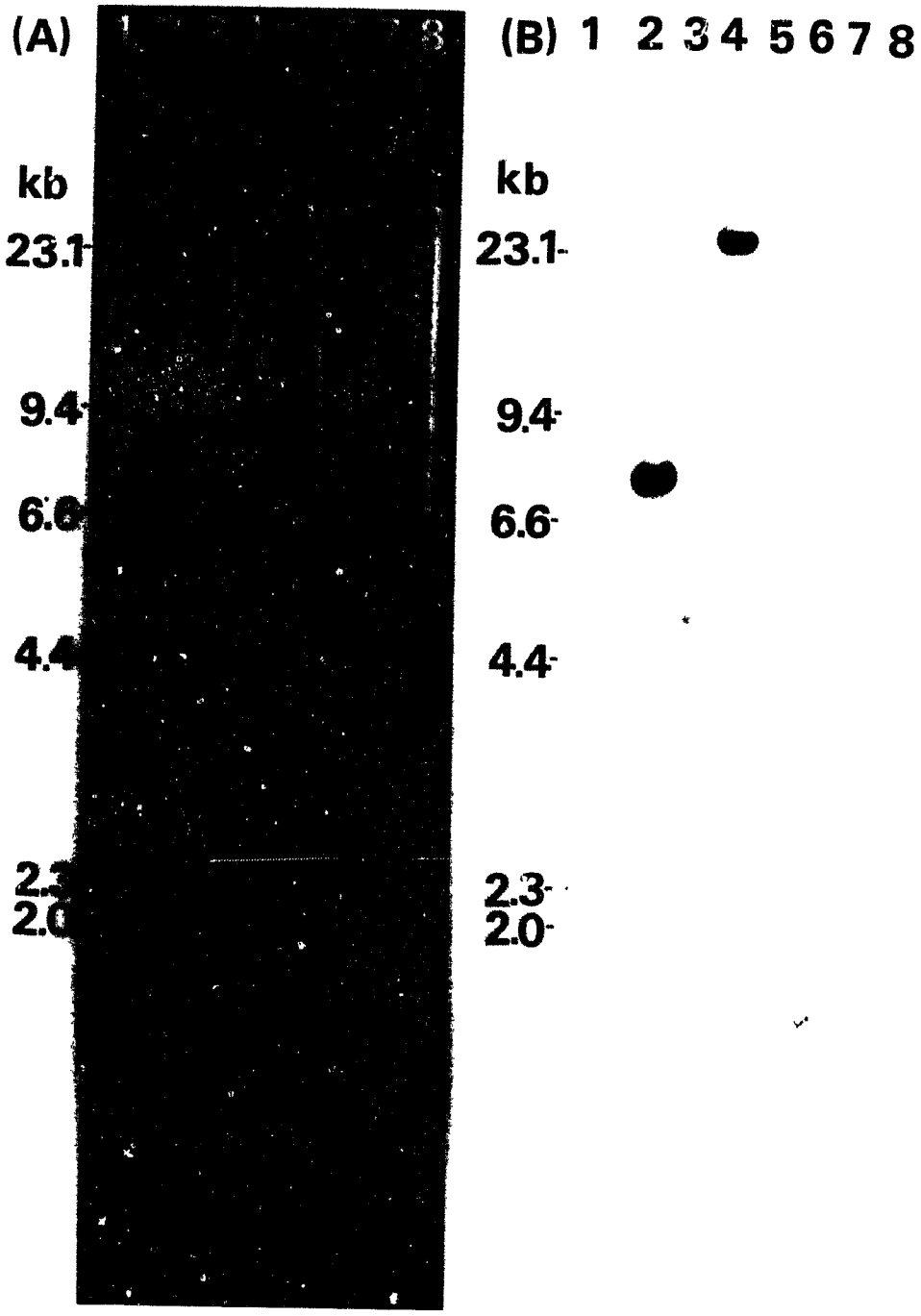


Fig. 21

K. lut genes are not located close to the β-lactam biosynthesis genes in S. clavuligerus

Since the secondary lysine catabolic pathway genes (AAA pathway genes) were clustered with other β-lactam biosynthesis genes (Fig. 21), it was of interest to test whether the primary lysine catabolic pathway genes (lut genes) were also clustered with the β-lactam biosynthesis genes. Lambda clones containing the S. clavuligerus β-lactam biosynthetic gene cluster along with extensive flanking sequences (Fig. 21) were digested to completion with SstI and the fragments were separated on an agarose gel. The fragments were transferred to a nylon membrane and probed with ^{32}P labelled pDQ401 DNA. pDQ401 did not hybridize to S. clavuligerus insert DNA in any of the lambda clones indicating that the lut genes, unlike lat, are not clustered with the β-lactam biosynthesis genes.

DISCUSSION

The role of lysine catabolism in growth and β-lactam biosynthesis has been the main focus of this investigation because lysine catabolism generates a precursor, AAA, for β-lactam production in actinomycetes (Kern et al., 1980). The pathway generating AAA, using LAT, is present only in β-lactam producing streptomycetes and the putative lat gene is clustered with other β-lactam biosynthesis genes. In addition, LAT is regulated like other β-lactam biosynthetic enzymes in many respects.

I. Lysine catabolism in streptomycetes

Lysine can be metabolized to AAA via piperideine-6-carboxylate through two alternate routes in aerobic bacteria (Fig. 4). One route proceeds via D-lysine and pipecolate in several steps; the other route involves two steps wherein lysine is first converted to piperideine-6-carboxylate which in turn is converted to AAA. The former pathway has been characterized in Pseudomonas (Miller and Rodwell, 1971b; Chang and Adams, 1971 and 1974) whereas the latter is present in several aerobes such as Flavobacterium (Soda and Misono, 1968; Soda et al., 1968), Pseudomonas (Fothergill and Guest, 1977) and streptomycetes (Kern et al., 1980). In Pseudomonas and Flavobacterium species, the second route is primarily used to assimilate lysine as the sole carbon and nitrogen source, whereas in streptomycetes the pathway generates AAA a precursor for β-lactam biosynthesis.

The possible role of the AAA pathway in supporting growth of streptomycetes has not previously been explored. The absence of the

pathway in β-lactam nonproducers and the presence of an entirely different lysine catabolic pathway in a variety of streptomycetes examined would suggest that the AAA pathway serves a secondary metabolic function and is unnecessary for growth. This information could be important for improving the yield of β-lactam antibiotics through a genetic approach since AAA has been identified as a limiting precursor (Inamine and Birnbaum, 1973).

To investigate the role of the AAA pathway in actinomycetes, apart from its fucti ı in supplying a precursor, lysine catabolism was investigated in the β-lactam-producing S. clavuligerus and a nonproducer, S. lividans. Analysis of the contents of the mycelium and the culture filtrate identified cadaverine and δ-aminovalerate, indicating that lysine was catabolized via these intermediates in these two streptomycetes. When culture filtrates were analysed cadaverine was the only ninhydrin-positive metabolite detected. Its accumulation correlated with the disappearance of lysine from the medium. Presumably cadaverine aminotransferase, the enzyme catabolizing cadaverine, is rate-limiting for lysine catabolism under these conditions. That streptomycetes catabolized lysine primarily via cadaverine was confirmed when lysine nonutilizing (Lut^-) mutants were isolated from both S. clavuligerus and S. lividans and characterized. Three types of mutants were found: type-1 mutants were probably blocked in the first step of lysine catabolism whereas type-2 were blocked in the second step or both early steps; mutants of a third type might bear multisite, polar or regulatory mutations because they could not utilize lysine or the intermediates in its catabolism. All S. lividans mutants including the

third type were able to use AAA as their sole nitrogen source indicating that lysine catabolism does not normally involve AAA in streptomycetes.

All S. clavuligerus Lut$^-$ mutants produced β-lactam antibiotics, indicating that the AAA pathway still functioned adequately. The inability of Lut$^-$ mutants to grow on lysine as the sole nitrogen source despite the presence of a fuctional AAA pathway suggested that AAA is not involved in primary lysine catabolism. In some of the cephamycin C nonproducing mutants of S. clavuligerus, lysine ε-aminotransferase, an enzyme catalyzing the first step in the AAA pathway of streptomycetes, could not be detected. These mutants were similar to wild type in their growth kinetics and were able to use several amino acids, including lysine, as the sole nitrogen source. This suggested that the AAA pathway might not have a function in primary metabolism. Thus some streptomycetes may have atleast two pathways for lysine metabolism. One pathway proceeds via cadaverine and δ-aminovalerate and is necessary for the growth of streptomycetes on lysine as the sole nitrogen source. The second pathway proceeds via 1-piperideine-6-carboxylate and AAA and is a secondary metabolic pathway supplying a precursor for β-lactam biosynthesis. Hence, the conversion of lysine to piperideine-6-carboxylate represents the first step in β-lactam biosynthesis.

The existence of separate pathways for primary and secondary metabolism of the same compound is by no means universal. In Pseudomonas aeruginosa, synthesis of the phenazine pigment, pyocyanin, uses the primary metabolic intermediate chorismate as a precursor. Chorismate is presumed to be converted to another primary metabolite, anthranilate (a precursor of tryptophan), before being incorporated into

pyocyanin. In P. aeruginosa anthranilate biosynthesis genes were found to be duplicated (Essar et al., 1990a and 1990b). One set of anthranilate synthase genes, trpE and trpG, form a part of the tryptophan biosynthetic pathway, whereas the second set, phnA and phnB, form a part of the secondary metabolic pathway that converts chorismate to anthranilate for pyocyanin biosynthesis. The two sets of genes from P. aeruginosa have been cloned and sequenced (Essar et al., 1990a and 1990b).

Unlike S. clavuligerus where the functions of the two lysine catabolic pathways are not interchangeable, the functions of the two anthranilate synthases are interchangeable under certain circumstances (Essar et al., 1990b). Mutants of phnA produced 22 to 34% phenazine compared to wild type indicating that anthranilate synthase coded by trpE and trpG was able to supply the precursor for phenazine biosynthesis. On the other hand, a trpE deletion mutation in P. aeruginosa could not be suppressed by phnA under normal circumstances. However, prototrophic variants in which expression of phnA was modified arose spontaneously. Analysis of mRNA in the variants showed that in the wild type strain phnAB mRNA gradually increased throughout growth, reaching a peak in stationary phase. The variants produced eight times more phnAB-specific mRNA than the wild type during early log phase.

In S. clavuligerus LAT activity peaks at the end of exponential phase under some culture conditions (S. Shapiro, unpublished results). The low activity of LAT during early exponential stage may contribute to its inability to the support growth of Lut$^-$ strains with lesions in the cadaverine pathway. Thus the evolution of the precursor biosynthetic

pathway in β-lactam-producing actinomycetes was different from that in pyocyanin-producing Pseudomonas. The former has a distinct secondary metabolic pathway to supply a precursor whereas the latter has a duplicate set of genes, one of which functions in secondary metabolism under modified regulation. A further example of functional gene duplication is found in Streptomyces hygroscopicus and is possibly of general occurrence in streptomycetes, namely glutamate synthase (kumada et al., 1990).

Another example is the valine catabolic pathway which supplies precursors for macrolide or polyether antibiotics in some streptomycetes. Unlike the AAA pathway in β-lactam producers, valine catabolism is also involved in primary metabolism to support the growth of streptomycetes on proline. As expected, in this role as a primary metabolic pathway for valine assimilation the first enzyme in this pathway, valine dehydrogenase, is induced by its substrate (Navarrete et al., 1990).

Attempts to isolate Lut$^-$ mutants from S. venezuelae yielded only presumptive regulatory mutants. Mutants unable to use cadaverine as the sole nitrogen source were still able to grow on lysine as the sole nitrogen source indicating that S. venezuelae catabolized lysine concurrently by two or more pathways, neither of which involves lat, since as shown in this thesis this gene is absent from S. venezuelae. Thus S. venezuelae is different from S. lividans and S. clavuligerus in that it has more than one primary lysine catabolic pathway. When a lysA mutant of S. venezuelae was grown on a minimal medium containing D-lysine as the sole nitrogen source, with limited L-lysine auxotrophic

supplementation at concentrations that do not provide sufficient nitrogen for growth, growth was visible within 24 h. However, when L-lysine was omitted from the medium, growth was only visible after 4-5 days. Interestingly, the lysA auxotroph was not leaky on minimal medium with asparagine as the nitrogen source and without lysine supplementation (no visible growth even after 5 days). This indicated that S. venezuelae has an enzyme activity which could slowly convert D-lysine to L-lysine to meet the auxotrophic requirement. Since the D- to L- conversion could be reversible, metabolism of L-lysine via D-lysine and subsequently via pipecolate might serve as an alternative route for lysine catabolism. The inability of presumptive regulatory Lut$^-$ mutants of S. venezuelae to use intermediates in the cadaverine pathway as well as D-lysine and intermediates in the D-lysine catabolic pathway, such as pipecolate and AAA, provided additional support for this conclusion. However, the possibility that lysine could be catabolized via δ-aminovaleramidase cannot be ruled out. Testing lysine-grown cultures for the activities of enzymes belonging to different lysine catabolic pathways would clearly define the routes available for lysine catabolism in S. venezuelae. The presence of more than one lysine catabolic pathway is not uncommon in aerobic bacteria. Some strains of Pseudomonas have two or more lysine catabolic pathways (Fothergill and Guest, 1977) and in these strains it is not possible to isolate different classes of Lut$^-$ mutants. The presence of a pathway, which does not involve lat, for lysine catabolism via pipecolate and AAA (Fig. 4) in streptomycetes is significant because it represents a possible alternative route for precursor biosynthesis in β-lactam producers.

However, this has to be tested in β-lactam producing streptomycetes. Although D-lysine is a very poor nitrogen source for the growth of wild-type S. clavuligerus, it might be possible to obtain mutants catabolizing D-lysine via pipecolate and AAA and show improved β-lactam production in D-lysine supplemented cultures.

II. Cadaverine and lysine aminotransferases

LAT activity was not detected in S. lividans under various conditions in which lysine utilization was expected to be mandatory. Since the same assay conditions were used for both β-lactam producers and nonproducers, it is unlikely that the enzyme activity would have escaped detection in β-lactam nonproducers. The possible presence of a highly active protease or strong enzyme inhibitor in cell extracts was ruled out by demonstrating enzyme activity in mixed cell extracts of S. lividans and S. clavuligerus. Cadaverine aminotransferase (CAT), an enzyme catalyzing the second step in the cadaverine pathway, was present in all actinomycetes tested whereas lysine ε-aminotransferase (LAT) was present only in the three β-lactam producers. This supports the hypothesis that the AAA pathway has no function in the growth of streptomycetes and hence constitutes a secondary metabolic pathway.

III. AAA catabolism in streptomycetes

There was no clear correlation between the ability to produce β-lactam antibiotics and failure to use AAA as the sole nitrogen source. However, three of seven species which do not produce detectable amounts of β-lactam antibiotics grew well on AAA, while three producers grew

poorly or not at all. Even though some β-lactam producers were able to catabolize AAA, the growth of these species was not comparable to the growth of β-lactam nonproducers on this amino acid. Therefore the relative or total inability of β-lactam producers to catabolize AAA may be a general phenomenon and a prerequisite for β-lactam production. The total inability of S. clavuligerus to use AAA as a nitrogen source might explain why this compound is excreted and accumulated in the culture medium (S. Shapiro, unpublished results). Not only do the β-lactam-producing streptomycetes have a distinct secondary metabolic pathway to generate AAA as a precursor but they also avoid its catabolism as a nitrogen source.

IV. Regulation of lysine catabolism

Studies on the regulation of primary and secondary lysine catabolic pathway enzymes by various nutritional factors indicated significant differences between the two pathways lending additional support to the results obtained in the biochemical and genetic studies on lysine catabolism.

A. Induction

LAT activity in S. clavuligerus was not influenced by lysine supplementation irrespective of the carbon source used and growth stage. CAT activity, on the other hand, was higher in lysine-supplemented cultures irrespective of the carbon source tested. Lysine increased CAT activity throughout growth of S. clavuligerus. When CAT activity was tested in cultures grown on lysine and intermediates in its catabolic

pathway, the highest activity was obtained in cadaverine supplemented cultures, and the next highest in lysine supplemented cultures. δ-Aminovalerate, on the other hand, had no effect on CAT activity. This suggested that the primary inducer of CAT was cadaverine and that the induction of CAT by lysine was due to catabolism of lysine to cadaverine. In Pseudomonas aeruginosa, CAT was also induced by δ-aminovalerate (Fothergill and Guest, 1977).

In Pseudomonas putida enzymes of the δ-aminovaleramide pathway for lysine catabolism, such as lysine oxygenase and δ-aminovaleramidase, are induced by lysine (Miller and Rodwell, 1971a). In addition, lysine oxygenase is activated by its substrate, lysine (Vandecasteele and Hermann, 1972). Since a critical concentration of lysine is needed for the activation of lysine oxygenase, this mechanism would prevent wasteful degradation of lysine under conditions of lysine limitation, even if the enzyme was synthesized. A similar mechanism probably operates in the Hut pathway of streptomycetes where histidase is post-translationally activated by a cascade mechanism (Kroening and Kendrick, 1989). The induction of CAT by cadaverine suggests that the actual inducer is cadaverine, but the situation could be analagous to that in the tryptophan catabolic pathway where enzymes are induced by an early pathway intermediate (Palleroni and Stanier, 1964). Since a critical concentration of the early intermediate is necessary for induction and since this concentration is achieved under conditions of excess tryptophan, this mechanism of induction prevents degradation of molecules present under all growth conditions and avoids wasteful expenditure of the energy reserves of cells (Palleroni and Stanier,

1964). The effect of cadaverine supplementation on activities of other lysine catabolic pathway enzymes has to be tested before any conclusion can be drawn on the similarity between the tryptophan catabolic pathway of Pseudomonas and the lysine catabolic pathway of Streptomyces.

Several amino acid catabolic enzymes of streptomycetes can be induced by their substrates. Proline dehydrogenase and arginase were induced by their substrates in S. clavuligerus (Bascaran et al., 1989). Lysine ϵ-aminotransferase was induced by lysine in P. aeruginosa where this enzymes forms a part of a catabolic pathway supporting growth on lysine as the sole nitrogen source (Fothergill and Guest, 1977). The evidence that LAT was not induced by lysine supplementation, irrespective of the culture conditions employed, is consistent with earlier results indicating that the AAA pathway is part of a secondary metabolic process. It might be argued that valine catabolism supplies a precursor for biosynthesis of macrolide or polyether antibiotics (Dotzlaf et al., 1984; Omura et al., 1983; Sherman et al., 1986), and yet valine dehydrogenase is induced by its substrate. However, this pathway is also involved in primary catabolic activity to support the growth of streptomycetes on proline (Navarrete et al., 1990).

B. Carbon catabolite regulation

Among the carbon sources tested, glycerol was the most depressive for both LAT and CAT activities. Glycerol was more depressive than starch at the 24 and 48-h sampling periods when biomass was being produced. Increasing the glycerol concentration in the medium from 0.2% to 1% did not alter the LAT activity, indicating that the source of

carbon, not its concentration, was the more important factor regulating LAT activity. In contrast "cephamycin C synthetase" (an enzyme system converting deacetylcephalosporin C to cephamycin C) and DAOCS activities were not only regulated by the carbon source but also by the concentration of the carbon source used (Lebrihi et al., 1988). Increasing the concentration of glycerol from 0.1% to 1.5% reduced the DAOCS activity by 70% whereas increasing glycerol concentration from 0.2% to 1.5% reduced "cephamycin C synthetase" activity by 80%. Increasing the concentration of starch on the other hand did not influence DAOCS activity whereas "cephamycin C synthetase" activity was mildly (40%) depressed.

Glycerol and starch and to a lesser extent maltose were depressive for CAT activity whereas histidine and succinate were not depressive. In this respect the lysine catabolic pathway was regulated like the histidine catabolic pathway of Bacillus subtilis (Chasin and Magasanik, 1968). Valine dehydrogenase of S. coelicolor is also depressed by readily metabolizable carbon sources such as glucose and glycerol (Navarrete et al., 1990). On the other hand, synthesis of the lysine catabolic pathway enzymes of P. putida (Vandecasteele and Hermann, 1972) and the histidine utilization enzymes of S. coelicolor (Kendrick and Wheelis, 1982) are not influenced by the carbon source employed. In both cases the enzyme activities are regulated by induction and activation.

Whether carbon catabolite depression of LAT and CAT activities is mediated by a common repression mechanism is not known but in a glucose catabolite derepressed mutant of P. chrysogenum simultaneous

derepression of β-galactosidase and penicillin production was observed (Barredo et al., 1988).

C. Phosphate regulation

Increasing the concentration of phosphate from 20 to 100 mM increased enzyme activity at all stages of growth, the effect being greatest at 72 h. These results contrast with those obtained by Lubbe et al. (1985) and Jhang et al. (1989), who found that phosphate decreases the formation of all four β-lactam synthases, ACVS, IPNS, IPN epimerase, and DAOCS in S. clavuligerus. Similar results were obtained in Cephalosporium acremonium (Zhang et al., 1988). In this respect, the regulation of LAT was different from that of other β-lactam biosynthesis enzymes. The effect of phosphate concentration on LAT activity may have two possible explanations. The increase in LAT activity may be due to increased synthesis of enzyme in response to phosphate. Alternatively, it may be due to less rapid turn over of the enzyme under the influence of high phosphate. The difference in LAT activity between 20 mM and 100 mM phosphate cultures was higher in 72-h culture compared to 24-h culture suggesting that probably there was a gradual increase in the synthesis of LAT or there was gradual accumulation of LAT in the 100 mM phosphate culture. The latter phenomenon could be anticipated if LAT is turned over rapidly under conditions favouring optimum β-lactam production (20 mM phosphate). There was a positive correlation between phosphate concentration and LAT activity in 48-h cultures of S. clavuligerus grown at different phosphate concentrations. Increasing the phosphate concentration also reduced biomass production.

The effect of phosphate on LAT activity can also be seen when starch is replaced with glycerol. On the other hand, CAT activity is not effected by phosphate concentration, indicating that the phosphate effect is specific to LAT. The importance of the phosphate effect on LAT and β-lactam production is hard to assess from the relatively limited data. It would be interesting to test whether there is an increase in the amount of AAA accumulated in the culture supernatant with increasing phosphate concentration. An increase in AAA accumulation in 100 mM phosphate would suggest that S. clavuligerus has a novel mechanism of regulation of precursor biosynthesis wherein AAA molecules are synthesized for future use under conditions which do not favour their use in β-lactam biosynthesis.

D. Nitrogen catabolite regulation

LAT and CAT significantly differ from each other in their responses to various nitrogen sources. In S. clavuligerus LAT activity was not influenced by the source of nitrogen. On the other hand, the activity of CAT was depressed by the presence of ammonium and glutamate in the culture medium containing a depressive carbon source such as glycerol. Replacing ammonium with glutamate in glycerol-lysine-ammonium medium depressed CAT activity whereas omitting ammonium from glutamate-lysine-ammonium medium had no effect. This suggested that the effect produced by ammonium may have been due to its assimilation to form glutamate, and that glutamate probably is the primary depressor of CAT. This is different from the regulation of Hut pathway enzymes of Bacillus subtilis by ammonium where a cascade mechanism involves ntr genes

(Magasanik, 1987). Glutamate mildly depressed CAT activity in cultures containing succinate as the carbon source. The severely reduced growth of S. clavuligerus in a medium containing glycerol, lysine and glutamate is intriguing. The simultaneous presence of two depressive nutrients in the culture would be expected to diminish CAT activity but this should not affect the metabolism of glycerol and glutamate. However, lysine and glutamate may interfere with each other's metabolism as nitrogen sources. The growth of B. subtilis was reduced when glucose, as a repressive carbon source, was used with histidine as the sole nitrogen source (Chasin and Magasanik, 1968). Histidine utilization enzymes of S. coelicolor (Kendrick and Wheelis, 1982), S. griseus (Kroening and Kendrick, 1987, 1989) and S. clavuligerus (Bascaran et al.,1989) and proline dehydrogenase of S. clavuligerus (Bascaran et al., 1989) were not influenced by ammonium supplementaion whereas arginase was depressed by ammonium in S. clavuligerus (Bascaran et al., 1989).

In summary, CAT is regulated primarily by induction and carbon and nitrogen catabolite depression. IAT on the other hand is regulated by carbon catabolite depression and a unique response to phosphate.

V. Cloning the gene for lysine ε-aminotransferase

A. Attempted cloning by suppression of a mutation in the cadaverine pathway in S. lividans

Even though the AAA and cadaverine pathways can both function as lysine dissimilatory pathways, their functions in streptomycetes are not interchangeable. AAA is a specific precursor for β-lactam biosynthesis

and probably cannot be replaced by intermediates of the cadaverine pathway. On the other hand the presence of a functional AAA pathway does not permit mutants of S. clavuligerus blocked in the cadaverine pathway to grow on lysine as the sole nitrogen source. S. lividans does not have the AAA pathway, but introduction of AAA pathway genes from S. clavuligerus on a high-copy plasmid should enable S. lividans mutants blocked in the cadaverine pathway to obtain glutamate from LAT and AAA aminotransferase reactions and thereby receive an adequate nitrogen supply. However, this strategy for cloning AAA pathway genes was not successful.

All three recombinant plasmids obtained from Lut^+ transformants retransformed the parent strain to the Lut^+ phenotype, but none of the transformed strains showed LAT activity. The Lut^+ phenotype could be attributed to complementation, rather than suppression. There are several possible explanations for the lack of suppression: 1) the presence of AAA pathway genes on a high copy-number plasmid and their expression from a heterologous promoter could be detrimental for cell growth, irrespective of the medium employed; 2) there might have been an SstI site within the gene, so that only part of the gene was cloned. This is unlikely because partially digested DNA was used in cloning and was shown later to be untrue when a 8.2-kb SstI fragment of S. clavuligerus containing the putative LAT gene directed the synthesis of LAT in S. lividans; 3) AAA pathway genes are specific for secondary metabolism and respond to different regulatory signals from those controlling primary metabolism needed for cell growth. Therefore, if the cloned genes were under the control of their own promoter in the

recombinant vector they might not have been expressed in the host strain at a high enough level during early exponential growth to provide the cells with sufficient nitrogen for growth.

B. Plasmid pDQ401

One of the three recombinant plasmids, pDQ401, was examined in detail to obtain information about the organization of lut genes in Streptomyces. The genomic fragments cloned in pDQ401 could not be retrieved by cleavage with SstI indicating that the plasmid had lost or modified the SstI cloning sites at each end of the insert. Restriction enzyme analysis of the plasmid DNA indicated that the a large amount of the DNA had been lost from the pIJ702 portion and maybe from the original SstI insert fragment as well during the generation of pDQ401. Interestingly, the plasmid was maintained stably despite apparent loss of a segment from pIJ702 encoding a Rep protein involved in plasmid replication (Kendall and Cohen, 1988). This suggests that the S. clavuligerus segment cloned in pDQ401 may have contained another DNA segment (origin of replication) which enabled pDQ401 to replicate. E. coli plasmids containing a chromosomal origin of replication are stably maintained in their host (Yasuda and Hirota, 1977). Similarly E. coli plasmids containing yeast sequences known as ARS (autonomously replicating sequences) are stably maintained episomally in Saccharomyces cerevisiae (Stinchcomb et al., 1979). ARS probably contain yeast origins of replication and a similar situation might exist in pDQ401. Since the restriction map of pDQ401 is uninterpretable, hybridization between pIJ702 and pDQ401 fragments should reveal which parts of pDQ401

are derived from S. clavuligerus and which parts (if any) of pLJ702 are present in pDQ401.

C. Complementation of cadaverine pathway mutations

pDQ401 may contain more than one lut gene. It complemented a type-1 lut mutation indicating that it might encode lysine decarboxylase, the enzyme catalyzing the first reaction in the cadaverine pathway. Mutations in two type-2 mutants (mutants blocked either in the second step or in both early steps) were also complemented by pDQ401 indicating that pDQ401 contained genes for more than one of the early steps in lysine catabolism via cadaverine or one gene encoding a multifunctional protein. The instability of pDQ401 in S. venezuelae was disappointing. The deletion of DNA segments when the plasmid was introduced in S. venezuelae suggests that the presence of lut or some other gene(s) on pDQ401 was lethal to the host.

The results obtained suggest that the genes governing lysine catabolism via cadaverine are probably clustered in Streptomyces. Histidine nonutilizing mutants of S. coelicolor have been characterized and mapped by conjugation. The genes were found to be clustered and mapped in the "silent region", close to agarase genes, at 9 o'clock on the genetic map of the circular S. coelicolor chromosome (Kendrick and Wheelis, 1982). The authors suggested that the failure to find auxotrophic and resistance markers in the silent region might be because this location is occupied by genes governing several catabolic pathways.

D. Southern hybridization of genomic DNA from Streptomyces

Hybridizing fragments detected by Southern analysis of SstI and BglII digested genomic DNA from S. clavuligerus with pDQ401 as the probe did not match those obtained by electophoresis of pDQ401 digested with SstI and BglII. The four internal SstI fragments and one BglII fragments of pDQ401 were not seen by Southern hybridization of similarly cleaved DNA from the S. clavuligerus genome. However,when SstI digested genomic DNA from several other streptomycetes was probed with pDQ401, hybridizing fragments again did not corresponded to any of the four internal SstI fragments of pDQ401. Therefore, there was no evidence that these fragments were from any of the other species tested, and no evidence to suggest confusion of the samples might have occurred. The explanation for this lack of correspondence between fragments of cloned DNA fragment of genomic DNA is not apparent and more investigation will be required to clarify these observations.

Although there was no direct size correspondence between fragments of the cloned S. clavuligerus DNA in pDQ401 and genomic DNA fragments, there was significant sequence similarity (hybridization) with various fragments of that genomic DNA and with DNA of several other β-lactam producing and nonproducing streptomycetes. This suggests that the genes present in the insert portion of pDQ401 were necessary for one or more ubiquitous metabolic fuctions.

E. Cloning by chromosomal walking

Since the phenotype of a mutation in the cadaverine pathway could not be suppressed by AAA pathway genes, an alternative strategy based on

chromosomal walking from a cloned β-lactam biosynthesis gene pcbC was explored. This strategy was based on the assumption that the gene coding for IAT would be clustered with other β-lactam biosynthesis genes. In support of this assumption, a 29.3-kb DNA fragment of S. cattleya directed the synthesis of cephamycin C in the heterologous host, S. lividans, in the absence of AAA supplementation (Chen et al., 1988). Thus the genes directing the synthesis of AAA were probably clustered with other β-lactam biosynthesis genes.

Restriction analysis of overlapping fragments of cloned DNA indicated that lat was indeed located between pcbC and cefE: it was positioned 10-12 kb from the pcbC probe sequence and 13-14 kb to the left of cefE. However, it is not known for sure whether the cloned fragment actually contains a structural gene for IAT; IAT activity in S. lividans theoretically could result from activation of a latent structural gene. There is a precedence to support this argument. Biosynthesis of actinomycin in S. antibioticus requires the activity of an enzyme phenoxazinone synthase. A DNA fragment from S. antibioticus introduced into S. lividans, an actinomycin nonproducer, directed the synthesis of phenoxazinone synthase. However, in vitro transcription and translation assay indicated that the fragment did not contain a structural gene for phenoxazinone synthase. This indicated that the DNA fragment contained an activator gene which activated a silent phenoxazinone synthase gene in S. lividans (Jones and Hopwood, 1984; Jones, 1989). Sequencing the cloned putative lat gene (currently underway) should provide evidence to distinguish between a structural and a regulatory gene. Regulatory proteins contain helix-turn-helix

motifs and this property can be used to distinguish between a structural and regulatory gene. Additionally, in vitro transcription and translation assays could be performed to confirm the presence of a structural gene. Thus, not only is α-aminoadipate biosynthesis regulated in many respects like later reactions in the β-lactam biosynthesis pathway, but the genes governing early and late reactions in the pathway are physically adjacent. Further evidence that α-aminoadipate biosynthesis is specifically part of a secondary metabolic pathway was obtained by Southern blot analysis of the genomic DNA from β-lactam-producing and nonproducing Streptomyces. Cloned DNA containing the putative lat gene hybridized only to the genomic DNA of β-lactam producers. However, this does not exclude the possibility that some untested non-producers might show hybridization with the lat probe, as found with pcbC probes (Cohen et al., 1990).

The results suggest that a pathway that fuctions in some aerobic bacteria such as Pseudomonas and Flavobacterium as a primary catabolic system has assumed a secondary metabolic function in β-lactam producing streptomycetes, and that its genes are clustered and regulated with other β-lactam biosynthesis genes. However, we do not know whether the lat genes from β-lactam producers and other aerobic bacteria are the same. Sequencing the S. clavuligerus lat gene would shed more light on its origin and relationship to the lat genes from other organisms. The reaction catalyzing the conversion of 1-piperideine-6-carboxylate to AAA has not been characterized in streptomycetes, but is presumed to be an oxido-reductase. Whether the gene encoding this enzyme is also clustered with β-lactam biosynthesis genes was not determined in the

present work.

The lat gene introduced into Lut⁻ mutants of S. lividans did not support the normal growth of these mutants on lysine as the sole nitrogen source. This could be expected if lat contained its own promoter permitting its regulation in the same manner as its regulation in S. clavuligerus. Suppression of the Lut⁻ phenotype of a cadaverine pathway mutant then would not occur because LAT activity is low in (S. clavuligerus) during early exponential phase under certain culture conditions when inoculum is transferred from a complex to a defined medium (S. Shapiro, unpublished results), and peak activity is attained only at the end of exponential phase.

E. Organization of β-lactam biosynthesis genes

Based on the results presented in this thesis and previously published information, several general conclusions can be drawn about the organization of genes governing β-lactam biosynthesis in Streptomyces. The enzymes in the pathway leading to synthesis of the penam ring system (ie. LAT, ACVS and IPNS) are specified by genes located at one end of the cluster, whereas late pathway genes (for DAOCS, IPN epimerase and probably the additional "processing" enzymes) are located at the opposite end of the gene cluster. In at least one other prokaryote, a Flavobacterium species, and in two eukaryotes, P. chrysogenum and A. nidulans, genes for the early pathway steps catalysed by IPNS and ACVS are apparently adjacent, although possibly not in the same orientation relative to other β-lactam biosynthesis genes (Smith et al., 1990b).

Weigel et al. (1988) suggested that transfer of a cluster of β-

lactam genes from a prokaryote (Streptomyces) to the progenitor of β-lactam-producing eukaryotes would explain the origin of β-lactam biosynthesis genes in fungi. P. chrysogenum and A. nidulans seem to have received or retained only the genes for early steps in the pathway which, along with an acyltransferase gene, enable them to make hydrophobic penicillins. The acyltransferase gene and the genes for ACVS and IPNS activities constitute a single cluster on one chromosome in P. chrysogenum (Diez et al., 1989; Smith et al., 1990a and b). Whether the cluster includes lat has not been investigated, but the ability of fungi to form α-aminoadipate as an intermediate in lysine biosynthesis may have allowed this function to be lost. The acyltransferase reaction is observed only in eukaryotes producing hydrophobic penicillins. This together with the fact that there is an intron in the gene encoding acyltransferase gene (Montenegro et al., 1990) suggests that the gene was of eukaryotic origin. Acremonium chrysogenum on the other hand has both early and late pathway genes. Since one early gene (for IPNS) and one late gene (for DAOCS) are located on different chromosomes (Skatrud and Queener, 1989), early and late pathway genes in this species may be grouped in clusters on the two chromosomes. A better understanding of gene organization, and information about nucleotide sequence similarities in the β-lactam biosynthesis genes of different producers, will allow more informed speculation on the possibilities and manner of horizontal transfer of these genes from prokaryotes to eukaryotes.

F. Organization of lysine catabolic pathway genes in actinomycetes

Genes governing two different lysine catabolic pathways are located in different regions of the S. clavuligerus genome. The AAA pathway genes are located as part of β-lactam biosynthesis gene cluster along with the genes for other early enzymes. The cadaverine pathway genes on the other hand are not adjacent to β-lactam biosynthesis genes. The exact location of the lut genes is not known. Some other catabolic pathway genes, such as those for histidine (Kendrick and Wheelis, 1982) and agar catabolism (Hodgson and Chater, 1981) have been located in the "silent" region of the S. coelicolor genome. Whether S. clavuligerus has a similar "silent" region in its chromosome is presently unknown.

Even though S. clavuligerus mutants deregulated in lysine biosynthesis produced more cephamycin C, production was not proportional to the amount of deregulation in these strains (Mendelowitz and Aharonowitz, 1983). In these strains probably some other steps, including AAA biosynthesis, were rate limiting. Since AAA biosynthesis was shown to be a rate limiting step in some β-lactam producers (Inamine and Birnbaum, 1973) it will be of interest to test whether introducing the AAA biosynthesis gene on a high copy-number plasmid into β-lactam producers would increase the yield of β-lactam antibiotics in wild type as well as in lysine biosynthesis deregulated mutants. However this would require the isolation of the gene encoding piperideine-6-carboxylate dehyrogenase, an enzyme catalyzing the second step in AAA biosynthesis, if not already present adjacent to lat

SUMMARY AND CONCLUSIONS

Biochemical and genetic analyses of lysine catabolism have revealed the presence of diverse lysine catabolic pathways in streptomycetes. The α-aminoadipate (AAA) pathway, present only in β-lactam producers, generates a precursor, α-aminoadipate, for β-lactam biosynthesis. Since it does not allow Streptomyces clavuligerus to grow on lysine as the sole nitrogen source, this pathway seems to be used exclusively to provide a precursor for β-lactam biosynthesis and thus is a secondary metabolic pathway. Not only do β-lactam-producing streptomycetes have a distinct pathway for generating AAA but they also avoid its further catabolism as a nitrogen source by lacking the necessary enzymes. The cadaverine pathway is present in all actinomycetes tested and is used to catabolize lysine as a nitrogen source. S. venezuelae is different in that it has an additional pathway for dissimilation of lysine. This is probably initiated by the conversion of L-lysine to D-lysine followed by the catabolism of D-lysine via pipecolate and AAA.

The primary and secondary lysine catabolic pathways are regulated differently. The cadaverine pathway enzyme, cadaverine aminotransferase (CAT), is induced by its substrate. It is also under carbon catabolite depression by carbon sources such as glycerol and starch which givw good growth. Additionally, CAT is strongly depressed by glutamate in glycerol-containing media, whereas it is mildly depressed in succinate containing media. Lysine ε-aminotransferase, the enzyme catalyzing the first step in the AAA pathway, is not induced by its substrate but is depressed by the readily assimilated carbon source glycerol. In these

respects the regulation of LAT resembles the regulation of other β-lactam biosynthetic enzymes. However, LAT differs significantly from other β-lactam biosynthetic enzymes in its response to phosphate. While the latter are repressed by high phosphate concentrations, there is a positive correlation between phosphate concentration and LAT activity.

The genes governing primary and secondary lysine catabolic pathways are organized at different locations on S. clavuligerus genome. The two lysine catabolic pathways are probably not interchangeable. It is not likely that the intermediates in the cadaverine pathway can replace AAA in β-lactam biosynthesis. Also, lat DNA did not suppress mutations in the cadaverine pathway even when introduced on a high copy-number plasmid.

The cadaverine pathway genes are probably clustered but have not been localized on the chromosome. The lat gene encoding lysine ε-aminotransferase activity is clustered with other β-lactam biosynthesis genes. It is located between pcbC and cefE genes: approximately 10-12 kb from pcbC and 12-14 kb from cefE. Thus, in streptomycetes the early β-lactam biosythesis genes are located at one end of the cluster whereas the late pathway genes are located at the other end of the cluster. The sequence apparently encoding LAT is present only in β-lactam producers, whereas the sequence containing putative cadaverine pathway genes is present in all streptomycetes tested. The limited distribution of lat is consistent with the AAA pathway functioning specifically in secondary metabolism, and, the conversion of lysine to piperideine-6-carboxylate by LAT is the first specific step in β-lactam biosynthesis in streptomycetes.

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